# IL-6 and $PGE_2$ regulate intestinal crypt homeostasis by autocrine and paracrine pathways

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# Abstract

The intestinal epithelium is the most rapidly renewing tissue in the body. Dynamic self-renewal in the intestine is achieved through continuous proliferation of intestinal stem cells, and is under the tight regulation of numerous signalling pathways. IL-6 and $PGE_2$ are pleiotropic cytokines, with well characterised roles in inflammation and intestinal tumorigenesis; however the roles for IL-6 and $PGE_2$ in intestinal renewal during homeostasis remain unknown.

The aim of this research was to determine the mechanisms through which autocrine and paracrine IL-6 and $PGE_2$ regulate tissue renewal in the small intestine and colon, respectively, during homeostasis. This work also aimed to investigate a potential source of IL-6 and $PGE_2$ in the intestinal lamina propria, the eosinophil.

This thesis demonstrates that in the mouse small intestine, IL-6 signalling induces pSTAT3 activation in Paneth cells, which was shown to be the site of IL-6 receptor expression. This induced an increase in crypt cell proliferation and ISC expansion of small intestinal crypts, most likely through IL-6-classic signalling, with involvement of the WNT signalling pathway. The colonic epithelium expresses COX enzymes for $PGE_2$ synthesis, and EP1-4 receptors for $PGE_2$ signal transduction. Autocrine $PGE_2$ signalling was required for colonic crypt cell proliferation during steady state renewal, which was mediated through the EP1/EP3 receptors. Paracrine signalling through the IL-6 and $PGE_2$ pathways also induced small intestinal and colonic crypt proliferation respectively. A potential paracrine source of IL-6 and PGE, that resides in the intestinal lamina propria during health is the eosinophil. A novel spatial relationship between eosinophils and the stem cell niche (site of renewal) and crypt top (site of regeneration) was identified, suggesting that eosinophils play a role in modulating epithelial cell renewal during homeostasis.

This work demonstrates that autocrine and paracrine IL-6 and $PGE_2$ signalling is required for the maintenance of intestinal homeostasis.

# Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University of to any other institution for a degree, diploma or other qualifications.

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PhD candidate

Chapter 3 includes work from the following jointly authored publication in The Journal of Immunology:

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Contributions from co-authors have been fully acknowledged in the body of the thesis.

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# Glossary

| | |
|---|---|
| 15-PGDH | 15-hydroxyprostaglandin dehydrogenase |
| ADAM | A Disintegrin and metalloproteinase domain-containing protein |
| ALPi | Alkaline Phosphate Intestinal |
| ANOVA | Analysis of Variance |
| APC | Adenomatous Polyposis Coli |
| APCs | Antigen Presenting Cells |
| APRIL | A Proliferation Inducing Ligand |
| BAC | Bacterial Artificial Chromosome |
| BMI1 | B cell-specific Moloney murine leukemia virus integration site 1 |
| BMP | Bone Morphogenetic Protein |
| CAMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| CCL | C-C motif Chemokine Ligand |
| CCR3 | CC-Chemokine Receptor 3 |
| CDC2 | Cell Division Cycle protein 2 |
| CMP | Common Myeloid Progenitor |
| COX | Cyclooxygenase |
| CRF | Corticotropin-releasing factor |
| CRISPR | Clustered Regularly Interspaces Short Palindromic Repeats |
| CXCL | C-X-C motif Chemokine Ligand |
| DAAM1 | Dishevelled-Associated Activator of Morphogenesis 1 |
| DAG | Diacyclglyceride |
| DKK-1 | Dickkopf-related protein 1 |
| DLL(1/4) | Delta-Like Ligand (1/4) |
| Dsh | Dishevelled |
| DSS | Dextran Sodium Sulfate |
| EAR | Eosinophil-Associated RNases |
| ECP | Eosinophil Cationic Protein |
| EDN | Eosinophil-Derived Neurotoxin |

Glossary

| | |
|---|---|
| EEC | Enteroendocrine cell |
| EET | Eosinophil Extracellular Trap |
| EGF | Epithelial Growth Factor |
| EP | E Prostanoid |
| EPO | Eosinophil Peroxidase |
| FAK | Focal Adhesion Kinase |
| FAP | Familial Adenomatous Polyposis |
| GFP | Green Fluorescent Protein |
| GI | Gastrointestinal |
| GMP | Granulocyte Macrophage Progenitor |
| GP130 | Glycoprotein 130 |
| GPCR | G-Protein Coupled Receptor |
| GSK | Glycogen Synthase Kinase |
| H&E | Hematoxylin & Eosin |
| HCCCM | Human Colonic Crypt Culture Media |
| HSC | Hematopoetic Stem Cell |
| IBD | Inflammatory Bowel Diseases |
| IEC | Intestinal Epithelial Cell |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-6 | Interleukin-6 |
| IL-6R | Interleukin-6 Receptor |
| IP3 | Inositol triphosphate |
| ISC | Intestinal Stem Cell |
| IWP2 | Inhibitor of WNT Protein II |
| JAK | Janus Kinase |
| JNK | c-Jun N-terminal Kinases |
| KO | Knockout |
| Lgr5 | Leucine-rich repeat-containing G-protein coupled Receptor 5 |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MACS | Magnetic Activated Cell Sorting |
| MAMP | Microbe-Associated Molecular Pattern |
| MAPK | Mitogen-Activated Protein Kinase |
| MBP | Major Basic Protein |
| MCCCM | Mouse Colonic Crypt Culture Media |
| M-CSF | Macrophage Colony-Stimulating Factor |
| MDSC | Myeloid-Derived Suppressor Cells |
| MEP | Megakaryocyte and Erythroid Progenitor |
| MMP | Metalloproteinase |
| MSC | Mesenchymal Stem Cell |
| MSICM | Mouse Small Intestinal Culture Media |
| Muc-2 | Mucin-2 |
| MyD88 | Myeloid Differentiation primary response protein 88 |
| NFAT | Nuclear Factor of Activated T-cells |
| NFκB | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| NK | Natural Killer |
| NLR | Nucleotide-binding site and Leucine-rich repeat-containing Receptors |
| NRH1 | Neurotrophin receptor homolog 1 |
| NSAID | Non-steroidal anti-inflammatory drug |
| PAMP | Pathogen-Associated Molecular Pattern |
| PCP | Planar Cell Polarity |
| PGE2 | Prostaglandin-E2 |
| PGES | Prostaglandin-E Synthase |
| PGH2 | Prostaglandin-H2 |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-Kinase |
| PKA | Protein Kinase A |
| PKC | Protein Kinase C |
| PLC | Phospholipase C |
| PRR | Pattern Recognition Receptor |
| PTK7 | Pseudo Tyrosine Kinase Receptor 7 |

| | |
|---|---|
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RANTES | Regulated on Activation, Normal T cell Expressed and Secreted |
| RLR | Retinoic acid inducible gene-I-Like Receptors |
| ROCK | Rho-associated Kinase |
| ROR2 | Receptor tyrosine kinase like Orphan Receptor 2 |
| RYK | Related to receptor tyrosine kinase |
| SI | Small intestine |
| sIL-6R | Secreted Interleukin-6 Receptor |
| SIGLEC-8 | Sialic acid-binding Immunoglobulin-like Lectin-8 |
| STAT | Signal Transducer and Activator of Transcription |
| TAK1 | Transforming Growth Factor –β Activated Kinase-1 |
| $T_C$ | Cytotoxic T Cell |
| TEB | Terminal End Buds |
| TGF | Transforming Growth Factor |
| $T_H$ | T Helper |
| TLR | Toll-Like Receptors |
| TPL2 | Tumor Progression Locus-2 |
| $T_{Reg}$ | T Regulatory |
| Trem2 | Triggering Receptor Expressed On Myeloid Cells 2 |
| UC | Ulcerative Colitis |
| Upd3 | Unpaired 3 |
| VEGF | Vascular Endothelial Growth Factor |
| WAE | Wound-associated Epithelial |
| WL | Wingless |
| WNT | Wingless-related integration site |
| WT | Wild type |

# Preface

The intestinal epithelium forms a vital barrier between the microbial contents of the gut lumen, and underlying host tissues. It is formed of a one-cell thick monolayer, which invaginates millions of times into structures called crypts. The small intestine has finger-like projections termed villi, which increase the surface area of the epithelium in order to fulfil the main function of the small intestine, absorption of nutrients from food. The intestinal epithelium is the most rapidly renewing tissue in the body, with the entire epithelium renewing itself every 5-7 days. Dynamic renewal is achieved by way of intestinal stem cells, which reside at the base of crypts, thought to be a relatively safe harbour away from the microbial contents of the gut lumen. Stem cells proliferate, differentiate (into all the cell types of the intestinal epithelium: goblet cells, Paneth cells, tuft cells, enteroendocrine cells, enterocytes), migrate up the crypt (-villus) axis, and are shed into the gut lumen (Barker et al., 2007). The renewal process is tightly regulated by a number of signalling pathways, which can be epithelial-derived (autocrine) or derived from immune cells residing in the underlying lamina propria of the mucosa (paracrine). The master regulator of intestinal renewal is thought to be WNT signalling, aberrant signalling of which induces intestinal polyposis and the development of cancers (Schepers and Clevers, 2012). A number of other signalling pathways contribute to the maintenance of gut renewal during homeostasis, including Bone Morphogenetic Protein (BMP), Transforming Growth Factor β (TGFβ), Epidermal Growth Factor (EGF) and Noggin (Vanuytsel et al., 2013; Reynolds et al., 2014); however, during stressful conditions, activation of inflammatory cytokine pathways also induces crypt regeneration. Two inflammatory cytokines which are emerging to have roles in crypt regeneration are Interleukin-6 (IL-6) though the Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway and Prostaglandin-$E_2$ ($PGE_2$).

IL-6 is a pleiotropic cytokine involved in a plethora of cellular and immune responses in health and disease (Mihara et al., 2012; Garbers et al., 2015; Hunter and Jones, 2015). Seminal work in *Drosophila* showed that the regenerative response to tissue injury or infection in the gut are modulated by JAK/STAT signalling in gut epithelial stem cells through the release of Unpaired 3 (Upd3), an IL-6-like cytokine (Jiang et al., 2009; Osman et al., 2012). In the mammalian gut, IL-6 and STAT3 have been shown to induce proliferation in the intestinal epithelium following tissue injury/inflammation (Pickert et al., 2009; Kuhn et al., 2014), and to promote the survival of normal epithelial cells during Inflammatory Bowel Diseases (IBD) and cancer (Bollrath et al., 2009; Grivennikov et al., 2009; Jin et al., 2010; Lee et al., 2012).

Similarly, in the colon, $PGE_2$ is another cytokine that has been implicated in cancer and IBD, with an emerging role in crypt cell proliferation and regeneration during homeostasis. The enzymes responsible for the synthesis of $PGE_2$ are Cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2), which are upregulated in colorectal cancer (Wang and Dubois, 2010). Therefore, COX enzyme inhibition represents an attractive target for the development of anti-cancer therapies. Indeed, the use of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit COX enzymes in patients with Familial Adenomatous Polyposis (FAP) was shown to reduce the incidence of intestinal polyps and tumour growth; however, this was associated with significant side effects (Bresalier et al., 2005; Solomon et al., 2005; Arber et al., 2006; Rostom et al., 2007). Recent studies have demonstrated that epithelial production of $PGE_2$ through COX-2 activation is required for epithelial regeneration in response to injury, which is vital for the maintenance of intestinal homeostasis (Chen et al., 2015). These findings demonstrate that IL-6 and $PGE_2$ are important for epithelial regeneration in conditions such as injury or cancer; however, the roles of IL-6 and $PGE_2$ in modulating crypt renewal during health remain largely unknown. Furthermore, the potential sources of IL-6 and $PGE_2$ in the intestines have not yet been fully investigated. Secretion of IL-6 and $PGE_2$ by the epithelium (autocrine) or immune cells in the underlying lamina propria (paracrine) represent potentially important sources of IL-6 and $PGE_2$ *in vivo*.

Immune cells also have an emerging role in the regulation of crypt renewal during homeostasis (Pull et al., 2005; Seno et al., 2009; Kuhn et al., 2014; Skoczek et al., 2014). The intestinal lamina propria underlying the epithelium is home to a number of immune cells in inflammation and during health. One immune cell subtype that is abundant in the lamina propria during health, which is emerging to have vital roles in the maintenance of gut immune homeostasis, is the eosinophil (Carlens et al., 2009; Chu et al., 2014). Eosinophils express a number of cytokines and receptors known to be important for crypt renewal and regeneration, including IL-6 and $PGE_2$ (Melo et al., 2013; Rosenberg et al., 2013). This suggests that eosinophils are a potential paracrine source of IL-6 and $PGE_2$ *in vivo* in the gut mucosa.

The sources, targets and influence of IL-6 and $PGE_2$ on intestinal homeostasis is the focus of this thesis. The general introduction will discuss the autocrine and paracrine factors known to modulate crypt renewal during homeostasis, and what is known about IL-6, $PGE_2$ and eosinophils in intestinal tissue renewal and regeneration in health and disease.

# 1 Chapter 1. General Introduction

## 1.1 The Gastrointestinal Tract

The gastrointestinal tract (GI tract) consists of a 10-meter long hollow tube that begins at the oral cavity, pharynx and oesophagus, continues through the stomach, small and large intestines to the rectum and anus (**Figure 1.1**). The GI tract is home to trillions of microorganisms and is an interface of constant challenge from microbial peptides from food. The main purpose of the GI tract is to digest and absorb nutrients from food. Food is ingested into the oral cavity and propelled along the in various organs of the GI tract by peristalsis, to the anus where it is expelled.

The GI tract wall is comprised of four distinct layers. The outermost layer, the serosa, consists of a layer of epithelial cells termed the mesothelium, and fat. Above the serosa is a smooth muscle layer called the muscularis externa, which is comprised of longitudinal and circular smooth muscle to aid the mechanical breakdown of food and peristalsis through the GI tract lumen. The next layer is the submucosa, which is comprised of connective tissue, blood vessels and lymphatics, which extend to the innermost layer, the mucosa. The mucosa itself is comprised of three layers, the epithelium, lamina propria and muscularis mucosa. The epithelium forms a physical barrier between the host and external environment. The lamina propria underlying the epithelium is comprised of a network of blood vessels, nerves, lymphoid tissue and glandular tissue, which is surrounded by a smooth muscle layer called the muscularis mucosa (**Figure 1.1**). The type of epithelial cells present in the mucosa varies dependent on the function of the region of the GI tract: for example the oral cavity is lined by squamous epithelial cells, designed to support any abrasion caused by passing food; whereas the stomach and intestines are lined by columnar epithelial cells to aid absorption and secretion (Tortora GJ, 2008).

The small and large intestines constitute the largest component of the GI tract; the epithelial surface covering approximately 400$m^2$ of surface area. The epithelium of both the small and large intestines are exquisitely organised into millions of invaginations called crypts, also known as the crypts of Lieberkühn (**Figure 1.1**). The major function of the small intestine is to absorb nutrients from food, and the large intestine is responsible for the resorption of water and formation of stools. The differences in function of the small and large intestines require a degree of variation in structure and cell type, as demonstrated by the presence of

villi in the small intestine which increases the surface area of the intestine for maximal nutrient absorption (Johnson, 2013). The epithelium also has a vital function as a physical barrier between the contents of the intestinal lumen and underlying host tissues.

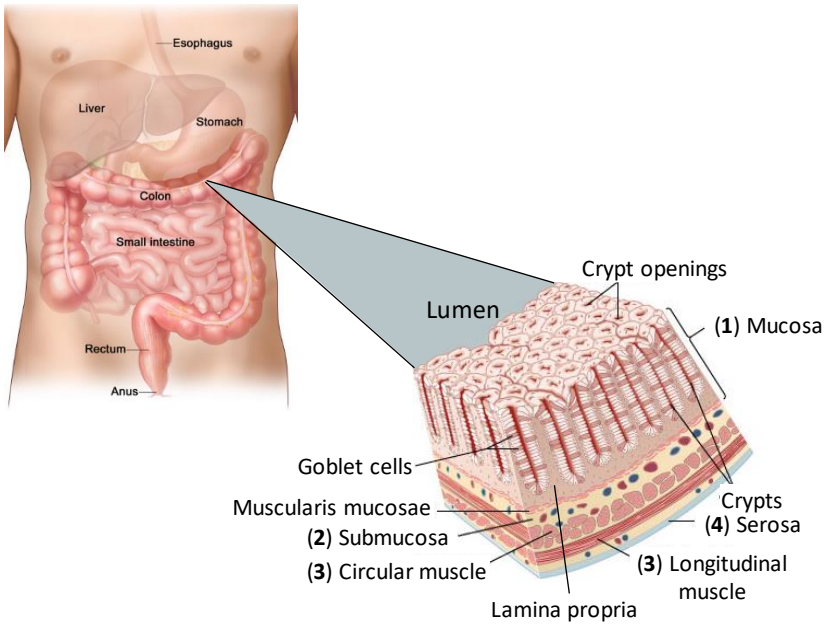


**Figure 1.1. Anatomy of the gastrointestinal tract.**

The GIT consists of a hollow tube beginning at the oral cavity, pharynx and oesophagus, continues through the stomach, small and large intestines to the rectum and anus. The GIT wall is comprised of 4 distinct layers: (**1**) Mucosa (**2**) Submucosa (**3**) Circular and longitudinal muscle (**4**) Serosa. The intestinal epithelium forms millions of invaginations called crypts, which form a vital barrier between luminal content and underlying tissues. Figure adapted from Encyclopaedia Britannica, Inc 2003

Within the intestinal lumen reside trillions of microorganisms; the total microbial load of the intestine is approximately $10^{13}$-$10^{14}$ microorganisms (Sartor, 2008). The intestinal microbiome is comprised of autochthonous (indigenous) and allochthonous (transient) flora. The indigenous flora reside in the intestines, whilst the transient flora colonizes the intestine following inflammation or stress (Hao and Lee, 2004). The bacteria present have a huge diversity of species thought to be from an estimated 15,000 to 36,000 individual species (Sartor, 2008). Human foetuses are born germ-free from the sterile environment in the uterus; however, the colonization of epithelial surfaces with bacteria begins immediately after birth. The majority of these bacteria are commensal, and form a beneficial relationship with the host. Commensal bacteria have major functions in the maintenance of normal gastrointestinal immune function through secretion of pro/anti-inflammatory signals, metabolism of non-digestible dietary components from which energy is supplied to the host,

and in the degradation of epithelial cells which have been shed into the gut lumen (Flint et al., 2012). However, despite the vast numbers of bacteria present in the intestinal lumen, the mucosal surface itself is maintained virtually sterile by way of protective mucus layers overlaying the epithelial barrier (Johansson et al., 2008).

### 1.1.1 The Epithelial Barrier

The intestinal epithelium forms a vital barrier between microbial content in the gut lumen and immune cells residing in the underlying lamina propria. The integrity of the barrier is dependent on epithelial cell integrity, epithelial paracellular permeability maintained by tight junctions, mucus layers overlying the epithelium and the presence of associated immune cells.

The intestinal epithelium forms a physical barrier between luminal microbes and the host tissues. When this physical barrier is breached, the microbial content of the gut lumen comes into direct contact with host tissues. This process leads to inflammation, and chronic exposure leads to the development of the Inflammatory Bowel Diseases (IBD): Ulcerative Colitis and Crohn's disease (McGuckin et al., 2009; Pastorelli et al., 2013).

Intestinal epithelial cells (IECs) are tightly bound together by intracellular junctional protein complexes, which regulate paracellular permeability (**Figure 1.2**) (Turner, 2009). The junctional complexes are comprised of tight junctions, adherens junctions, gap junctions and desmosomes. Adherens junctions are involved in cell-cell adhesion and intracellular signalling (Perez-Moreno and Fuchs, 2006). Gap junctions regulate intracellular communication (Sosinsky and Nicholson, 2005) whilst desmosomes are involved in cell-cell adhesions (Garrod and Chidgey, 2008). Of particular interest are tight junctions, which specifically seal the paracellular space to prevent paracellular diffusion of microbes, whilst allowing selective absorption of nutrients across the epithelial barrier. They are highly dynamic structures, which are constantly remodelled in response to external stimuli, including both commensal and pathogenic bacteria. Commensal and probiotic bacteria have been shown to enhance epithelial barrier function *via* redistribution of zonula occluding proteins (Ulluwishewa et al., 2011), which connect the transmembrane domains of tight junctions to the perijunctional actomyosin ring (Fanning et al., 1998). When intracellular junctional protein complexes are incorrectly formed, increased epithelial permeability and susceptibility to colitis is observed (Vetrano et al., 2008).

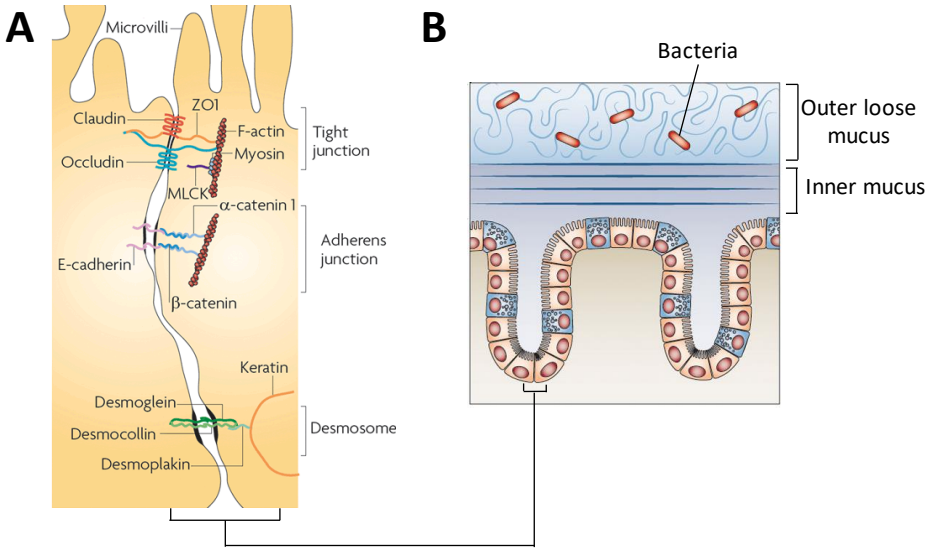


**Figure 1.2. The Epithelial Barrier.**

(**A**) IECs are bound together by intracellular junctional protein complexes comprised of tight junctions, adherens junctions and desmosomes, which regulate paracellular permeability. (**B**) The intestinal epithelium is coated with protective layers of mucus, the inner firmly adherent layer, which is devoid of bacteria, and outer loose mucus layer (colon only), where commensal bacteria reside. Figure adapted from (Turner, 2009; Johansson et al., 2013).

The intestinal epithelium is coated with a protective layer of mucus (**Figure 1.2**) (Johansson et al., 2013). The inner firmly adherent mucus layer is continuously replenished by goblet cells of the epithelium and is thought to be sterile to the luminal microbial content (Hansson and Johansson, 2010). The outer layer, present in the large intestine only, is more loosely associated with the epithelium and creates an environment where commensal bacteria can reside (Johansson et al., 2008). The mucus of the small and large intestines is primarily comprised of Mucin-2 (Muc-2). This belongs to a family of four gel-forming mucins, which are highly glycosylated glycoproteins with numerous O-glycans attached to domains rich in proline, threonine and serine (Gum et al., 1994). The loosely adherent characteristic of the outer layer of mucus is due to cleavage of Muc-2 thus enabling bacterial penetration. Deletion of Muc-2 in mouse studies leads to direct physical contact between bacteria and the intestinal epithelium. These mice developed loose stools, diarrhoea containing blood and fail to thrive (Johansson et al., 2008). This suggests an integral role for mucus in the maintenance of intestinal barrier homeostasis.

However, it is important that in the event that bacteria penetrate the inner mucus layer and come into contact with the epithelium, IECs must be able to detect microbial patterns in

order to mount an immune response. IECs therefore express receptors on their cell surface membrane for the detection of microbial peptides, including Toll-Like Receptors (TLRs; signalling discussed in detail later), Nucleotide-binding site and Leucine-rich repeat-containing receptors (NLRs) and Retinoic acid inducible gene-I-Like Receptors (RLRs). In response to microbial challenge IECs are able to release anti-microbial molecules, and secrete cytokines and chemokines in order to link the innate and adaptive immune system (Rescigno, 2011). This suggests that IECs are able to initiate inflammatory pathways in response to microbial sensing.

The presence of innate immune cells underlying the intestinal mucosa forms an important part of the intestinal barrier. These immune cells have roles in microbial clearance following pathogenic challenge and breach of the mucosal barrier, modulation of the adaptive immune response, as well as emerging roles in modulating renewal in the gastrointestinal tract.

### 1.1.2 Intestinal Tissue Renewal

The gut epithelium is the most rapidly renewing tissue in the body. The duration of the renewal cycle is 5-7 days, with approximately 10 billion cells shed into the gut lumen every day. These cells are continually replaced by Intestinal Stem Cell (ISC) progeny residing at the bases of intestinal crypts (**Figure 1.3**); ISC are vital for the self-renewal capacity of the intestines (Barker et al., 2007). ISC maintenance and proliferation is dependent on a number of factors: WNT signalling is vital for steady state crypt renewal (Korinek et al., 1998; Pinto et al., 2003; Kuhnert et al., 2004); the WNT agonist Rspondin-1 which causes crypt hyperplasia *in vivo* (Kim et al., 2005); Epithelial Growth Factor (EGF) signalling which is important for crypt growth in culture (Dignass and Sturm, 2001); and Noggin which induces expansion of crypt numbers *in vivo* (Haramis et al., 2004). Pluripotent ISCs reside at the crypt base, thought to be a relatively stable environment, away from the contents of the gut lumen. The kinetics of ISC division are best modelled by symmetric cell division (Snippert et al., 2010), however there is some evidence to suggest that ISCs also undergo asymmetric cell division (Potten et al., 2002; Quyn et al., 2010). How and when asymmetric cell division occurs remains to be fully elucidated. Following ISC division, one daughter cell is fated to remain a pluripotent ISC, the other will differentiate into a multipotent progenitor cell and enter the transit amplifying zone of the crypt. These cells continue to proliferate and differentiate into one of five lineages: enterocytes, enteroendocrine cells, goblet cells, Paneth cells (small intestine only) and the recently described tuft cells (Snippert et al., 2010; Gerbe et al., 2012). Following the differentiation process, enterocytes, enteroendocrine

cells, goblet cells and tuft cells all migrate towards the top of the crypt, or villus in the small intestine. In contrast, Paneth cells migrate to the base of the crypts in the small intestine and reside in the stem cell niche (Barker et al., 2007).

When cells reach the apex of the crypt (or villus in the small intestine), they undergo apoptosis, detach from the epithelium *via* anoikis and are shed into the gut lumen where they are degraded by residing commensal bacteria. The loss of cells into the lumen through cell shedding was initially thought to cause a breach or 'gap' in the epithelial barrier (Kiesslich et al., 2007); however, it is now widely accepted that epithelial cells are lost into the lumen whilst maintaining the integrity of the epithelial barrier (Watson et al., 2005; Marchiando et al., 2011). This is dependent on complex interplay between microtubules, microfilaments and redistribution of apical junction complex proteins, which form intercellular barriers to maintain mucosal function (Marchiando et al., 2011).

This hierarchy of tissue renewal is thought to minimise the possibility of molecular damage to the continuously proliferating stem cell niche.

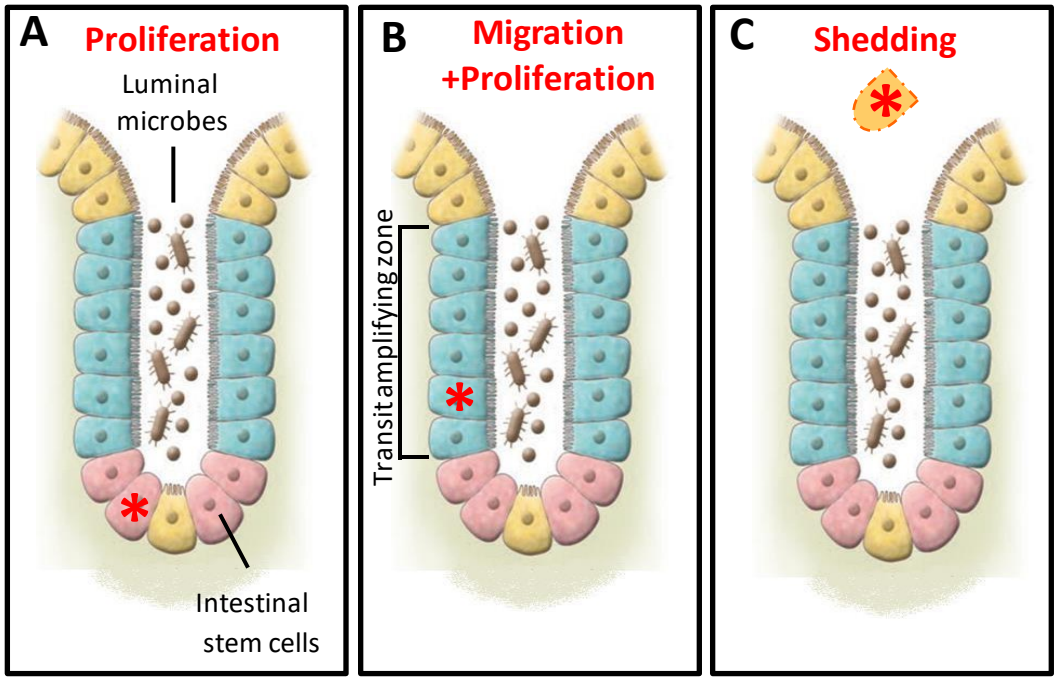


**Figure 1.3. Colonic crypt renewal.**

(**A**) Proliferation is initiated by intestinal stem cells residing at the base of crypts. (**B**) Stem cell progeny migrate up the crypt axis and differentiate into goblet cells, enterocytes, enterendocrine cells or tuft cells. (**C**) When cells reach the top of crypts, they detach from the epithelium through anoikis and are shed into the gut lumen, where they are removed by residing commensal microbes.

#### 1.1.2.1 Epithelial Cell Types of the Small and Large Intestine

As mentioned above, when ISC differentiate they give rise to all cell types present within the intestinal crypt (summarised in **Figure 1.4**), which have distinct functions.

**Enterocytes** make up approximately 80% of all cells in the intestine (Cheng and Leblond, 1974). The primary function of enterocytes is nutrient absorption hence their hyperpolarised nature. They are essential for maintaining the polarised nature of crypt cells, which is vital for the integrity of the epithelial barrier. Luminal antigens are taken up by enterocytes through fluid-phase endocytosis and processed in one of two ways; enzymatic degradation of the antigens by lysosomes, or released into the interstitial space where they enter the systemic circulation. The expression of Major Histocompatibility Complex class I and class II allows enterocytes to present processed antigens directly to T cells which may elicit an immune response (Snoeck et al., 2005).

**Enteroendocrine cells** (EEC) are hormone-secreting cells comprising approximately 1% of the intestinal epithelial cells. They are usually located sporadically, interspersed between other types of epithelial cells (Buffa et al., 1978). The frequency of EECs in the intestines falls from the duodenum to the colon and then rises again in the rectum, where you occasionally see clusters of EECs (Cristina et al., 1978). A common characteristic of EECs are apical cytoplasmic processes with microvilli that extend towards the lumen, however basal processes extending towards adjacent epithelial cells have also been described in the large intestine (Sjolund et al., 1983). These processes act as sensors of the luminal contents. EECs are also characterised by the presence of secretory vesicles which are able to secrete peptide products which impact on colonic motility and secretion and exert effects on distant sites in the GI tract, including gastric emptying (Gunawardene et al., 2011).

As described above, the functional integrity of the intestinal epithelium is largely dependent on the mucus layer overlying the epithelial cells (Kim and Ho, 2010). **Goblet cells** are responsible for the secretion and constant replenishment of the mucus layer, which provides the first line of defence against invasive microbes arising from ingested food. The main component of the mucus secreted by goblet cells is mucin, formed of large glycoproteins with a polymeric protein backbone structure linked to numerous oligosaccharide side-chains (Andrianifahanana et al., 2006). The intestine is the site of colonization of a wide variety of bacteria, both commensal and pathogenic. These bacteria get trapped in the mucus layer

secreted by goblet cells and are eliminated by peristalsis before they reach the epithelial surface (Lievin-Le Moal and Servin, 2006; Dharmani et al., 2009).

**Paneth cells** are an important part of the innate immune system in the small intestine, and secrete anti-microbial peptides including lysozyme and defensins (Bevins and Salzman, 2011). Defensins are antimicrobial cationic peptides which can disrupt bacterial membranes, leading to microbial death. In the small intestine, following interactions between Pathogen-Associated Molecular Patterns (PAMPs) and the intestinal epithelium, α-defensins are produced by Paneth cells residing in the stem cell niche (Ayabe et al., 2000). As they are produced by Paneth cells, there are no α-defensins in the large intestine. However, β-defensins are produced endogenously throughout the GI tract (Vora et al., 2004).

The migration of Paneth cells to the stem cell niche following maturation suggests a role for Paneth cells in the maintenance of the stem cell niche. Paneth cells were first described as slow-cycling or quiescent stem cells. Following injury, Paneth cells switch to a proliferative phenotype, gaining gene expression features characteristic of stem cells, and losing their Paneth cell identity. This process is thought to potentially underlie the regenerative response of the crypt (Roth et al., 2012). During homeostasis, Paneth cells express EGF, TGFα, WNT3 and the Notch-ligand Delta-like ligand 4 (DLL4), all of which are essential for proliferation of stem cells in culture. This was confirmed by *in vivo* genetic deletion of Paneth cells, which resulted in a loss of intestinal stem cells. Although Paneth cells are not present in the large intestine, in the colonic crypt stem cell niche resides a pool of CD24+ secretory type cells, distinct from mature goblet cells; these cells are thought to be the colonic Paneth cell equivalent (Sato et al., 2011).

Although intestinal **tuft cells** were previously acknowledged on many occasions, a lack of molecular markers meant that the functional characteristics of tuft cells were not defined until relatively recently (Gerbe et al., 2012). A number of genetically modified mouse lines, as well as genetic modification of cell lines have allowed further information regarding the function of tuft cells within the intestine to be elucidated. Tuft cells were identified to be the main source of endogenous intestinal opioids and a unique source of COX enzymes (discussed later) in the intestinal epithelium (Gerbe et al., 2011).

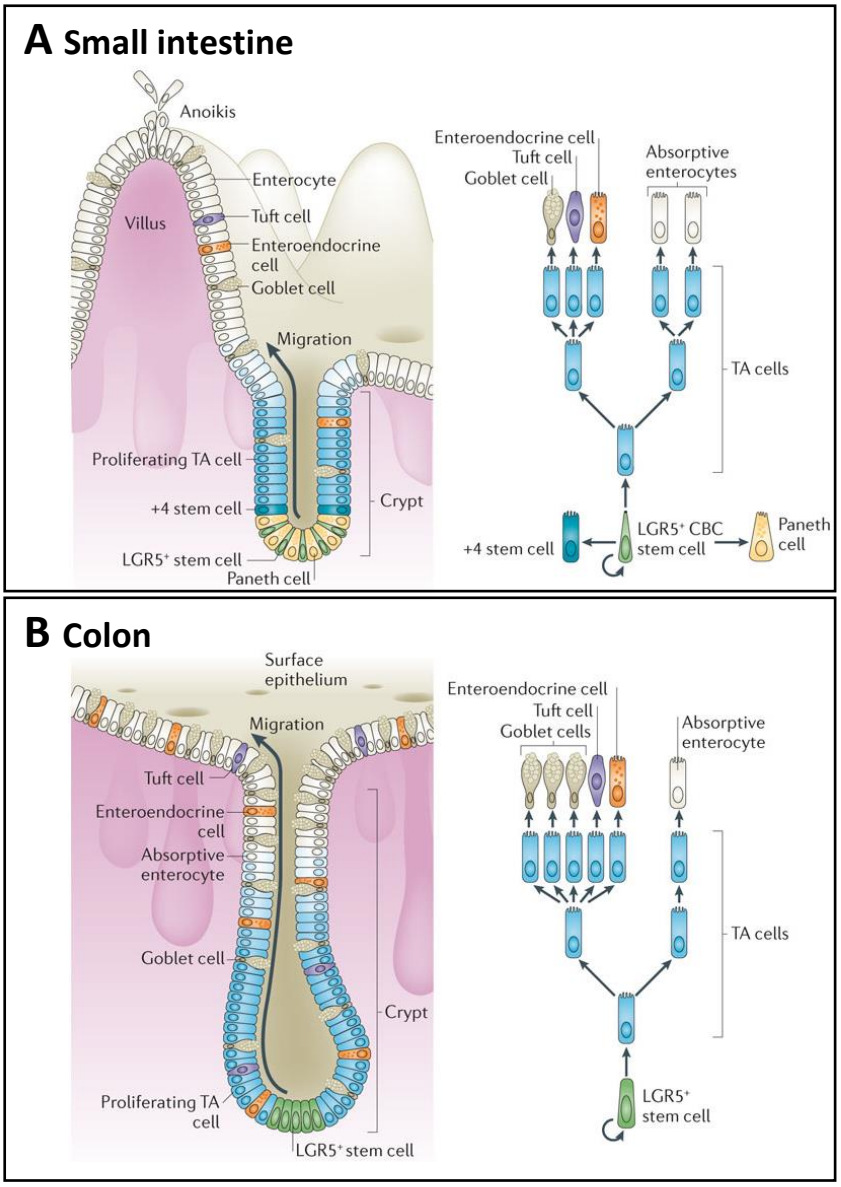


**Figure 1.4. Characterisation of intestinal epithelial cell lineages in crypts and villi.**

(**A**) In the small intestine, Lgr5+ stem cells reside at the base of crypts. Lgr5+ stem cells, proliferate and give rise to rapidly proliferating transit-amplifying (TA) cells, which differentiate into the following cell lineages: Paneth cells, which migrate to the base of crypts; slow cycling +4 stem cells, which reside at the +4 position; goblet cells, enterocytes, enteroendocrine cells and tuft cells which all migrate up the crypt villus axis. When cells reach the villus tip, they are shed into the gut lumen by anoikis. (**B**) The colonic epithelium are comprised of Lgr5+ stem cells, which proliferate, give rise to rapidly proliferating TA cells, and differentiate into goblet cells, enterocytes, enteroendocrine cells and tuft cells. Cells migrate up the crypt-axis and are shed into the gut lumen by anoikis. Figure from (Barker 2014)

#### 1.1.2.2 Characterisation of Intestinal Stem Cells

As mentioned previously, ISC are vital for the self-renewing capacity of the intestinal epithelium. However, until the important publications by Clevers' group in 2007 (Barker and Clevers, 2007; Barker et al., 2007), no stem cells had been identified in the colon. Previously, long-term DNA label retention studies had suggested cycling +4 position crypt cells were the stem cells of the small intestine (Potten et al., 2002). However, the functional identity of small intestinal stem cells remained unknown until relatively recently due to a lack of specific markers and stem-cell assays.

A number of intestinal stem cell markers have since been characterised. Seminal work from the Clevers laboratory identified the stem cell marker Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5). The WNT signalling pathway was known to be crucial for the regulation of crypt stem cells in the intestines (Reya and Clevers, 2005). It is for this reason that a panel of WNT target genes were hypothesised to be specifically expressed in intestinal stem cells. A unique expression pattern of Lgr5 in murine small intestinal crypts but not in the villi was observed. Lgr5+ cells were also present at all crypt bases, and appeared to mark the cycling crypt base columnar cells between Paneth cells in the small intestine. Development of an inducible Cre knock-in allele and the *Rosa26-LacZ* reporter strain (**Figure 1.5**), allowed lineage-tracing experiments of Lgr5+ cells to be undertaken. In this system, Cre DNA recombinase is fused to a mutant estrogen ligand-binding domain; which, upon binding of tamoxifen, is able to enter the nucleus. This complex excises an upstream stopper cassette, which allows expression of β-galactosidase or fluorescent protein (Snippert and Clevers, 2011). Once activated, expression is observed in stem cells, and all daughter cells. Lineage-tracing of a single Lgr5+ cell revealed that within 5 days, Lgr5+ cells were able to differentiate into all the cell types of the colonic epithelium suggesting that Lgr5+ cells are the true stem cells of the intestines (Barker et al., 2007). Lgr5-EGFP-ires-CreERT2 mice were utilised in this thesis to enable the visualisation of Lgr5 stem cells in the intestine through fluorescence microscopy (**Figure 1.5**).

Evidence has since revealed the presence of two subtypes of stem cells, defined by their location and cycling dynamics. Lgr5+ stem cells are fast-cycling, and are interspersed between Paneth cells at the base of crypts, which support them by secreting factors including WNT and DLL4 (Barker et al., 2007; Sato et al., 2011). Slower-cycling cells represent a rarer population of stem cells, reside at the +4 positive above Paneth cells in the crypt, and are marked by expression of BMI1 (B lymphoma Moloney-murine leukaemia virus insertion

region 1 homolog) (Sangiorgi and Capecchi, 2008). Following genetic ablation of Lgr5 cells, it was shown that BMI1 slow-cycling stem cells are able to repopulate crypts and give rise to all the cell types of the epithelium, including Lgr5+ stem cells (Tian et al., 2011). More recent studies have determined that quiescent crypt cells are secretory precursors which express Lgr5 and are committed to mature into Paneth and enteroendocrine cells; however following intestinal injury, these cells are capable of extensive proliferation and give rise to all epithelial cell types (Buczacki et al., 2013).

The ability of other cells within the crypt to act as reserve stem cells has also been tested (reviewed in (Philpott and Winton, 2014; Beumer and Clevers, 2016)). Delta-like ligand 1 (DLL1) is a Notch ligand expressed by secretory progenitors of the crypt. When Lgr5+ stem cells were ablated by irradiation, DLL1 cells were shown to de-differentiate to a stem cell state, where they are able to replenish all cells within the crypt (van Es et al., 2012). The DLL1 secretory progenitors form only a small proportion of cells within the crypt, therefore it was hypothesised that should the cells of the highest abundance within crypts have the ability to retain stemness, this would increase the pool of 'reserve stem cells' significantly. Similarly to secretory cells, alkaline phosphate intestinal (ALPi)-expressing enterocytes were also shown to replenish all cells within the crypt following Lgr5 stem cell ablation (Tetteh et al., 2016).

A number of laboratories are now taking advantage of the ability of the fast-cycling, self-renewal properties of intestinal stem cells in order to grow stem cells in *in vitro* culture (Sato et al., 2009; Jung et al., 2011; Sato et al., 2011; Skoczek et al., 2014; Parris and Williams, 2015). Tissue samples from the mouse and human intestine are broken down into individual stem cells or whole crypts are isolated, which grow in culture as organoids or crypts in a culture media containing WNT, R-spondin1, EGF and Noggin in a laminin-rich matrigel. Mouse intestinal organoids grow as budding cysts, containing crypt (and villus)-like domains. These organoids grow and expand in culture for several months or even years, maintaining all cell types of the epithelium observed *in vivo* and retaining genomic stability (Barker et al., 2008; Sato et al., 2011). Human whole crypt culture, as developed by the Williams laboratory, recapitulates the topological hierarchy of the intestinal epithelium to permit the study of native polarised epithelial cells (Reynolds et al., 2014; Parris and Williams, 2015).

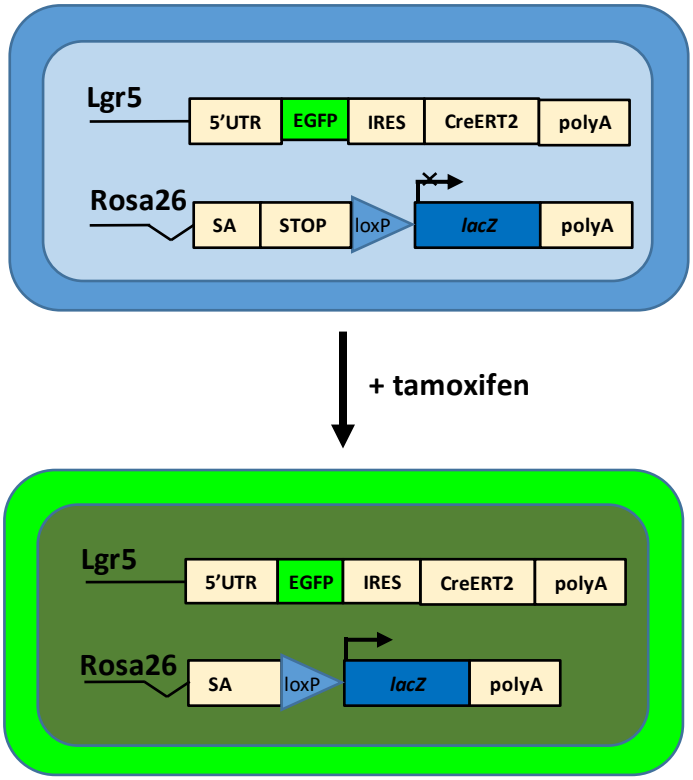


**Figure 1.5. Generation of mice expressing EGFP and creERT2 through Lgr5 gene knock-in.**

In order to visualise Lgr5, an EGFP-IRES-creERT2 cassette was inserted into the Lgr5 gene locus. A fluorescent protein or LacZ is inserted in the locus of the ubiquitously expressed Rosa26 gene. Expression is prevented by an upstream STOP cassette. The EGFP-IRES-creERT2 allele was crossed with the Cre-inducible Rosa26-*lacZ* reporter. The addition of tamoxifen allows Cre recombinase fused to a mutant estrogen ligand-binding domain to enter the nucleus. The STOP cassette is excised, and the LacZ reporter is expressed irreversibly in Lgr5+ cells, allowing for lineage tracing of Lgr5 stem cells. UTR, untranslated region; FP, fluorescent protein; IRES, internal ribosome entry site; CreERT2, Cre recombinase fused to mutant estrogen ligand-binding domain; polyA, polyadenylation sequence; SA, splice acceptor. Adapted from (Barker, van Es et al. 2007)

### 1.1.3 Autocrine and Paracrine Regulation of Epithelial Renewal

As described above, through the secretion of WNT, EGF, TGFα and DLL4, CD24+ cells maintain the intestinal stem cell niche in a crypt autonomous manner (Sato et al., 2011). In addition to secretion by Paneth cells, EGF and the EGFR are expressed endogenously throughout the epithelium, suggesting that cells are able to respond to EGF in an autocrine fashion. EGF is a potent activator of crypt cell proliferation and also has roles in intestinal healing and cancer (Dignass and Sturm, 2001; Normanno et al., 2006).

The WNT signalling pathway has been described as the master regulator of intestinal tissue renewal (Schepers and Clevers, 2012). Paneth cells are the major source of WNTs in the intestine. Co-culturing of Paneth cells with sorted Lgr5+ stem cells induced a marked improvement in organoid forming efficiency, which was also induced by addition of exogenous WNT to culture media in the absence of Paneth cells (Sato et al., 2011; Farin et al., 2016). The importance of WNT signalling in epithelial renewal was investigated through depletion of the gene for Tcf-4 (a major transcription factor activated by WNT signalling), which leads to the development of an epithelium which is comprised solely of differentiated, non-dividing cells. The lack of a proliferative compartment in intestinal crypts suggests that Tcf-4 maintains the crypt stem cells of the intestine (Korinek et al., 1998). WNT signalling was confirmed to be important in the maintenance of adult intestinal stem cells by transgenic expression of the WNT inhibitor Dickkopf-related protein 1 (DKK1) in adult mice, which induces the complete loss of crypts (Reya and Clevers, 2005). Constitutive activation of WNT signalling using Rspondin-1, a potent WNT signal enhancer induces massive proliferation in crypts (Kim et al., 2005). R-spondin-1 binds Lgr5 with high affinity and was shown to be vital for WNT-mediated self-renewal of intestinal stem cells (Yan et al., 2017).

All WNT proteins have a covalent lipid modification which tether them to the cell membrane. Porcupine is the enzyme responsible for the WNT protein lipid modification (Kadowaki et al., 1996); inhibition of porcupine by Inhibitor of Wnt Production-2 (IWP2) is widely used as an *in vitro* WNT signalling inhibitor (Chen et al., 2009; Koo et al., 2012). The transmembrane protein Wingless (WL) binds lipidated WNTs and conveys them to the plasma membrane for secretion (Najdi et al., 2012). Secreted WNT may then be incorporated into secretory vesicles, where they remain bound to WL, until they are presented to Frizzled receptors (Gross et al., 2012). Binding of WNT3 to Frizzled receptors on Paneth cells at the base of intestinal crypts tethers WNT3 to the basal membrane of crypts. Tethered WNT3 is transferred to neighbouring Lgr5+ stem cells, which then distribute the WNT ligand to daughter cells through cell division (Farin et al., 2016). The WNT3 signal is therefore diluted along the crypt-axis, inducing a WNT signalling gradient from the base to the top of crypts, which has previously been described (Davies et al., 2008; Reynolds et al., 2014).

Upon WNT receptor activation, three different signalling transduction pathways are known to be activated: the canonical WNT signalling cascade, and two non-canonical WNT pathways, the planar cell polarity pathway and the WNT/calcium pathway (reviewed in Nusse (2012)). In the canonical WNT pathway, in the absence of WNT ligand, a destruction

complex of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3 (GSK3) phosphorylates β-catenin, which continually targets it for degradation by the proteasome (Rubinfeld et al., 1993; Siegfried et al., 1994; Ikeda et al., 1998). In the presence of WNT, WNT ligand binds Frizzled, or the transmembrane co-receptor Lrp5/6 (inducing a complex formation with Frizzled), which instils a conformational change in the receptor to enable phosphorylation. This inhibits GSK3 and binds Axin, preventing interaction with APC, which inhibits the formation of a destruction complex with β-catenin. Translocation and subsequent accumulation of β-catenin in the nucleus occurs, where it induces transcription factors Tcf/Lef (**Figure 1.6**) (reviewed in Nusse (2012)). The APC gene is known to be mutated in the hereditary cancer syndrome familial adenomatous polyposis (FAP) whereby innumerate pre-cancerous intestinal polyps are formed in the intestine, which eventually leads to the formation of malignant adenocarcinoma (Nishisho et al., 1991). Loss of both APC alleles also occurs in the majority of sporadic colon cancers (Kinzler and Vogelstein, 1996). Mutated APC induces stabilisation and accumulation of β-catenin in the nucleus, leading to inappropriate transcription of Tcf reporter constructs, which in turn activates a genetic program to maintain cell proliferation in crypt stem and progenitor cells (Korinek et al., 1997). Confirmation of this paradigm was provided by Barker et al., (2009), where the APC gene was deleted in Lgr5+ stem cells, which induced formation of a rapidly growing (but not differentiating) microadenoma in the proliferative compartment of the crypts. In these crypts, constitutive activation of WNT signalling lead to conservation of the proliferative nature of stem cells and prevention of differentiation (Barker et al., 2009).

Non-canonical WNT signalling is independent of β-catenin and instead requires the recruitment of Dishevelled (Dsh). There are two defined non-canonical WNT signalling pathways, the planar cell polarity (PCP) pathway, and the WNT/Calcium pathway (**Figure 1.7)**. In the PCP pathway, WNT binds to Frizzled and either Neurotrophin receptor homolog 1 (NRH1), Ryk (related to receptor tyrosine kinase), Pseudo Tyrosine Kinase 7 (PTK7) or Receptor tyrosine kinase like Orphan Receptor 2 (ROR2) as a co-receptor. The receptor complex then recruits Dsh and forms a complex with Dishevelled-associated activator of morphogenesis 1 (DAAM1). DAAM1 activates Rho, which activates Rho-associated kinase (ROCK). Dsh also forms a complex with Ras-related C3 botulinum toxin substrate 1 (Rac1), which activates c-Jun N-terminal kinases (JNK). DAAM1 also activates the actin-binding protein Profilin. Signalling from Profilin, ROCK and JNK lead to actin polymerization and other cytoskeletal changes required to regulate cell polarity and gastrulation (**Figure 1.7A**) (Komiya and Habas, 2008). Aberrant non-canonical WNT signalling through the PCP pathway is also

known to induce malignant phenotypes of cancer through abnormal tissue polarity, cell invasion and metastasis.

The WNT/Calcium signalling pathway depends on the interaction of G proteins with Dsh to activate phospholipase C (PLC). This then leads to accumulation of inositol triphosphate (IP3) and diacyclglyceride (DAG) which induces intracellular calcium release. Elevated intracellular calcium activates $Ca^{2+}$/calmodulin-dependent protein kinase II (CamKII) and calcineurin (Krausova and Korinek, 2014). CamKII activates TGF–β Activated Kinase-1 (TAK1), which inhibits the function of β-catenin/TCF. Calcineurin activates nuclear factor of activated T cells (NFAT) to regulate ventral cell fates. DAG also activates protein kinase C (PKC), which activates CDC42 for mediation of tissue separation during gastrulation (**Figure 1.7B**) (van Amerongen, 2012).

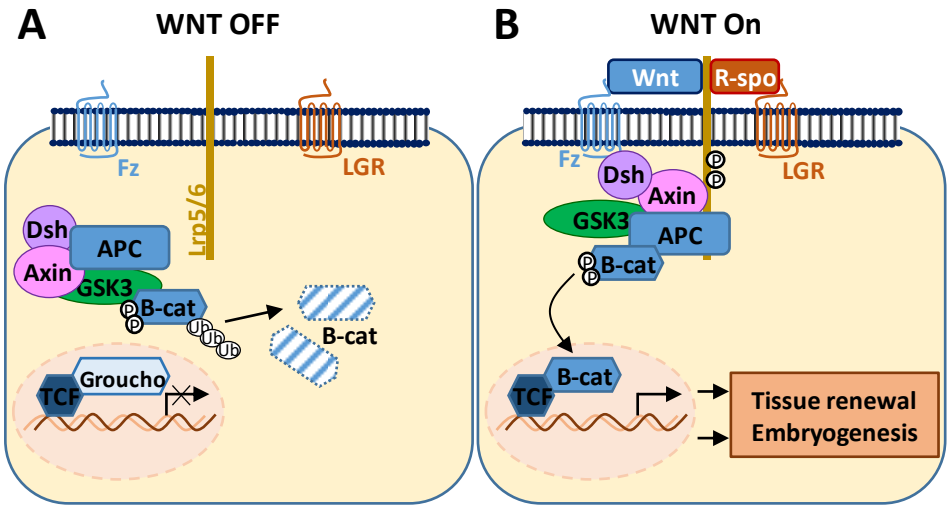


**Figure 1.6. The canonical WNT signalling transduction pathway.**

(**A**) In the absence of WNT, a destruction complex of Axin, APC and GSK3 phosphorylates β-catenin, which targets it for degradation by the proteasome. (**B**) In the presence of Wnt and R-spondin, Wnt ligand binds Frizzled and Lrp5/6 which induces phosphorylation. This inhibits GSK3 and binds actin, which inhibits formation of a destruction complex with β-catenin. This leads to an accumulation of β-catenin in the nucleus where it induces transcription factors Tcf/Lef, which have a number of roles in intestinal tissue renewal and embryogenesis.

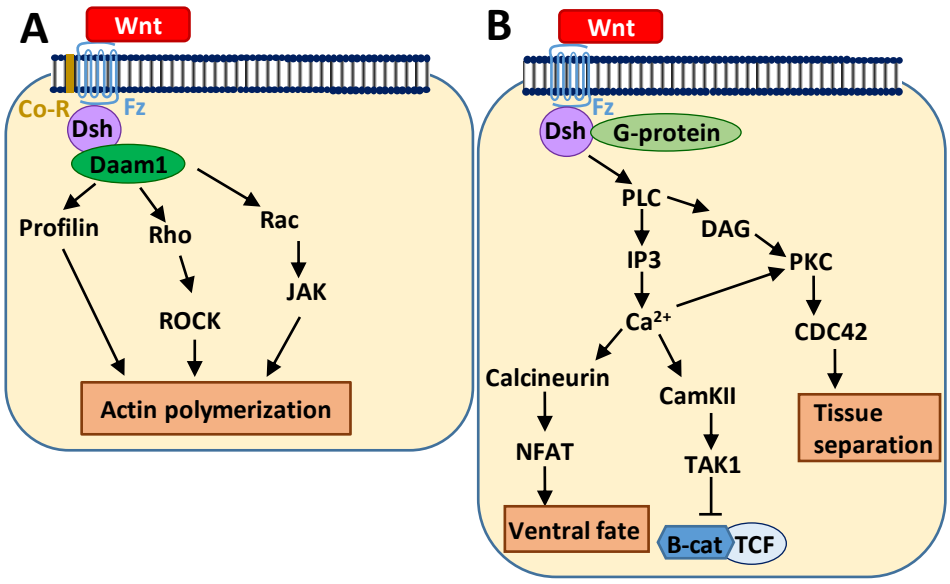


**Figure 1.7. The non-canonical WNT signalling transduction pathways.**

(**A**) The planar cell polarity pathway. WNT binds to Fr and a Co-receptor (gold) and recruits Dsh. Dsh forms a complex with Daam1, Daam1 activates Rho, which activates ROCK**.** Dsh also forms a complex with Rac1 which activates JNK. Profilin activation by Daam1, ROCK and JNK lead to actin polymerization. (**B**) The WNT/calcium pathway. WNT binds Fr and recruits Dsh to activate PLC. This leads to accumulation of IP3 and DAG which induces intracellular calcium release and PKC. $Ca^{2+}$ activates calcineurin and CamK11 which activate NFAT and inhibit β-catenin/TCF.

Recent studies suggest that WNT signals are modulated by cross talk with other signalling pathways including BMPs and TGFβ. Reduced WNT signalling impeded cell proliferation, but migration and shedding remained unaffected, resulting in diminished crypts, suggesting that WNT signalling alone is insufficient to maintain renewal of the epithelium. The combination of canonical WNT activation, with suppressed TGFβ/BMP signalling maintained steady state renewal of the human colonic epithelium (Reynolds et al., 2014). BMP signalling is a negative regulator of intestinal stem cell renewal through the suppression of WNT signalling (He et al., 2004). Activation of BMP signalling induces phosphorylation of SMAD proteins, which translocate to the nucleus and induce transcription of BMP target genes (Haramis et al., 2004). In contrast to the expression of WNT by the intestinal epithelium, BMP signals are increased towards the top regions of crypts (Reynolds et al., 2014), where they have a role in crypt cell positioning, differentiation and apoptosis. BMP signalling is crucial for the maintenance of intestinal crypt homeostasis by preventing excessive proliferation of intestinal stem cells and therefore reducing the risk of carcinogenesis (Vanuytsel et al., 2013). Noggin is a potent antagonist of BMP signalling, which is expressed by intestinal

myofibroblasts at the base of crypts and is required for organoid growth in culture (Haramis et al., 2004; Sato et al., 2009). This suggests that *in vivo*, BMP signalling inhibition is mediated by paracrine secretion of noggin by myofibroblasts.

WNT signalling is augmented by Prostaglandin-$E_2$ ($PGE_2$) (Goessling et al., 2009), a growth factor known to induce progression of colorectal cancer (Wang and Dubois, 2010). Tuft cells of the intestinal epithelium are an endogenous source of opioids including COX enzymes which synthesise $PGE_2$ (Gerbe et al., 2012). The roles for $PGE_2$ in the progression of colorectal cancer have been well characterised; however, $PGE_2$ is also known to induce crypt cell proliferation during homeostasis (Wang and Dubois, 2010). The expression of COX enzymes is also observed in myofibroblasts and immune cells residing in the underlying lamina propria of the gastrointestinal mucosa (Roulis et al., 2014). This suggests that the intestinal epithelium synthesises and responds to $PGE_2$ in an autocrine and paracrine manner (described in detail in **Section 1.4**).

Roles are emerging for immune cells residing in the underlying lamina propria of the intestinal mucosa in the modulation of crypt renewal (Pull et al., 2005; Seno et al., 2009; Kuhn et al., 2014; Skoczek et al., 2014). Skoczek et al., (2014) showed that luminal microbial addition to the air apical interface of colonic explants induces migration of monocytes and eosinophils towards to the stem cell niche. This immune cell migration was associated with an increase in proliferation and stem cell number in colonic crypts. The physical interaction and microbial sensing of the luminal content through Myeloid Differentiation primary response 88 (MyD88) signalling was shown to be required for monocyte-induced crypt cell proliferation and stem cell expansion (Skoczek et al., 2014). This suggests that immune cells are able to induce stem cell driven tissue renewal in a paracrine manner, although the mechanism through which monocytes are able to induce crypt renewal remains to be seen. The role of eosinophils in modulating human colonic crypt cell proliferation will be addressed in this thesis.

The residing immune cells in the intestinal lamina propria are known to secrete a variety of factors, including cytokines, which have a number of downstream functions in health and disease (Rosenberg et al., 2013; Hunter and Jones, 2015; Villarino et al., 2015). Epithelial cells also secrete a variety of cytokines and chemokines and express receptors for cytokines (reviewed by Peterson and Artis (2014)). Roles are emerging for paracrine and autocrine cytokine secretion in the regulation of intestinal tissue renewal from infiltrating inflammatory cells and from epithelial cells themselves. Of particular interest is Interleukin-

6 (described in detail in **Section 1.3**), which signals through the JAK/STAT signalling pathway and has been associated with crypt regeneration following tissue injury or damage in both the *Drosophila* and mammalian gut (Jiang et al., 2009; Kuhn et al., 2014).

In summary, the complex interplay between growth factors and signalling pathways is required for the finely tuned regulation of epithelial renewal (summarised in **Figure 1.8**) (reviewed in Vanuytsel et al. (2013)). Aberrant signalling through the pathways responsible for epithelial renewal, specifically WNT signalling, leads to the development of adenocarcinomas in the intestinal epithelium (Schepers and Clevers, 2012; Nusse and Clevers, 2017). Epithelial cells produce and respond to cytokines and growth factors in an autocrine manner, and immune cells residing in the lamina propria underlying the epithelium deliver signals to maintain epithelial renewal through paracrine signalling (**Figure 1.8**). This thesis aims to address the roles of IL-6, $PGE_2$ and eosinophils in epithelial renewal during homeostasis.

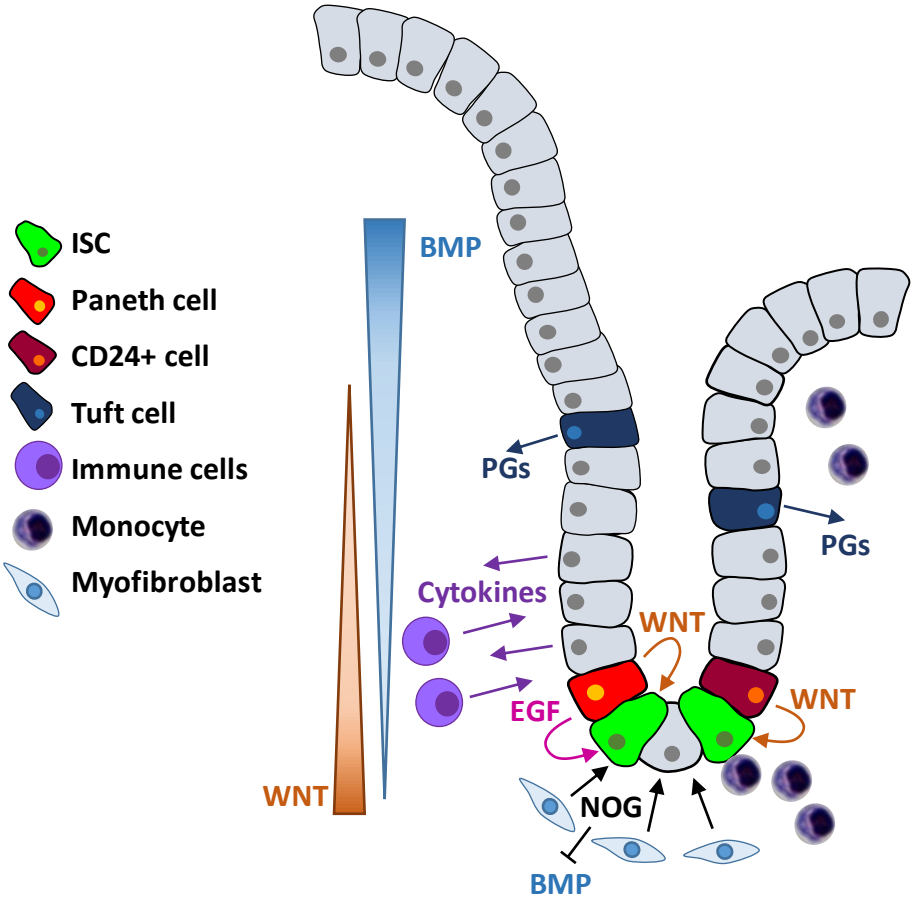


**Figure 1.8. Paracrine and autocrine regulation of intestinal tissue renewal.**

WNT signalling by Paneth cells (small intestine only) and CD24+ cells (colon) at the base of crypts induces ISC proliferation. BMP signalling is a negative regulator of epithelial renewal; however, secretion of Noggin by myofibroblasts antagonises BMP. Epithelial cells express and respond to EGF signalling in an autocrine manner to enhance cell proliferation. Tuft cells express the enzymes responsible for Prostaglandin (PG) secretion. Immune cells in the underlying lamina propria and epithelial cells secrete cytokines, which have pleiotropic roles in epithelial renewal. Monocytes enhance ISC expansion and proliferation through physical interactions with ISC. Adapted from (Vanuytsel et al., 2013).

## 1.2 The Intestinal Immune System

The largest number of immune cells of anywhere in the body are contained within the intestine, of which 20-40% are eosinophils (Carlens et al., 2009). The intestinal epithelium is continually exposed to dietary antigens and commensal microbiota and forms a vital barrier between the contents of the gut lumen and underlying immune cells. A plethora of immune cells reside in the lamina propria, which are able to mount an immune response in order to clear pathogens in the event of a breach of the epithelial barrier (Mowat and Agace, 2014).

Following bacterial penetration of the barrier, innate immune cells, including monocytes, macrophages, dendritic cells, neutrophils and eosinophils form the first line of defence, and directly or indirectly destroy invading pathogens. Cells of the innate immune system also secrete cytokines which leads to the recruitment of adaptive components (including T cells and IgA+ B cells), which are able to specifically recognize and remember bacterial antigens to enable a more efficient response in the event of repeat exposure to pathogens (**Figure 1.9**) (Mowat and Agace, 2014).

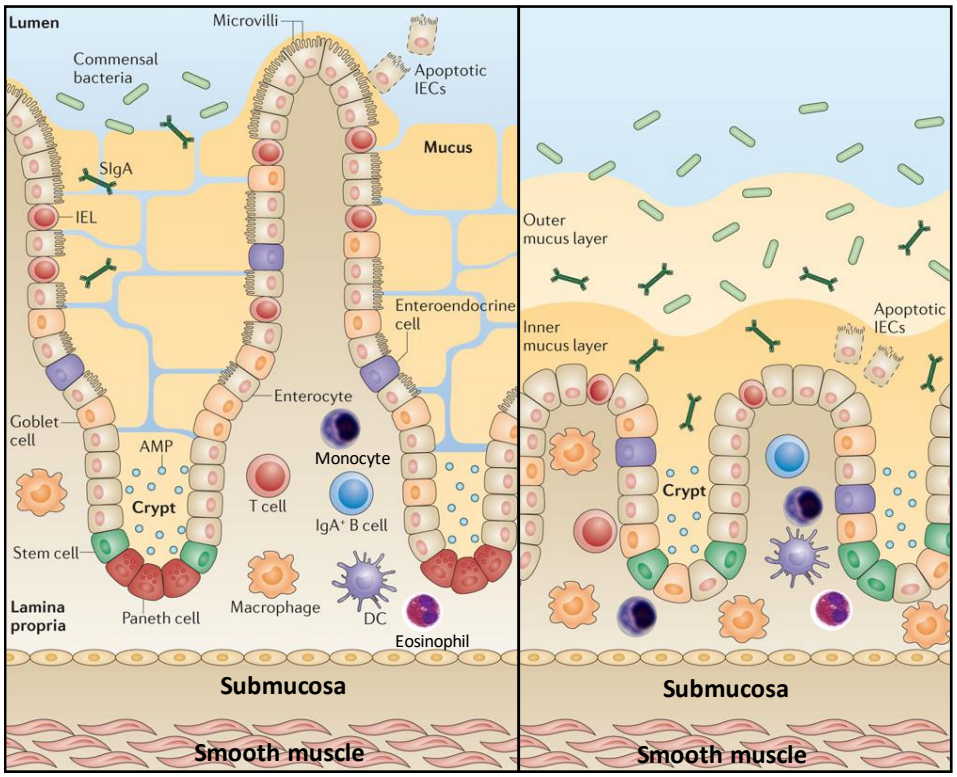


**Figure 1.9. The intestinal immune system.**

The small intestinal (LEFT) and colonic (RIGHT) epithelium form a vital barrier between microbial content of the gut lumen and immune cells in the underlying lamina propria. Protective mucus layers prevent commensal microbes from coming into contact with the epithelium. In the event of a breach of the epithelial barriers, immune cells in the underlying LP mount an immune response to destroy invading pathogens. Adapted from (Mowat and Agace, 2014).

### 1.2.1 Innate Immunity

The innate immune system is the first line of defence against microbial pathogens. The human innate immune system is comprised of immune cells, epithelial cells and mucosal barriers, which are able to produce an immediate initial response against invading pathogens (Mowat and Agace, 2014).

The cellular components of innate immunity are vital in the cross-activation of the adaptive immune system. The majority of these cells are of myeloid origin: monocytes, macrophages, mast cells, dendritic cells and granulocytes (neutrophils, eosinophils and basophils). However, some lymphoid cells such as natural killer cells, innate lymphoid cells and other lymphocytes are also involved in innate immunity.

Innate immune cells are derived from hematopoietic stem cell (HSC) which firstly differentiate into a common myeloid progenitor (CMP). CMPs then differentiate into either megakaryocyte and erythroid progenitor (MEP) or granulocyte and macrophage progenitor (GMP). MEPs terminally differentiate into erythrocytes or platelets. GMPs then differentiate further into a monocyte progenitor which gives rise to monocytes (can be further derived into dendritic cells or macrophages), or a granulocyte progenitor which gives rise to the granulocytes: neutrophils, eosinophils and basophils. Collectively, the process of differentiation of HSCs to all lineages of blood cells is called haematopoiesis (**Figure 1.10**) (Laiosa et al., 2006).

The major role for cell-mediated immunity is the phagocytosis of invading pathogens, which are then presented on the cell surface inducing inflammation and triggering the adaptive immune system. Following activation by microbial sensing, monocytes differentiate into macrophages or dendritic cells. Macrophages directly phagocytose pathogens; both macrophages and dendritic cells are then able to become Antigen Presenting Cells (APCs) through uptake of bacterial antigens and migrating to the lymph nodes in order to present surface antigen to naïve T and B cells, in order to mount an adaptive immune response. Neutrophils and eosinophils are potent effector cells; neutrophils engulf the pathogen and destroy it through the release of antimicrobial toxins, whereas eosinophils release cytoplasmic granule content upon activation, which is cytotoxic to pathogens.

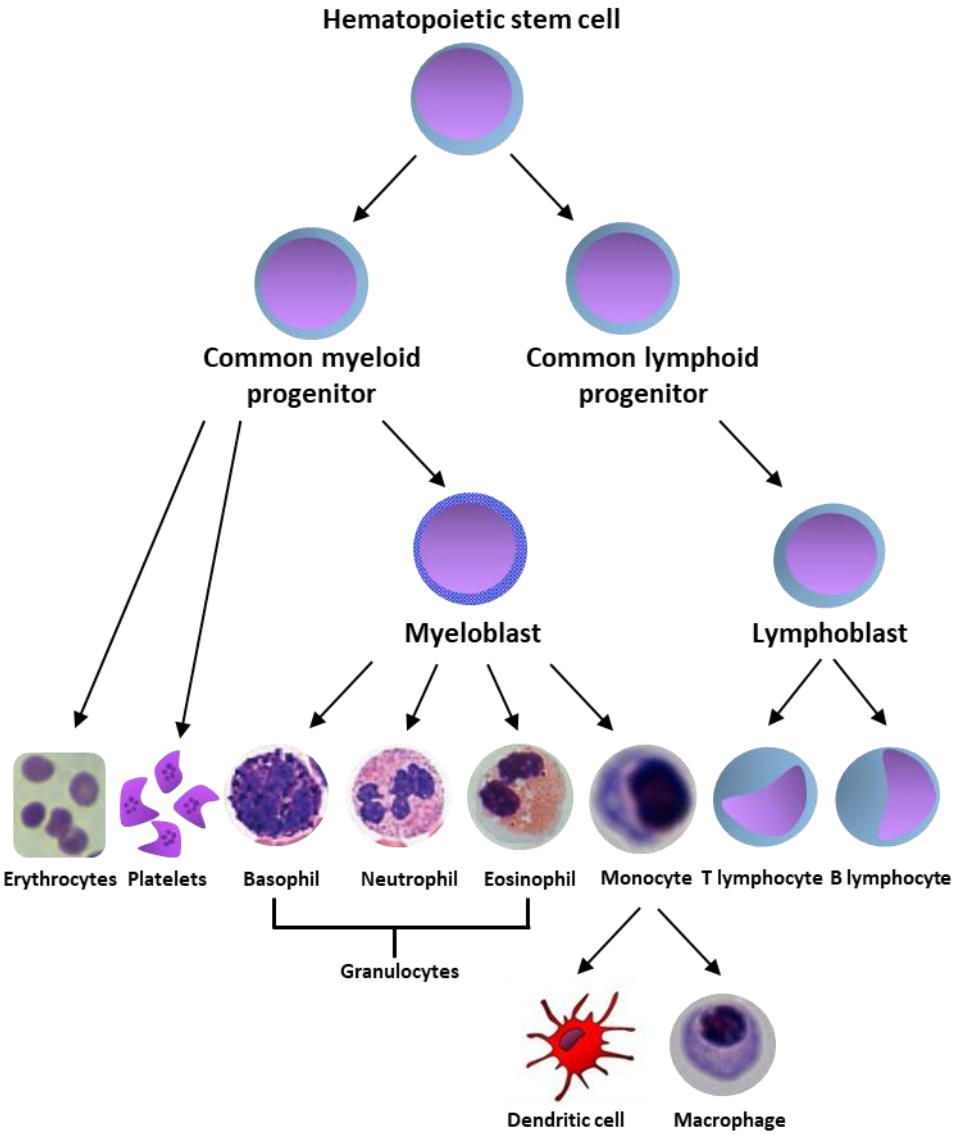


**Figure 1.10. Haematopoiesis.**

A multipotent stem cell gradually differentiates into mature blood cells: first into a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP); CLPs differentiate into the cells of the adaptive immune system; CMPs differentiate into either erythrocytes, platelets of myeloblasts. Myeloblasts then differentiate further into granulocytes, basophils, neutrophils or eosinophils – or monocytes, which further differentiate into dendritic cells or macrophages (adapted from OpenStax, Anatomy & Physiology)

In addition to the vital role of immune cells in pathogen clearance, evidence is beginning to emerge for a role of immune cells in the regulation of intestinal tissue renewal (Pull et al., 2005; Seno et al., 2009; Kuhn et al., 2014; Skoczek et al., 2014). Indeed, immune cells express a number of factors known to be important for epithelial renewal and the abundance of immune cells present in the lamina propria in the absence of pathogenic challenge suggests that they may play a role in epithelial renewal during homeostasis (Lee et al., 2010; Rosenberg et al., 2013). Of particular interest to this thesis are eosinophils, which have a number of functions not only in host defence against pathogens, but also in immune cell regulation and the maintenance of homeostasis, discussed in detail in **Section 1.5**.

### 1.2.2 Adaptive Immunity

The adaptive immune response is activated by APCs of the innate immune system, and characterised by its ability to recognize and remember specific pathogens and is constituted of two main cell types: T-lymphocytes and B-lymphocytes.

Once activated, T-lymphocytes undergo clonal expansion and differentiate into a number of subsets of T cells, which convey different functions. Helper T cells ($T_H$ cells) release cytokines to activate macrophages and induce differentiation of cytotoxic T cells and B cells (Garay and McAllister, 2010). $T_H$ cells can be further sub-divided into $T_{H1}$, $T_{H2}$ or $T_{H17}$ cells; $T_{H1}$ cells are important for clearance of intracellular pathogens, whereas $T_{H2}$ cells enhance the clearance of extracellular microorganisms (Diehl and Rincon, 2002). The more recently defined $T_{H17}$ cells have been implicated in the induction of autoimmune diseases through the secretion of IL-17 (Bettelli et al., 2006); however, they also play a role in bridging the innate and adaptive immune system through the activation of neutrophils through granulocyte-colony stimulating factor secretion (Stockinger and Veldhoen, 2007). Cytotoxic T cells ($T_C$ cells) form a synapse with infected cells to induce direct lysis (Marshall and Swain, 2011). The generation of memory T cells, which are preserved following resolution of inflammation, enables the immune system to mount a rapid, specific response when exposed to the same pathogenic antigen again (Mahnke et al., 2013). Regulatory T cells ($T_{reg}$ cells) are important to suppress inappropriate T cell responses and prevent autoimmune responses (Kleinewietfeld and Hafler, 2014).

Following activation, B-lymphocytes differentiate into plasma cells, which secrete antibodies specific to the invading antigen. The antibodies bind to target antigens to form immune complexes which are able to activate complement or target them for phagocytosis by macrophages. B cell activation is thought to be mainly induced by $T_H$ cells, however a role is

emerging for eosinophil-mediated generation of plasma cells in the bone marrow and intestinal lamina propria (Chu et al., 2011; Chu et al., 2014).

### 1.2.3 Induction of Cytokine Secretion

In order for an immune response to be elicited, it must be possible for mammalian cells to recognise pathogens at the mucosal surfaces. Pattern recognition receptors (PRRs) on the surface of immune cells allow mammalian cells to distinguish between harmless antigens and potentially pathogenic microbial components (Medzhitov and Janeway, 2000). These receptors are present on a variety of innate immune cells including macrophages, eosinophils, neutrophils and dendritic cells. An example of PRRs are TLRs, which enable mammalian cells to recognise PAMPs such as lipopolysaccharides which are present on the membrane of gram-negative bacteria, which are the dominant bacteria present in the colon (Raetz and Whitfield, 2002). TLRs can also induce an immune response by binding to a toll Interleukin-1 receptor domain. Activation of this domain stimulates an association with MyD88 which induces downstream activation of the Nuclear Factor κ-light-chain-enhancer of activated B cells (NFκB) signalling pathway (Baumgart and Carding, 2007), which plays a major role in the regulation of cytokine secretion.

In response to antigen challenge, cytokines are produced by immune cells and epithelial cells. Cytokines have immunomodulatory functions and chemoattractant properties: activation of cytokines induces recruitment of immune cells of the innate and adaptive immune system (Jung et al., 1995; Maaser and Kagnoff, 2002; Abreu et al., 2003). Two cytokines which are known to induce inflammation in the intestinal epithelium are IL-6 and $PGE_2$. In the next sections, IL-6 and $PGE_2$ signalling transduction pathways and emerging roles for IL-6 and $PGE_2$ in intestinal homeostasis will be discussed.

## 1.3 Interleukin-6

Interleukin-6 is a pleiotropic cytokine involved in a plethora of cellular and immune responses in health and disease (Mihara et al., 2012; Garbers et al., 2015; Hunter and Jones, 2015). As well as playing a pivotal role in the activation of immune cells, IL-6 is known to play a role in the regulation of metabolism, the maintenance of bone homeostasis and in pathological pain responses (Scheller et al., 2011). Furthermore, recent studies have implicated IL-6 in epithelial regenerative responses following injury. Seminal work in the *Drosophila* midgut demonstrated that following acute stress such as enteric infection,

enterocytes are stimulated to produce Upd3, an IL-6-like cytokine, which activates JAK/STAT signalling in intestinal stem cells to promote cell division and therefore regeneration (Jiang et al., 2009; Osman et al., 2012). In the mammalian gut, STAT3 and IL-6 are known to play a role in regeneration of the intestinal epithelium following colonic tissue injury (Pickert et al., 2009; Kuhn et al., 2014) and to promote the survival of epithelial cells (Grivennikov et al., 2009; Jin et al., 2010) during inflammation and IBD (Bollrath et al., 2009; Lee et al., 2012). Whilst a role for IL-6 in health is beginning to emerge, little is known about the influence of IL-6 in the maintenance of intestinal homeostasis.

### 1.3.1 IL-6 and Homeostasis

A role for IL-6 in the survival of normal epithelial cells has recently been described. IL-6 secreted by lamina propria myeloid cells was shown to promote the survival of pre-malignant colonic epithelial cells. This was shown to be mediated by STAT3 activation (Grivennikov et al., 2009). Following addition of IL-6, pSTAT3 activation was observed in enterocytes of villi but not in crypts. An increase in the induction of anti-apoptotic factors and decreased caspase activity was observed, resulting in a prolonged life span of enterocytes. When these mice were subsequently challenged with ischemic reperfusion, IL-6 treated mice exhibited increased barrier function and reduced small intestinal injury (Jin et al., 2010). These data suggest that IL-6 is able to promote the survival of normal tissue during homeostasis and in small intestinal and colonic tissue injury.

IL-6 also has important roles in the regulation of the immune system, which is required to maintain intestinal homeostasis; a pivotal role for IL-6 in the switch from innate to adaptive immunity has been described (Scheller et al., 2011). Acute inflammation is characterised by a rapid infiltration of neutrophils, which are replaced by monocytes and T cells in order to prevent tissue damage from secreted products of neutrophils. Secretion of chemokines (including IL-6) leads to the recruitment of neutrophils, proteolytic cleavage of the IL-6 receptor then drives IL-6 trans-signalling in resident tissue cells which induces switching from neutrophil recruitment to monocyte recruitment (Kaplanski et al., 2003). Monocytes differentiate into either macrophages or dendritic cells, upregulation of Macrophage-Colony Stimulating Factor (M-CSF) expression by IL-6 trans-signalling has been shown to skew differentiation of monocytes towards the macrophage lineage (Chomarat et al., 2000). The resolution of inflammation following pathogenic challenge in the intestine is vital for the maintenance of the epithelial barrier. The apoptosis of neutrophils following infiltration is key for the resolution of inflammation, and this has been shown to be driven by IL-6

(Kaplanski et al., 2003). IL-6 also has a critical role in the differentiation of B cells, which are integral for intestinal immunity. In fact, IL-6 was first characterised for its role in enhancing antibody production of a B cell line (Hirano et al., 1985). The promotion of antibody production by B cells was shown to be dependent on IL-6-induced IL-21 production of T cells (Dienz et al., 2009; Eddahri et al., 2009). Recent studies have defined a role for eosinophil-mediated IL-6 secretion in the maintenance of plasma cells in the bone marrow and lamina propria, which is crucial for the maintenance of long term immunity (Chu et al., 2011; Chu et al., 2014). However, the role of IL-6 in epithelial cell renewal during homeostasis remains largely unknown.

IL-6 can signal through classic or trans signalling, the two signalling events having divergent functions (Scheller et al., 2011). The roles for IL-6 in homeostasis are thought to be associated with IL-6 classic signalling, whilst IL-6 trans signalling is thought to promote inflammation and cancer growth (summarised in **Figure 1.11**). The next section will discuss the IL-6 classic and trans signalling transduction pathways.

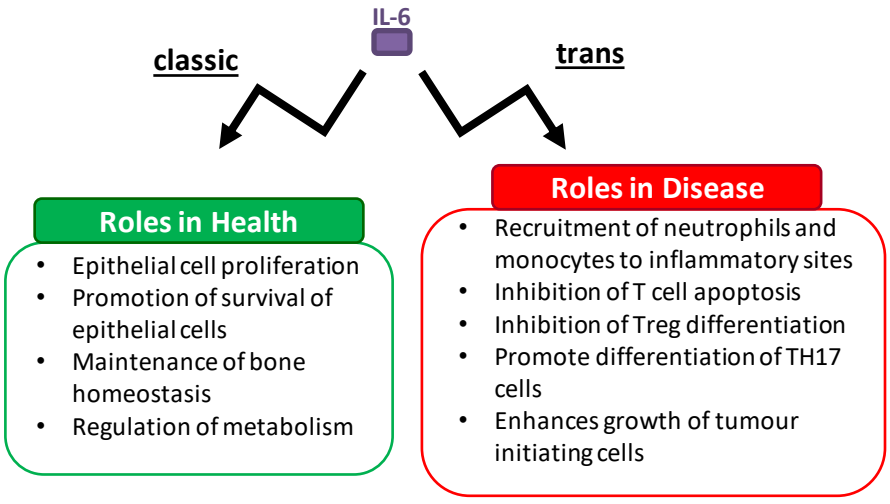


**Figure 1.11. The roles of IL-6 signalling in health and disease.**

IL-6 signalling is mediated by classic or trans signalling, which have divergent functions in health and disease. Classic IL-6 signalling is associated with epithelial proliferation and survival and the maintenance of bone and metabolic homeostasis. IL-6 trans signalling has a number of roles in inflammation, including recruitment of immune cells to inflammatory sites, where IL-6 promotes survival and differentiation of T cells. IL-6 trans-signalling has also been associated with tumorigenesis. Adapted from (Scheller et al., 2011).

### 1.3.2 The IL-6 Signalling Transduction Pathway

The signal transduction of IL-6 is induced through binding of IL-6, complexed to the IL-6 receptor (IL-6R), to the β-receptor Glycoprotein 130 (gp130) which is expressed ubiquitously on all cells throughout the human body (Garbers et al., 2015). Classical IL-6 signalling is traditionally associated with homeostasis (Scheller et al., 2011) and occurs through binding of IL-6 to the membrane-bound IL-6R, which then complexes to the ubiquitously expressed gp130 receptor. This results in activation of a number of downstream signalling cascades including the JAK/STAT pathway. Jak1 phosphorylates tyrosine residues of the gp130 receptor, which leads to recruitment and phosphorylation of STAT3 (Garbers et al., 2015) (**Figure 1.12**). Several mouse models have identified a role for IL-6 classic signalling in the activation of anti-inflammatory pathways on target cells, including apoptosis of neutrophils for the resolution of inflammation (Kaplanski et al., 2003). Furthermore, IL-6 classic signalling has been shown to be essential for the regeneration of intestinal epithelial cells following Dextran Sodium Sulfate (DSS)-induced tissue injury (Kuhn et al., 2014).

The pro-inflammatory effects of IL-6 signalling are thought to be mediated through IL-6 trans-signalling (Rose-John, 2012). In IL-6 trans-signalling, metalloproteinases termed A Disintegrin and metalloproteinase domain-containing protein (ADAM) 10 (Matthews et al., 2003) and/or ADAM17 (Chalaris et al., 2010) cleaves the IL-6R from the membrane, resulting in soluble IL-6R (sIL-6R) which is able to bind directly to IL-6. The IL-6/sIL-6R complex can then activate IL-6 signalling in any cell expressing the gp130 protein, which is constitutively expressed by the intestinal epithelium (**Figure 1.12**). The trans-signalling pathway has been associated with inflammatory disorders including Crohn's disease and rheumatoid arthritis, and cancer (Jones et al., 2005; Scheller et al., 2011). Genetic overexpression of the soluble form of the gp130 protein (sgp130Fc), which binds IL-6/sIL-6R complex and therefore inhibits IL-6 trans-signalling has been shown to prevent inflammation (Rabe et al., 2008), without blocking IL-6 classical signalling through the membrane bound IL-6R (Jostock et al., 2001).

Conflicting roles for IL-6 in the regulation of metabolic homeostasis have been described; IL-6 is secreted by muscle cells in the post-exercise period when insulin action is enhanced (Pedersen and Febbraio, 2008), but IL-6 has been associated with obesity and reduced insulin action (Skurk et al., 2007). However, although one study demonstrated induction of obesity in IL-6 Knockout (KO) mice (Wallenius et al., 2002), this has not been reproduced by other studies (Di Gregorio et al., 2004). Detailed analysis of wild type and IL-6KO mice has since

shown that a lack of IL-6 leads to insulin resistance and glucose intolerance as well as liver inflammation (Matthews et al., 2010). Interestingly, mice with a hepatocyte IL-6R deletion alone exhibited increased glucose tolerance and sensitivity to insulin with no marked effects on liver inflammation (Wunderlich et al., 2010). Activation of IL-6 trans-signalling in mice overexpressing IL-6/sIL6R lead to a significant reduction in body mass and body fat, accompanied by an induction of proliferation and differentiation of inflammatory cells in the liver (Peters et al., 1997). These studies suggest that IL-6 classical-signalling plays a role in the control of glucose tolerance, and IL-6 trans-signalling is involved in hepatic inflammatory responses.

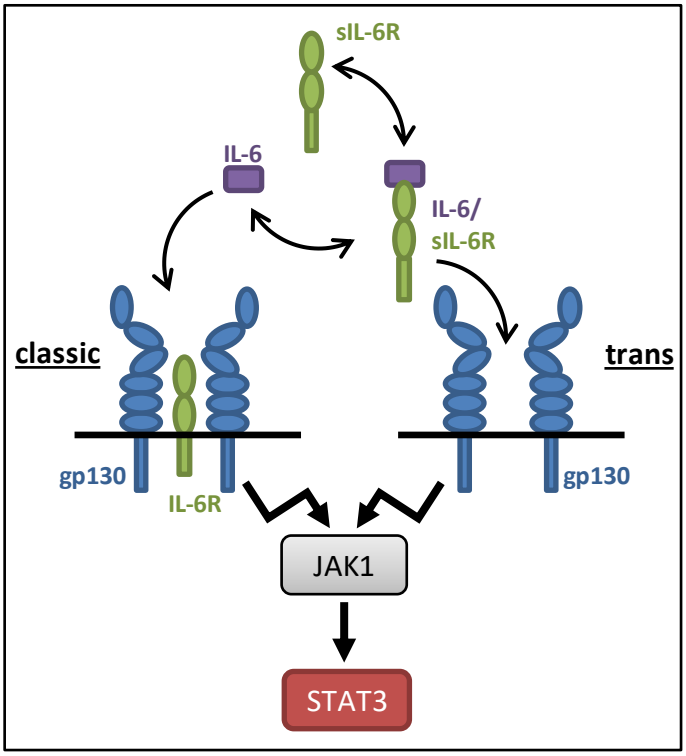


**Figure 1.12. Classical and trans IL-6 signalling.**

Cells that express gp130 and the IL-6R can respond to IL-6 (classic signalling). Cells which express gp130 can still respond to IL-6 by trans-signalling; sIL-6R binds to IL-6 and activates signalling in any cell expressing gp130. Figure adapted from (Scheller et al., 2011, Garbers et al., 2015).

### 1.3.3 IL-6 in Inflammation and Cancer

IL-6 signalling plays a major role in the regulation of inflammation. Classical IL-6 signalling, through activation of the IL-6R on naïve T cells has been shown to enhance the differentiation of naïve T cells into $T_{H17}$ cells, whilst also inhibiting the formation of $T_{reg}$ cells, which protect tissues from autoimmune injury (Bettelli et al., 2006). Interestingly, the IL-6R is proteolytically cleaved from the membrane of $T_{H17}$ cells upon activation, suggesting that IL-6 trans-signalling promotes the maintenance of $T_{H17}$ cells, which induces downstream tissue injury (**Figure 1.13**) (Jones et al., 2010). Cleavage of the IL-6R from other IL-6R expressing immune cells such as neutrophils and eosinophils may also promote IL-6 trans signalling. Neutrophil infiltration is a key process in inflammation, and the resolution of inflammation is dependent on neutrophil apoptosis. Induction of apoptotic pathways in neutrophils was previously shown to induce IL-6R cleavage from the membrane of neutrophils, which drives IL-6 trans signalling in neighbouring cells, and promotes the recruitment of phagocytic mononuclear cells which are involved in the removal of apoptotic neutrophils. In this study, early apoptosis of pre B cells (transfected with gp130 and the IL-6R) also induced cleavage of the IL-6R from the membrane (Chalaris et al., 2007). This suggests that apoptosis is a natural inducer of IL-6R cleavage from other immune cells which subsequently may promote IL-6 trans signalling in inflammation.

Following bowel injury induced by biopsy or bacterial triggered colitis, inhibition of IL-6 decreased epithelial proliferation and resulted in impaired wound healing in mice (Kuhn et al., 2014). Use of an IL-6-/- mouse demonstrated that whilst there was no change in the basal rate of proliferation in the intestine of IL-6KO mice, a significant decrease in proliferation was observed following DSS exposure compared to wild type, suggesting that IL-6 is required for proliferation following induction of inflammation. IL-6 was also shown to promote tumour growth by enhancing proliferation of tumour initiating cells (Grivennikov et al., 2009; Jin et al., 2010).

The roles of IL-6 in tumorigenesis is widely accepted in a number of cancers including lung, melanoma, breast and colorectal (reviewed in Wang and Sun (2014)). IL-6 expression by tumour cells and in serum is indicative of increased tumour stage, size, metastasis and reduced overall survival of patients with colorectal cancer (Knupfer and Preiss, 2010; Hara et al., 2017). These effects were observed in patients with inflammatory conditions such as IBD, who are significantly predisposed to develop colorectal cancer, and also in sporadic colorectal cancer mediated through IL-6 trans-signalling (Yamamoto and Rose-John, 2012).

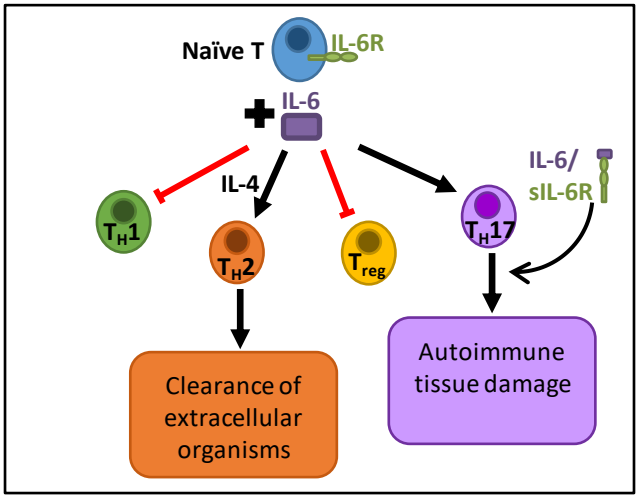


**Figure 1.13. The roles of IL-6 in T cell differentiation.**

IL-6 inhibits $T_H1$ and $T_{reg}$ cell differentiation (red arrows). Classical IL-6 signalling promotes IL-4-induced $T_H2$ cell differentiation to enhance clearance of extracellular organisms. $T_H17$ cell differentiation is promoted by IL-6 classical signalling, membrane bound IL-6R is cleaved from $T_H17$ cells and trans-IL-6 signalling promotes maintenance of $T_H17$ cells and downstream tissue damage.

### 1.3.4 STAT3 Signalling in Health and Disease

The STAT pathway was originally described in the context of downstream IL-6 signalling. Activation of the gp130 by the IL-6/IL-6R signalling complex induces activation of a number of downstream signalling cascades including the JAK/STAT pathway. However, it has since emerged that STAT signalling can be activated by a number of cytokines as well as G Protein-coupled receptors (GPCRs), TLRs and miRNAs; tight regulation of which is crucial for many cancers. STAT3 signalling, and downstream gene expression is known to modulate tumour cell proliferation, survival, invasion and inflammation (reviewed in Yu et al. (2014)). STAT3 is able to directly activate transcription factors such as c-Myc, CyclinD1, CyclinB1 and Cell division cycle protein 2 homolog (Cdc2), which promote cell proliferation (Bromberg et al., 1999; Bowman et al., 2001; Masuda et al., 2002). Alongside activation of proliferative genes, STAT3 also regulates transcription of anti-apoptotic genes Bcl-$X_L$, Bcl-2, Bcl-w and Mcl-1 and activates the cell survival marker Survivin (Zushi et al., 1998; Bromberg et al., 1999; Stephanou et al., 2000). In addition, the induction of vascular endothelial growth factor (VEGF) expression by STAT3 induces angiogenesis, and invasion of tumour cells is mediated

by expression of tissue-degrading metalloproteinases MMP-2 and MMP-9 (Jarnicki et al., 2010).

A number of cytokines are able to activate the STAT3 pathway, including IL-6, IL-10/IL-22 and IL-17/IL-23 families (Ernst et al., 2014). As described in the previous sections, IL-6 activates downstream STAT3 to induce a number of functions in health and disease. Another member of the IL-6 family, IL-11 has been shown to be more potent than IL-6 in the context of promoting gastric and colon cancer progression. Pharmacological inhibition of IL-11 reduced STAT3 activation and suppressed tumour growth (Putoczki et al., 2013). STAT3 activation is observed following DSS-induced experimental colitis in mice, and this was shown to be dependent on IL-22, rather than IL-6 (Pickert et al., 2009). In addition, IL-22 induced pSTAT3 activation in intestinal stem cells, which was required for organoid formation and epithelial regeneration (Lindemans et al., 2015).

STAT3 activation induced by IL-6 also has defined roles as a regulator of $T_{H17}$ cell differentiation and function, which was dependent on IL-17. IL-17 is the major cytokine produced by $T_{H17}$ cells, and they are most abundant in the small intestinal lamina propria (Ivanov et al., 2006), where they respond to pathogen invasion (Ivanov et al., 2009). Complex interplay between IL-17 and IL-6 contributes to the development of arthritis and autoimmune disease. IL-6-induced STAT3 activation drives T cell development to $T_{H17}$ lymphocytes. $T_{H17}$ cells secrete IL-17, which stimulates stromal cells to produce IL-6 and induce further inflammation (reviewed in Camporeale and Poli (2012)). Furthermore, STAT3 is also known to regulate other T cell subtypes, including Natural Killer T (NKT) cells and mucosal-associated invariant T cells, which are important for pathogen clearance through activation of the IL-23R and IL-21R (Wilson et al., 2015).

In the intestinal epithelium, STAT3 is required to maintain mucosal barrier integrity. The secretion of antimicrobial proteins RegIII-β, RegIII-γ and β-defensins by Paneth cells is stimulated by STAT3 (Wolk et al., 2004). Mucus is a major component of the epithelial barrier and prevents luminal microbes from coming into direct contact with the epithelium, therefore preventing inappropriate immune responses (Hansson and Johansson, 2010). STAT3 has been shown to directly induce expression of the mucin genes including Muc-1, which is expressed by intestinal epithelial cells (Li et al., 2009). The roles of epithelial STAT3 during steady state epithelial renewal remain to be elucidated.

Another inducer of JAK/STAT signalling during cancer is $PGE_2$, which indirectly activates the JAK/STAT pathway through cross-activation of the EGF signalling pathway (Oshima et al., 2011). Berberine, an alkaloid used in traditional Chinese medicine, has been shown to have anti-tumour efficacy in the treatment of a number of cancers including colorectal cancer. The mechanism of action for Berberine was shown to be through inhibition of COX-2/$PGE_2$ signalling, and downstream JAK2/STAT3 activation (Liu et al., 2015).

The convergence of a number of cytokines on the STAT3 signalling pathway (IL-11, IL-22, IL-17) highlights the need for investigation into the effects of IL-6-mediated STAT3 activation in intestinal tissue renewal. A number of unanswered questions remain in relation to the roles of IL-6/STAT3 signalling in intestinal homeostasis: the epithelial and non-epithelial sources of IL-6, characterisation of IL-6 receptor expression on epithelial cell types, cellular localisation of pSTAT3 activation in response to IL-6 signalling, and the downstream effects in intestinal renewal and stem cell expansion. This thesis aims to address these questions.

## 1.4 Prostaglandin-$E_2$

The focus of this thesis is the regulation of epithelial renewal by autocrine and paracrine signalling. Another cytokine that is known to induce epithelial renewal during inflammation and cancer is Prostaglandin-$E_2$, however $PGE_2$ also has emerging roles in induction of proliferation during health (Wang and Dubois, 2010). Recent studies have shown that epithelial production of $PGE_2$ through COX-2 activation is required for epithelial regeneration in response to injury, which is vital for the maintenance of intestinal homeostasis (Chen et al., 2015). High levels of $PGE_2$ have been associated with exacerbation of the inflammatory response, promotion of tumour growth *via* stimulation of angiogenesis, cell invasion, cell growth and inhibition of apoptosis (Wang and Dubois 2006).

COX-1 and COX-2 enzymes are responsible for the synthesis of endogenous $PGE_2$, which are upregulated in colorectal cancer and associated with unfavourable survival (Wang and Dubois 2010). Constitutive activation of COX-1 is observed throughout the epithelium in tuft cells (Gerbe et al., 2011), but COX-2 has been shown to be activated when the tissue is under stress conditions such as in DNA damage (Wang and Dubois 2010). Inhibition of COX enzymes utilising NSAIDs has previously been targeted for the prevention of colorectal cancer initiation and development. Regular use of NSAIDs for 10-15 years has been shown to reduce the risk of developing colorectal cancer by 40-50% (Flossmann et al., 2007; Rostom et al., 2007; Chan et al., 2008), particularly when tumours express high levels of COX-2 (Chan et al.,

2007). In addition, the use of NSAIDs in patients with colorectal at stage I, II and III improves overall survival (Chan et al., 2009). These findings prompted initial clinical trials for the prevention of adenoma formation in patients with FAP where treatment with NSAIDs lead to regression of pre-existing adenomas (Rostom et al., 2007), and the prevention of recurrence of adenoma formation (Bresalier et al., 2005; Solomon et al., 2005; Arber et al., 2006). However, this was associated with a significant increase in the risk of cardiovascular thromboembolic events. It is possible that side effects following NSAID use could be due to inhibition of $PGE_2$ signalling required for normal tissue renewal and stem cell maintenance, therefore targeting of specific downstream $PGE_2$ signalling components could potentially lead to the development of anti-cancer therapies with reduced side effects.

The identification of a novel cell type within the epithelium which expresses COX enzymes (Gerbe et al., 2011), and previous studies demonstrating the presence of $PGE_2$ receptors E-Prostanoid (EP) receptors 1-4 mRNA in the intestinal epithelium (Shoji et al., 2004; Sugimoto and Narumiya, 2007), suggests that the epithelium may be able to produce and respond to $PGE_2$ signalling in an autocrine manner. The importance of autocrine $PGE_2$ signalling in stem cell maintenance has previously been described in other systems. Self-renewal of mesenchymal stem cells (MSCs) derived from cord blood or adipose tissue was shown to be dependent on autocrine $PGE_2$ signalling through activation of the EP2 receptor on the cell membrane of MSCs (Lee et al., 2016). In non-small cell lung cancer, autocrine (and paracrine) $PGE_2$ signalling through the EP4 receptor was shown to mediate MMP-2/CD44-dependent invasion (Dohadwala et al., 2002). This suggests that the $PGE_2$ signalling-axis forms an autocrine loop and activates differential EP receptor subtypes in order to induce different functions in homeostasis and disease. Whilst the roles of differential EP receptor activation/inhibition in tumorigenesis is becoming better defined, (described in detail below) the role of EP receptor activation/inhibition in normal tissue renewal remains largely unknown.

### 1.4.1 Prostaglandin-$E_2$ Synthesis

$PGE_2$ is synthesised *via* the arachidonic acid pathway (**Figure 1.14**). Arachidonic acid is released from cellular membranes through Phospholipase A2. The enzymes COX-1 and COX-2 oxidize arachidonic acid to prostaglandin-$G_2$, which is rapidly converted to prostaglandin-$H_2$ ($PGH_2$). $PGH_2$ is then converted to $PGE_2$ through the action of Prostaglandin E Synthase (PGES) (Smith et al., 2000). $PGE_2$ can then be degraded by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) which oxidises the 15-hydroxyl group on prostanoids to a ketone,

and therefore abrogating binding of ligands to the prostaglandin receptors (Tai et al., 2002; Myung et al., 2006). The healthy colonic mucosa expresses high levels of 15-PGDH, however 15-PGDH is lost in colorectal cancers and FAP. Recent studies suggest that inhibition of 15-PGDH accelerates hematopoietic recovery in mice following bone marrow transplant, and promotes tissue regeneration in mouse models of colitis and liver injury (Zhang et al., 2015).

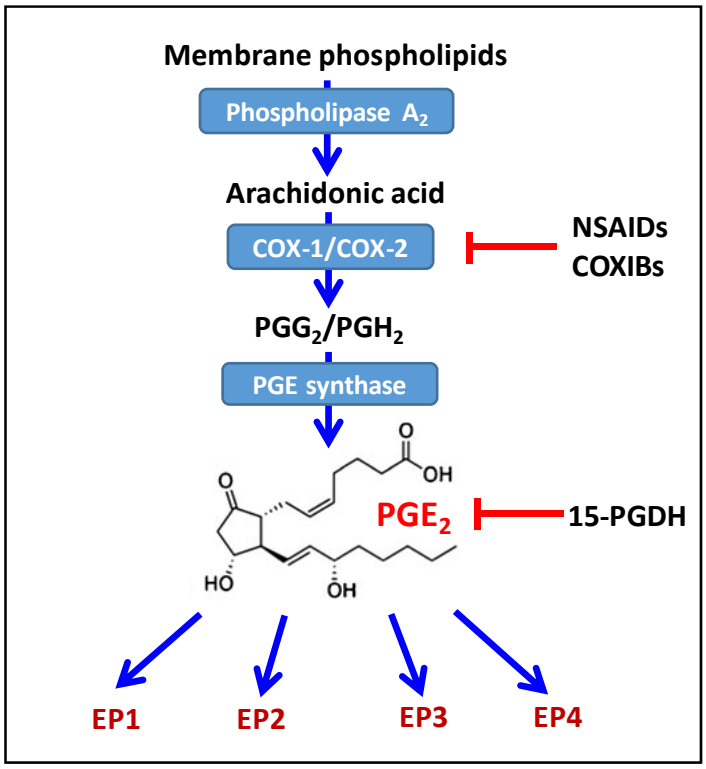


**Figure 1.14. Synthesis of PGE2.**

Following release from cell membrane through phospholipase $A_2$, arachidonic acid is converted to $PGG_2$ and rapidly $PGH_2$ through the action of Cyclooxygenase enzymes Cox-1 and Cox-2. $PGH_2$ is then converted to $PGE_2$, which is degraded by 15-PGDH. $PGE_2$ signals through four GPCRs, EP1, EP2, EP3 and EP4.

$PGE_2$ mediates actions by binding to one of four G protein-coupled receptors EP1-4. The distinct signalling transduction pathways of the EP receptor subtypes allows for the diverse functionality of $PGE_2$ activation (**Figure 1.15**). The EP1 receptor is coupled to $G_{\alpha q}$ proteins, which mediate signalling through activation of PLC. This leads to the accumulation of intermediates including IP3 and DAG, which induce an increase in intracellular $Ca^{2+}$, activating PKC. This induces the expression of NFAT, NFκB and Mitogen-Activated Protein Kinase (MAPK) (Sugimoto and Narumiya, 2007). Intracellular calcium signals are known to regulate a number of cellular processes, including proliferation and apoptosis in all mammalian cell types (Roderick and Cook, 2008). EP2 and EP4 receptors are coupled to adenylate cyclase stimulation *via* $G_s$. This increases intracellular cyclic AMP and activates Protein Kinase A (PKA). EP2 and EP4 can also activate Phosphoinositide 3-kinase (PI3K) through the β-arrestin pathway, and EP2 activates the GSK3/β-catenin pathway, which increases expression of many cancer- associated genes (Fujino et al., 2002). The EP3 receptor is coupled to $G_i$ and $G_{\alpha s}$; activation of $G_i$ leads to adenylate cyclase inhibition, whereas binding of $G_s$ activates adenylate cyclase signalling and cyclic AMP production (Namba et al., 1993).

The generation of mouse models deficient in each EP receptor subtype, and the development of EP receptor selective agonists and antagonists has allowed for the physiological role of each EP receptor subtype to begin to be investigated.

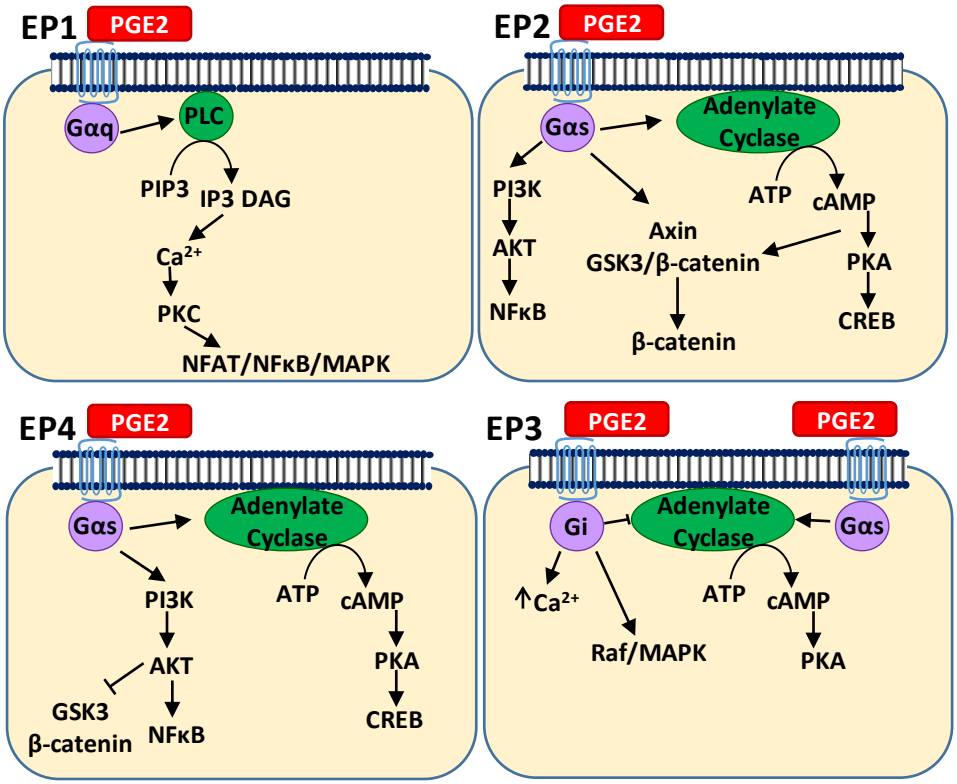


**Figure 1.15. The distinct signalling transduction pathways of EP receptor subtypes.**

(**A**) EP1 receptor is coupled to $G_{\alpha q}$ proteins, and activate PLC. This leads to accumulation of IP3 and DAG to increase intracellular calcium, activating PKC and inducing expression of NFAT, NFκB and MAPK. (**B**) EP2 is coupled to $G_{\alpha s}$ protein, and induces adenylate cyclase activation to increase intracellular cAMP, which in turn activates PKA and CREB. EP2 can also activate PI3K, and the GS3K/β-catenin pathway. (**C**) EP4 is coupled to $G_{\alpha s}$ and adenylate cyclase activation, and also activates the PI3K pathway to induce AKT and NFκB expression. (**D**) The EP3 receptor can bind either $G_i$ or $G_{\alpha s}$ proteins. $G_i$ inhibits adenylate cyclase and activates Raf/MAPK and calcium signalling. Binding of $G_{\alpha s}$ activates adenylate cyclase, to induce cAMP and PKA expression. Adapted from (O'Callaghan and Houston 2015).

#### 1.4.1.1 EP1 Receptor

EP1 receptor (mRNA) expression has previously been shown to be restricted to the lung, stomach and kidney (Watabe et al., 1993), where activation of EP1 receptor has a role in mediating stress responses (Matsuoka et al., 2003; Matsuoka et al., 2005) and promoting chemical carcinogenesis (Mutoh et al., 2002). However, more recent studies observed EP1 receptor expression at the mRNA level in the colonic mucosa (Shoji et al., 2004; Olsen Hult et al., 2011). Selective pharmacological inhibition of the EP1 receptor in APC1309 mice (mice deficient in APC) reduced polyp formation (Kitamura et al., 2003). Furthermore, a systemic genetic deletion of the EP1 receptor in mice inhibits colon tumour growth induced by

treatment with azoxymethane and induces apoptosis of tumour cells (Kawamori et al., 2005). EP1 receptor signalling is also thought to promote carcinogenesis through activation of signalling pathways which mediate migration and invasion, which is key for tumour metastasis (Yang et al., 2010; Kim et al., 2011; Zhang et al., 2014). EP1-mediated $PGE_2$ signalling induces focal adhesion kinase (FAK) expression in cancer cells, which activates pathways key for regulation of cell migration and proliferation, namely PKC/c-Src and EGFR (Bai et al., 2013). Signalling through the EP1 receptor is also thought to play a role in the suppression of the immune system, which is associated with tumour progression. $PGE_2$ is secreted by tumour cells in response to Fas binding to Fas ligand, constitutive expression of which is key for optimal tumour growth. This is associated with the infiltration of tumours by potent immunosuppressive immune cells, Myeloid-Derived Suppressor Cells (MDSCs) which help tumours to overcome the immune response of the host (Zhang et al., 2009). Furthermore, EP1-receptor mediated $PGE_2$ signalling has also been associated with decreased barrier function of Caco-2 cells, which is a key characteristic of IBD (Rodriguez-Lagunas et al., 2010).

#### 1.4.1.2 EP2 Receptor

The EP2 receptor subtype is the least abundant EP receptor mRNA detected in mouse tissue (Katsuyama et al., 1995), but expression is highly inducible following stimulation with lipopolysaccharide (LPS) (Ikegami et al., 2001). EP2 is most highly expressed in the uterus of mice, where it plays a role in ovulation and fertilisation (Hizaki et al., 1999). However, EP2 has also been shown to be expressed by the colonic epithelium (Shoji et al., 2004; Olsen Hult et al., 2011), and mediate $PGE_2$ signalling in APC (Delta716) mice, where an upregulation of COX-2 enzyme induces intestinal polyposis (Sonoshita et al., 2001). The EP2 receptor is also highly expressed by tumour cells in colon cancer (Gustafsson et al., 2007). The role of EP2-mediated $PGE_2$ signalling in cancer is thought to be associated with tumour angiogenesis; EP2 receptor activation induces expression of the pro-angiogenic factor VEGF (Sales et al., 2004; Chang et al., 2005), as well as regulating endothelial cell motility and survival (Kamiyama et al., 2006). The increase in cyclic AMP associated with EP2 (and EP4) receptor activation has also been shown to play a major role in inhibition of the anti-tumour immune response (Kalinski, 2012), by inhibition of T helper cells (Harris et al., 2002) and promoting development of T regulatory cells which suppress the immune response (Sharma et al., 2005).

#### 1.4.1.3 EP3 Receptor

Expression of the EP3 receptor is widely distributed, and was detectable in almost all tissues examined including kidney, uterus, stomach and ileum (Sugimoto et al., 1992). Three splice variants have been identified for the EP3 receptor subtype, which exhibit identical binding properties but differ in their functions. EP3α and β are exclusively coupled to adenylate cyclase inhibition through $G_i$, whereas the EP3γ variant is coupled to both $G_i$ and $G_s$, leading to adenylate cyclase inhibition at low $PGE_2$ concentrations, and stimulation at high $PGE_2$ concentrations (Namba et al., 1993). EP3-mediated $PGE_2$ signalling has been described in the mediation of fever (Ushikubi et al., 1998), pain responses (Minami et al., 2003; Takasaki et al., 2005) and more recently, in inflammatory vascular permeability by activation on mast cells (Morimoto et al., 2014). The roles of EP3 receptor activation in tumorigenesis remain unclear. Deletion of the EP3 receptor in APC (Delta716) mice had no effects on polyp formation (Sonoshita et al., 2001). However, in a study using azoxymethane-treated mice and rats, EP3 receptor activation was shown to play a role in suppression of growth and deletion of EP3 enhanced colon carcinogenesis suggesting an anti-tumorigenic role for the EP3 receptor in this model. Furthermore, expression of the EP3 receptor was shown to be downregulated in tumour cells compared to epithelial cells from the normal mucosa (Shoji et al., 2004). In addition, Macias-Perez et al. (2008) demonstrated a decrease in tumorigenesis *in vivo* following EP3 receptor activation, and a reduction in tumour cell proliferation (Macias-Perez et al., 2008). In contrast, other studies have eluded to a role for EP3 in angiogenesis associated with tumour growth by enhancing expression of VEGF (Amano et al., 2003) and MMP9 (Amano et al., 2009; Ogawa et al., 2009).

#### 1.4.1.4 EP4 Receptor

The EP4 receptor is expressed abundantly in a diverse range of tissues, with high expression levels observed in the ileum, thymus and uterus (Ikegami et al., 2001). The EP4 receptor has also been well characterised for its role in tumorigenesis. Ablation of EP4 receptor on myeloid cells of APCMin/+ mice showed marked inhibition of adenoma size and number (Chang et al., 2015). Activation of the EP4 receptor has been associated with transactivation of the EGFR signalling pathway (Buchanan et al., 2006; Oshima et al., 2011), which leads to downstream activation of a number of pathways including MAPK, PI3K/AKT, STAT and PLC which have been defined for their roles in proliferation, differentiation, migration and cell survival (Normanno et al., 2006). In HT-29 colorectal cancer cells, enhanced EP4-mediated $PGE_2$ signalling was associated with resistance to apoptosis and increase in anchorage-

independent growth (Hawcroft et al., 2007); of note, in this system the EP4-mediated signalling did not trans-activate EGFR signalling.

Signalling through the EP4 receptor subtype has also been associated with a number of pro-tumorigenic immune responses, inducing the development of T regulatory cells (Sharma et al., 2005), and immune-suppressing MDSCs (Sinha et al., 2007; Obermajer and Kalinski, 2012). However, EP4 receptor signalling has also been associated with wound healing following mucosal injury. Using a biopsy injury system to generate wounds in the mouse colon, it has been shown that mice deficient in the EP4 receptor exhibit multiple deficiencies in tissue repair including incomplete wound covering with wound associated epithelial cells (WAE) (Manieri et al., 2012; Manieri et al., 2015). In this system, the authors identified an induction of EP4 receptor expression in mesenchymal cells in the underlying lamina propria (Manieri et al., 2012). More recently, Miyoshi et al (2017) showed that EP4 receptor signalling induced the differentiation of WAE through direct inhibition of GSK3β and therefore nuclear β-catenin activation, independent of canonical WNT signalling (Miyoshi et al., 2017).

In agreement with a role for EP4 in epithelial repair following injury, EP4 receptor signalling has also been shown to ameliorate severe colitis and mucosal damage in mice treated with DSS by downregulating the immune response (Kabashima et al., 2002). However, using a different model of inducible colitis in mice, where naïve T cells are transferred into mice deficient in recombinase-activation gene 2, transfer of EP4+/- or EP4-/- T cells was shown to induce a weaker inflammatory response compared to transfer of T cells from wild type controls (Yao et al., 2013).

### 1.4.2 Paracrine Signalling

Previous studies have suggested that paracrine $PGE_2$ signalling is important for promoting intestinal tissue repair and regeneration following tissue injury, which is vital for the maintenance of epithelial homeostasis. The use of COX-2 conditional knockout mice to analyse the role of COX-2 expression in distinct cell types revealed that deletion of COX-2 in myeloid cells including macrophages and granulocytes (eosinophils and neutrophils) exacerbated DSS-induced murine colitis. Deletion of COX-2 in epithelial cells had no effects on DSS-induced colitis compared to littermate controls. (Ishikawa et al., 2011). Furthermore, paracrine $PGE_2$ signalling by myofibroblasts in the intestinal lamina propria play a major role in the response to innate/inflammatory signals initiated following epithelial injury (Roulis et

al., 2014). Myofibroblasts sense inflammatory signals which induces the expression of Tumor progression locus-2 (Tpl2), which has previously been described to be activated in response to signalling pathways with contrasting roles in homeostasis and inflammation (Fukata et al., 2005; Roulis et al., 2011). The expression of Tpl2 by myofibroblasts induces COX-2 synthesis of $PGE_2$, which was shown to mediate the epithelial homeostatic proliferation response to injury (Roulis et al., 2014).

Eosinophils are myeloid cells present in the lamina propria of the colonic and small intestinal mucosa, which are emerging as an immune cell type with differential functions in health and disease (Melo et al., 2013; Rosenberg et al., 2013). Phenotypic differences have been described between circulating eosinophils in the blood, and activated eosinophils which are recruited into tissues where they gain/induce expression of additional receptors (Carlens et al., 2009; Wallon et al., 2011), and have an increased survival (Wu et al., 2011). Eosinophils are known to express a number of the components required for $PGE_2$ signalling. Eosinophils secrete $PGE_2$ in lipid bodies present in the cytoplasm, and EP2 and EP4 receptor expression is observed on the cell surface of eosinophils (Rosenberg et al., 2013), suggesting that eosinophils may be a good candidate for the source of paracrine $PGE_2$ signalling in the colonic epithelium, whilst also secreting and responding to $PGE_2$ in an autocrine manner. The differential activation of EP receptors have been shown to have various functions in health and disease (Sugimoto and Narumiya, 2007). Activation of the EP2 receptor has been shown to induce epithelial cell proliferation and tumour invasion through VEGF (Kamiyama et al., 2006; Lee et al., 2016), and EP4 receptor activation has a number of roles in tumorigenesis but also in amelioration of inflammation in mouse models of colitis (Kabashima et al., 2002; Chang et al., 2015). This suggests that autocrine $PGE_2$ signalling in eosinophils may have varying roles in inflammation and cancer. The relationship between paracrine $PGE_2$ secretion by eosinophils and epithelial renewal and regeneration has not yet been described.

Eosinophils also express IL-6, and the IL-6R (Melo et al., 2013), suggesting that they are able to induce autocrine IL-6 signalling. Evidence has also previously been presented for a role of paracrine IL-6 signalling by eosinophils on the maintenance of plasma cells in the bone marrow (Chu et al., 2011), and in the generation and maintenance of IgA expressing B cells in the small intestinal lamina propria (Chu et al., 2014). The next section explores the emerging roles of eosinophils in the maintenance of homeostasis and immune cell regulation.

## 1.5 The Eosinophil

Eosinophils are potent effector cells, which are recruited to sites of inflammation, however they also constitute an abundant population under homeostatic conditions in tissues such as the GI tract. Eosinophils are most commonly associated with helminth infection, allergic asthma and IBD; however, the large numbers of eosinophils present along the GI tract from birth and in quiescent IBD have prompted initial experiments into the roles of eosinophils in homeostasis (reviewed in Rosenberg et al. (2013)). As such, eosinophils are emerging as regulators of tissue remodelling, inflammation and the maintenance of epithelial barrier function.

### 1.5.1 Structure and Function

Eosinophils are specialised granulocytes which were first described by Paul Ehrlich in 1979 for their affinity to acidophilic dyes. They are present from birth and develop from pluripotent progenitors in the bone marrow. Eosinophils enter the peripheral circulation in a mature state, where they can circulate for up to 18h (Steinbach et al., 1979) before they are activated and recruited into tissues in response to stimuli such as eotaxin (the major chemokine for eosinophils) or Interleukin-5 where they have a prolonged survival (Hogan et al., 2008; Blanchard and Rothenberg, 2009). During homeostasis, eosinophils reside in the thymus and along the GI tract (Lamouse-Smith and Furuta, 2006).

The eosinophil is formed of a bilobed nucleus surrounded by a characteristic cytoplasmic accumulation of specific granules (**Figure 1.16**). Each granule is comprised of a dense crystalline core surrounded by a less-dense matrix, enclosed by a trilaminar matrix. The major granule components are as follows: cationic proteins such as major basic protein (MBP) and eosinophil peroxidase (EPO); and eosinophil-associated RNases (EARs) such as eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (reviewed in Rosenberg et al. (2013)). These proteins are toxic to microbial pathogens, as well as host tissues. Therefore, the uncontrolled degranulation of eosinophils could lead to epithelial damage. A novel mechanism has been described which minimises the risk of damage to surrounding tissues. Eosinophils are now known to expel a matrix of mitochondrial DNA, known as an eosinophil extracellular trap (EET), which captures the cytotoxic granule proteins and invading bacteria. This process has not yet been described in the GI tract but has been characterised in the asthmatic airway and in atopic dermatitis (Yousefi et al., 2008; Yousefi et al., 2012). Other non-cytotoxic components of the specific granules include a

number of cytokines, chemokines and growth factors. One of the growth factors secreted with eosinophilic granules is EGF, which is vital for intestinal crypt cell proliferation. Another prominent feature of eosinophils is the presence of lipid bodies, where prostaglandins and leukotrienes are synthesised (reviewed by Rosenberg et al. (2013)).

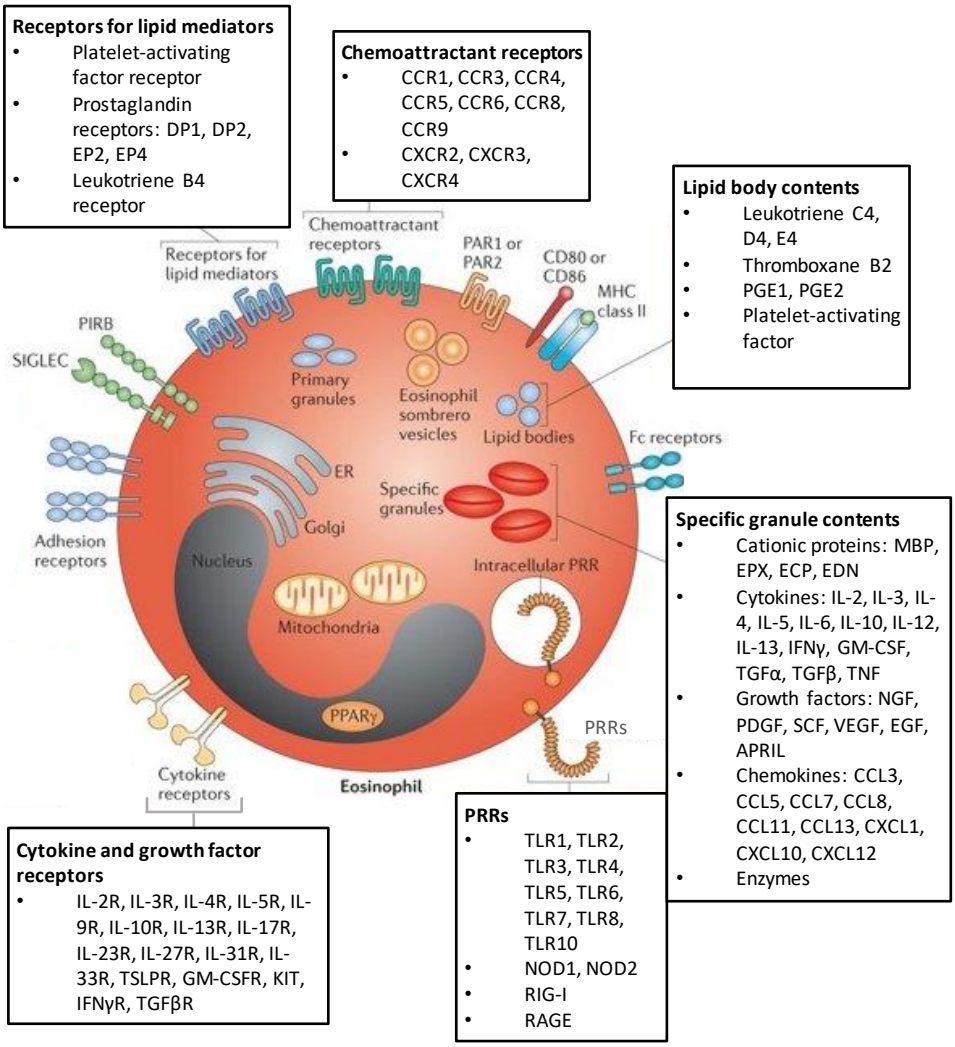


**Figure 1.16. Eosinophils express a number of receptors and factors for health and disease.**

Schematic diagram of an eosinophil showing a characteristic bi-lobed nucleus and expression of receptors with a number of different functions. Eosinophils degranulate when activated and release granule contents including MBP, ECP, EDN, GM-CSF, VEGF, EGF and APRIL. Eosinophils secrete $PGE_2$ in their lipid bodies and express EP2 and EP4 receptors on their surface. Expression of chemoattractant, cytokine and growth factor receptors as well as pattern recognition receptors is observed on the cell surface of eosinophils.

On their cell surface membrane, eosinophils express a variety of receptors for ligands specific for degranulation, growth, chemotaxis, adhesion and cell-cell interactions (summarised in **Figure 1.16**). Of note, eosinophils express CC-chemokine receptor 3 (CCR3), which mediates eosinophil chemotaxis in response to binding of eotaxins, Interleukin-5 receptor subunit-α (IL-5Rα) and the sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8). Of particular importance is IL-5Rα, which responds to IL-5. IL-5 is important for all aspects of eosinophil development, activation and survival. IL-5 is produced by $T_{H2}$ cells, mast cells, natural killer (NK) cells, NKT cells and by eosinophils themselves. Activation of IL-5Rα induces eosinophil development from progenitors, activation of eosinophils and prolonged eosinophil survival in the periphery and tissues (reviewed in Rosenberg et al. (2013)).

Eosinophils also express a number of PRRs, as discussed earlier, including TLR7 and TLR5. This suggests that eosinophils are able to mount an immune response following the recognition of PAMPs.

### 1.5.2 Roles of Eosinophil in Homeostasis

Eosinophils are able to respond to signals from other leukocytes, namely secreted IL-5, as well as playing a major role in modulating the function of other leukocytes to regulate inflammation. The expression of components on the eosinophil cell surface specific to antigen presentation, such as Major Histocompatibility Complex class II molecules, allows eosinophils to process antigens and stimulate T cell proliferation and cytokine release in response to specific antigens (Wang et al., 2007). Eosinophils are able to promote recruitment of $T_{H2}$ cells in response to production of chemoattractants such as C-C motif chemokine ligand 17 (CCL17) and CCL22 (Jacobsen et al., 2008). Eosinophils also interact with other innate immune cells. Dendritic cell maturation is induced by eosinophils activated by the pathogen-derived molecular pattern CpG DNA (Lotfi and Lotze, 2008), and the eosinophil granule protein EDN also promotes dendritic cell maturation (Yang et al., 2003; Yang et al., 2008). The eosinophil granule protein MBP also acts on neutrophils, inducing an oxidative burst, IL-8 secretion and increasing expression of the integrin complement receptor 3 on the neutrophil cell surface (Haskell et al., 1995).

Eosinophils have also been shown to prime B cells for the production of IgM, and support the survival of plasmablasts in the bone marrow via secretion of IL-6 and A Proliferation Inducing Ligand (APRIL) (Chu et al., 2011). Plasma cells are crucial for long-term immune protection through their secretion of antigen-specific antibodies. Mature long-lived

plasmablasts migrate to the bone marrow following B cell differentiation, where their survival is prolonged *via* secretion of cytokines and chemokines (Dilosa et al., 1991; Blink et al., 2005; Radbruch et al., 2006). Expression of the chemokine C-X-C motif chemokine 12 (CXCL12) by stromal cells induces bone marrow migration of plasmablasts as well as supporting long-term survival (Hargreaves et al., 2001; Hauser et al., 2002; Nie et al., 2004). *In vitro* studies have identified a role for APRIL, IL-6, Tumor Necrosis Factor, IL-10, IL-4 and IL-5 in plasma cell survival (Cassese et al., 2003). Further to these experiments, APRIL was shown to be crucial for the long-term survival of plasmablasts in the bone marrow (Belnoue et al., 2008). Eosinophils were shown to be the major source of APRIL and depletion of eosinophils considerably reduced the numbers of plasma cells in the bone marrow (Chu et al., 2011). Recent studies have also revealed a role for eosinophils in the generation and maintenance of IgA expressing plasma cells in the intestinal lamina propria. Use of eosinophil-deficient mouse models showed that a loss of eosinophils induced a reduction in the number of IgA+ plasma cells and secreted IgA in the small intestinal lamina propria, and defects in the epithelial barrier (Chu et al., 2014). This data demonstrates a crucial role for eosinophils in the modulation of the intestinal immune system.

Eosinophils also play an important role in mammary gland formation. Initially, a mouse model depleted of leukocytes and restored with a bone marrow transplant identified a role for recruitment of eosinophils and macrophages to the growing terminal end buds (TEB) of the mammary gland during postnatal development. Using mouse models homozygous for a null mutation in the gene for eotaxin, eosinophil recruitment around the TEB was depleted. This led to reduced mammary gland branch and TEB formation. Furthermore, macrophage depletion with a Colony-Stimulating Factor-1 null mutation also lead to reduced mammary gland formation (Gouon-Evans et al., 2000). This data highlights an important role for eosinophils and macrophages in development.

In addition, alternatively activated macrophages play a major role in recruitment of eosinophils to tissues *via* release of a chitinase-like selective eosinophil chemoattractant, YM1. In parallel, eosinophil are able to recruit alternatively activated macrophages through release of IL-4 and IL-13 and prolong their viability in adipose tissues (Wu et al., 2011). It has also been suggested that eosinophils may aid macrophages in apoptotic cell clearance in the thymus (Kim et al., 2010). Mast cells and eosinophils reside in close proximity under homeostatic conditions in the GI tract, as well as in inflammation in the allergic lung and diseased gut in patients with Crohn's disease. The eosinophil granule proteins, MBP, ECP and

EPO induce histamine release by mast cells and prolong mast cell survival (Munitz and Levi-Schaffer, 2004).

Although, as yet, the roles for eosinophils in epithelial homeostasis remain largely unknown, these data have demonstrated a crucial role for eosinophils in homeostasis, through regulation of the immune system and in development. The abundance of eosinophils amongst other immune cells in the intestinal lamina propria highlights the possibility of a role for eosinophils in the maintenance of intestinal homeostasis, either by regulation of other immune cells, or as a direct influence on the intestinal epithelium.

### 1.5.3 Roles of Eosinophils in Disease

Both beneficial and pathological roles of eosinophils have been described in the IBDs, Crohn's disease and Ulcerative Colitis. IBD is characterised by an infiltration of leukocytes, including eosinophils, neutrophils, monocytes and lymphocytes, into the intestinal mucosa. Although eosinophils only represent a small proportion of the infiltrating leukocytes (Walsh and Gaginella, 1991), the level of eosinophils in the colonic mucosa has been shown to correlate with disease severity and a poorer prognosis (Desreumaux et al., 1999). High levels of eosinophil degranulation products, i.e. MBP, ECP, EDN and EPO, have been associated with adult IBD, and these levels correlated with disease severity (Raab et al., 1998; Carlson et al., 1999). Levels of eotaxin-1, were also shown to be elevated in the serum of adult IBD patients (Chen et al., 2001; Mir et al., 2002). Studies using DSS-induced mouse models of colitis demonstrated a crucial role for eotaxin-1 for the recruitment of eosinophils to the colonic mucosa, and deletion of eotaxin-1 led to a decrease in the number of eosinophils in the colonic mucosa, and attenuation of symptomatic colitis in mouse models (Ahrens et al., 2008). A mechanism for this response was later described in 2011. Paediatric genome-wide studies implicated the 17q12 loci, encoding eotaxin-1, in early-onset IBD. In mouse models, recruitment of inflammatory monocytes to the colon was observed following DSS treatment. These monocytes were shown to express eotaxin-1, which lead to the recruitment of eosinophils to the colon. Furthermore, depletion of inflammatory macrophages using a CCR2/- mouse showed decreased eotaxin-1 expression and decreased eosinophilic inflammation in the colon (Waddell et al., 2011).

Eosinophil accumulation in IBD is also associated with an altered epithelial barrier function. In a recent study, colonic samples from patients with Ulcerative Colitis showed increased permeability to antigens compared with control. This was shown to be associated with an

increase in corticotrophin-releasing factor (CRF), which alongside mast cells, regulates intestinal barrier permeability. In this study, colonic eosinophils were shown to express the muscarinic receptors M2 and M3 on their cell surface. This is in contrast to peripheral eosinophils, which did not express M2 or M3 receptors. Furthermore, from co-culture studies it was shown that eosinophil activation with carbachol induced CRF production, which lead to increased T84 cell permeability (Wallon et al., 2011).

The eosinophil granule protein MBP was shown to have similar effects on epithelial barrier function. Using a co-culture of human eosinophils or AML14.3D10 eosinophil myelocytes with T84 epithelial cells it was shown that accumulation of eosinophils led to decreased transepithelial resistance and increased transepithelial flux. Cell-free co-culture supernatant experiments revealed that this was dependent on MBP (Furuta et al., 2005).

Eosinophils are also commonly associated with allergic exacerbation in the asthmatic airway. During the inflammatory response, eosinophils are recruited to the lungs by $T_{H2}$-secreted cytokines, such as IL-5, and chemokines including eotaxins. Eosinophilic accumulation and degranulation causes epithelial damage due to the release of toxic granule proteins such as MBP which leads to airway hypersensitivity (Walsh, 2001). The use of mouse models of allergic airway disease, whereby mice are sensitized usually with ovalbumin or Aspergillus, has allowed for the detailed study of the allergic asthmatic exacerbation response. In these mice, allergic antigen sensitization induces an accumulation of eosinophils in the airway, increased numbers of $T_{H2}$ cells, excess mucus secretion and airway hypersensitivity.

Findings have also suggested a role for eosinophil-epithelial cell interactions in the resolution of epithelial inflammation following an asthma exacerbation. Apoptosis of inflammatory cells is a key factor in the resolution of inflammation. Studies investigating induced sputum of patients following asthma exacerbations have shown that treatment with steroids induced eosinophil apoptosis which are recognised and phagocytosed by macrophages (Woolley et al., 1996). Eosinophils have also been shown to migrate into the airway lumen where they are subsequently cleared (Uller et al., 2001). Furthermore, it has been shown that epithelial cells have the ability to phagocytose apoptotic eosinophils (Walsh et al., 1999). The phagocytic capacity of epithelial cells was shown to be dependent on integrin, lectin and phosphatidylserine membrane receptors and enhanced by the corticosteroid dexamethasone (Sexton et al., 2001).

### 1.5.4 Eosinophil-Epithelial Cell Interactions

Interactions between epithelial cells and eosinophils have been extensively reviewed (Sexton and Walsh, 2002). Epithelial cells have the potential to secrete and respond to a variety of inflammatory mediators including cytokines, chemokines and lipid and peptide products (Peterson and Artis, 2014). The expression of the major chemokine for eosinophils, eotaxin, on airway epithelial cells has been described (Conroy and Williams, 2001). Eotaxin expression in the airway is regulated by inflammatory cytokines including IL-13, IL-4, Tumor Necrosis Factor-α and IL-1 (Lamkhioued et al., 1997; Lilly et al., 1997; Fujisawa et al., 2000; Jedrzkiewicz et al., 2000). Epithelial cells in the airway also express functional CCR3, which is the receptor for eotaxin (Stellato et al., 2001). Preliminary results from the Sobolewski laboratory have also shown expression of CCR3 by the colonic epithelium (El Hadi, personal communication). The expression of CCR3 may be a mechanism of regulating chemokine production, whereby secreted eotaxin can be sequestered by the epithelium and presented to infiltrating eosinophils to enhance their activation. Epithelial cells have also been shown to support eosinophil survival and activation through secretion of eosinophil activation factors including IL-5 and GM-CSF (Motojima et al., 1996; Salvi et al., 1999).

Other cytokines have also been implicated in eosinophil-epithelial interactions in inflammation. Addition of IL-9 to HBE4-E6/E7 human epithelial cells in culture led to release of the T cell chemoattractants IL-16 and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) (Little et al., 2001), which induced increased expression of eosinophil IL-5R, leading to enhanced activation and prolonged survival (Gounni et al., 2000). The use of a polarized *in vitro* epithelial model of inverted BEAS-2B human bronchial epithelial cells demonstrated the importance of the polarity of epithelial cells for their ability to influence eosinophil behaviour. Purified eosinophils were added into co-culture, and eosinophil transmigration was only observed from the basal to the apical side of the monolayer; this was shown to be modulated by eotaxin, RANTES and the presence of functional CCR3 on the eosinophil surface membrane. Transmigration was only partially inhibited by removing eotaxin and RANTES and blocking CCR3, suggesting that epithelial secretion of cytokines and/ or chemokines are also involved in eosinophil transmigration. When BEAS-2B epithelial cells were co-cultured with activated human eosinophils, an increased biosynthesis of cysteinal leukotrienes was observed (Jawien et al., 2002). Cysteinal leukotrienes play a major role in the pathogenesis of asthma, including bronchoconstriction, mucus hypersecretion, airway

remodelling, accumulation of eosinophils and epithelial and endothelial damage. These data suggest epithelial cells are able to actively promote eosinophil attraction and activation.

The components of eosinophil granules are cytotoxic to microbial cells. The eosinophil granule protein MBP has also been shown to induce tissue damage of intestinal epithelial cells (Gleich et al., 1979) and bronchial epithelial cells (Kato et al., 2012) *in vitro*. The exertion of EETs by eosinophils *in vivo* in response to microbial pathogens is thought to minimise the cytotoxic effects of eosinophil granule proteins on surrounding tissues (Yousefi et al., 2012). However, degranulation of eosinophils in the colon is thought to be associated with epithelial damage in patients with colitis, although the mechanism remains unknown (Levy et al., 2001).

The presence of a number of innate immune cell subtypes, including macrophages, monocytes and neutrophils, in the colonic lamina propria suggest that a complex interplay between different innate immune cells contributes to regeneration and renewal of the colonic epithelium. Immune cells have been shown to relocalize in response to epithelial injury and induce epithelial regeneration (Pull et al., 2005; Brown et al., 2007; Seno et al., 2009). WNT signalling is vital for intestinal crypt proliferation (Pinto et al., 2003) and macrophages have been shown to promote WNT/β-catenin activity through secretion of Tumor Necrosis Factor α (Oguma et al., 2008). Macrophages activated by microbial challenge have been shown to be necessary for the colonic crypt regenerative response to injury (Pull et al., 2005). Furthermore, Triggering Receptor Expressed On Myeloid Cells 2 (Trem2) signalling by macrophages induced expression of IL-4 and IL-13 in colonic mucosal injury, which was required for increased epithelial proliferation and wound repair (Seno et al., 2009). Previous work by Skoczek et al. (2014) showed using an explant culture system, that addition of luminal microbes to the apical surface of explants induces migration of monocytes to the stem cell niche. Physical interactions between monocytes and intestinal stem cells were shown to induce crypt cell proliferation and increase stem cell number. Furthermore, in this system, following luminal microbial input, CCR3+ eosinophils were also shown to move closer to the stem cell niche, suggesting that eosinophils may play a similar role in modulation of crypt renewal during homeostasis (Skoczek et al., 2014).

In the study by Skoczek et al. (2014), a novel protocol for the co-culture of monocytes and isolated colonic crypts was also developed. However, the use of an eosinophil-crypt co-culture model in the human has not yet been described. Previous studies into the functional effects of eosinophils have relied on the promyelocytic cell line HL-60 Clone 15 cells (which

differentiate into a stable eosinophil phenotype following addition of Butyric acid), AML14.3d10 eosinophil myelocytes, or eosinophils isolated from blood (Fischkoff, 1988; Sedgwick et al., 1996; Jawien et al., 2002; Farahi et al., 2007; Bates et al., 2010; Akuthota et al., 2012). Investigations into the role of eosinophils in epithelial cell permeability have utilised the T84 epithelial cell line, where eosinophils were shown to reduce barrier function through secretion of MBP (Furuta et al., 2005). Whilst eosinophils isolated from the blood represent a good system for investigation of eosinophils, differences in function between peripheral and tissue resident eosinophils have been described (Hogan et al., 2008; Blanchard and Rothenberg, 2009). Therefore, the ideal model system for investigation of tissue resident eosinophil function would be eosinophils isolated from the intestinal mucosa.

## 1.6 Rationale

In the laboratory, we are interested in the regulation of epithelial renewal by autocrine epithelial-derived factors and by immune cell-derived paracrine factors. Under stressful conditions, i.e. tissue injury or cancer, regeneration of the colonic epithelium is modulated by cytokines including IL-6 and $PGE_2$ (Wang and Dubois, 2006; Wang and Dubois, 2010; Kuhn et al., 2014). There is also a large body of evidence to show that activation of cytokine signalling pathways modulates epithelial renewal during homeostasis (reviewed in Vanuytsel et al. (2013)). However, no studies have fully investigated the roles of IL-6 and $PGE_2$ in epithelial renewal during health through autocrine and/or paracrine signalling. Therefore the research questions of this thesis asks what are the autocrine sources of IL-6 in health in the intestinal epithelium, what are the cellular locations of the IL-6 receptor and what are the effects of IL-6R activation on small intestinal renewal during health. Whilst the epithelium is known to be a source of $PGE_2$, this thesis asks whether an autocrine role for $PGE_2$ exists in the colonic epithelium, and whether differential EP receptor activation induces crypt renewal during health (**Figure 1.17**). The regulation of intestinal renewal by IL-6 and $PGE_2$ constitute the main focus and findings of this thesis.

Another, less well defined, focus of the thesis is the potential paracrine sources of crypt regenerative factors in the intestinal mucosa. Previous work in the Sobolewski laboratory has shown that *in vitro* eosinophils regulate crypt renewal in a paracrine manner in the mouse colonic epithelium (unpublished, AS, DS); initial experiments suggests that a similar paradigm exists in the human colonic mucosa (unpublished, Sobolewski lab). Eosinophils are a paracrine source of $PGE_2$ and IL-6, and the eosinophil is resident in abundance in the human gut mucosa during health, where eosinophils are known to modulate gut immune

homeostasis (**Figure 1.17**) (Rosenberg et al., 2013; Chu et al., 2014). This begs the question of the role of human eosinophils in the maintenance of crypt homeostasis. For these reasons, the development of an eosinophil-crypt co-culture model was also attempted and is described in the latter part of the thesis.

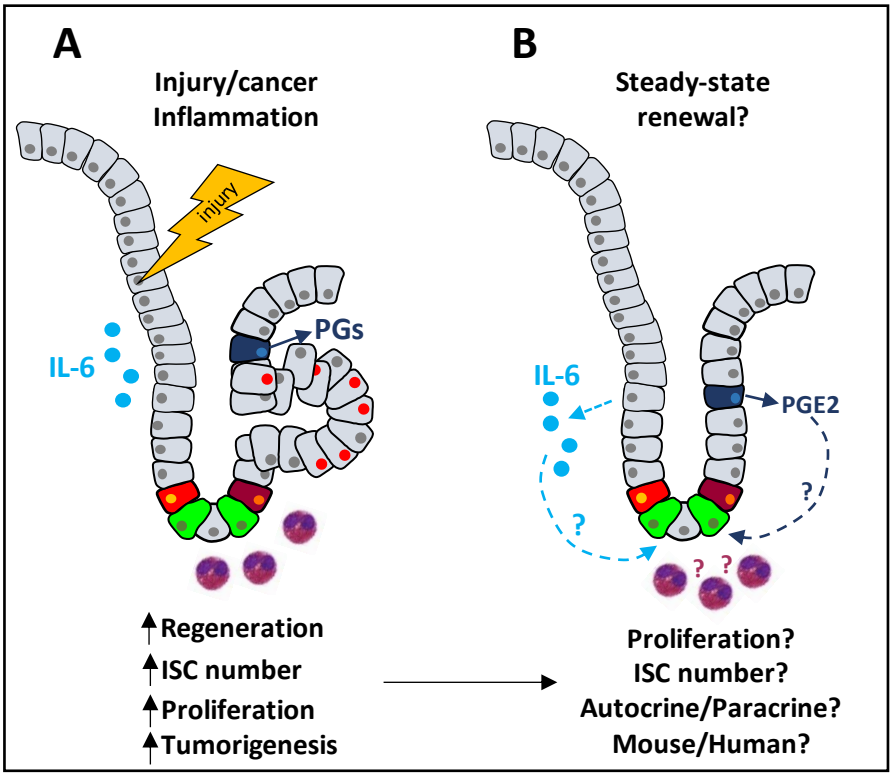


**Figure 1.17. Graphical representation of rationale.**

**(A)** IL-6 has been shown to be important for small intestinal crypt regeneration following injury. In colorectal cancers and tissue injury, autocrine and paracrine $PGE_2$ signalling has been shown to induce proliferation and carcinogenesis. Initial experiments suggest that eosinophils induce mouse colonic crypt cell proliferation. **(B)** This thesis aims to determine the roles of autocrine and paracrine IL-6 and $PGE_2$ in steady-state renewal of the small intestine and colon respectively, and to examine the spatial relationship between eosinophils and colonic crypts in the human.

## 1.7 Hypothesis

Intestinal tissue renewal is modulated by autocrine and paracrine Interleukin-6 and Prostaglandin-$E_2$ signalling during homeostasis.

## 1.8 Aims

1. Determine the role of IL-6 signalling in crypt renewal during homeostasis
2. Examine the role of $PGE_2$ in colonic crypt renewal, and determine the contribution of differential EP receptor activation in modulating crypt cell proliferation
3. Evaluate the spatial characteristics of eosinophil distribution along the colonic crypt axis, and develop and characterise an eosinophil-crypt co-culture system to investigate the effects of eosinophils on colonic crypt homeostasis.

# 2 Chapter 2. Materials and Methods

## 2.1 Reagents and buffers

### 2.1.1 Chemicals and reagents

**Table 2.1. List of Chemicals and Reagents**

| **Chemical or Reagent** | **Supplier** |
|---|---|
| Advanced DMEM | Invitrogen |
| Ammonium Chloride ($NH_4Cl_2$) | Fisher Scientific |
| Bovine Serum Albumin | Sigma |
| Calcium Chloride ($CaCl_2$) | VWR International |
| Collagenase A | Roche |
| Click-IT reaction kit | Fisher Scientific |
| D-Glucose | Fisher Scientific |
| Dimethyl Sulfoxide (DMSO) | Sigma |
| DNaseI | Sigma |
| Donkey Serum | Sigma |
| EDTA | Sigma |
| Ethanol | Sigma |
| Fetal Bovine Serum (FBS) | Sigma |
| FITC-conjugated *Ulex europaeus*Lectin (UEA-1 FITC) | Sigma |
| Goat Serum | Abcam |
| Growth factor reduced matrigel | VWR international |
| HEPES | Fisher Scientific |
| Hoescht | Life Technologies |
| IMDM | Invitrogen |
| Iso-pentane | Fisher Scientific |
| Low Melting Point Agarose (LMA) | Sigma |
| Magnesium Chloride (MgCl2) | Fluka (Sigma) |
| Mouse IL-6 | Peprotech |
| Murine recombinant epidermal growth factor | Peprotech |
| Murine recombinant noggin | Peprotech |
| Murine recombinant R-spondin1 | R&D systems |

| | |
|---|---|
| O.C.T Compound | Tissue-Tek |
| Paraformaldehyde | Sigma |
| Percoll | Sigma |
| Phosphate buffered saline | OXOID |
| Potassium Chloride (KCl) | Fisher Scientific |
| Quik-Diff staining kit | Reagena |
| RPMI | Invitrogen |
| Sodium Bicarbonate ($Na_2HPO_4$) | Fisher Scientific |
| Sodium Chloride (NaCl) | Fisher Scientific |
| Sodium Dodecyl Sulfate (SDS) | Melford |
| Triton-X-100 | Roche |
| Vectashield | Vector laboratories |
| Xylene | Sigma |

### 2.1.2 Buffers

**Table 2.2. List of buffers used**

| **Buffer** | **Formula** |
|---|---|
| Citrate Buffer (pH 6.0) | Tri-sodium Citrate 2.94g in 1L of $H_2O$ (10mM) |
| HEPES Buffered Saline (HBS) (pH 7.4) | NaCl (140mM), KCl (5mM), Hepes (10mM), d-glucose (5.5mM), Na2HPO4 (1mM), MgCl2 (0.5mM), CaCl2 (1mM) |

### 2.1.3 Primary and Secondary Antibodies

**Table 2.3. List of primary and secondary antibodies used in the study**

| **Antibody** | **Species Origin** | **Clonality** | **Working concentration** | **Supplier** |
|---|---|---|---|---|
| anti-BrdU | Rat | Monoclonal | 1:100 | Abcam |
| anti-CCR3 | Rabbit | Monoclonal | 1:50 | Abcam |
| Anti-CD14 | Rabbit | Polyclonal | 1:100 | LSBio |
| anti-Chromagranin-A | Mouse | Monoclonal | 1:100 | Abcam |
| anti-Cleaved caspase 3 | Rabbit | Polyclonal | 1:100 | Cell signaling |
| anti-COX-1 | Goat | Polyclonal | 1:50 | Santa Cruz |
| anti-COX-2 | Goat | Polyclonal | 1:50 | Santa Cruz |
| anti-COX-2 | Mouse | Monoclonal | 1:50 | Santa Cruz |

| | | | | |
|---|---|---|---|---|
| anti-E-cadherin | Goat | Polyclonal | 1:100 | R&D Systems |
| anti-E-cadherin | Mouse | Monoclonal | 1:100 | Abcam |
| anti-EP1 | Rabbit | Polyclonal | 1:50 | Cayman |
| anti-EP2 | Rabbit | Polyclonal | 1:50 | Cayman |
| anti-EP3 | Rabbit | Polyclonal | 1:50 | Cayman |
| anti-EP4 | Rabbit | Polyclonal | 1:50 | Cayman |
| anti-IL-6 neutralising | Rat | Monoclonal | 58 µg/g *in vivo*, 100µM *in vitro* | BioXcell |
| anti-IL-6R blocking | Rat | Monoclonal | 58 µg/g *in vivo*, 100µM *in vitro* | BioXcell |
| anti-Ki67 | Rabbit | Polyclonal | 1:100 | Abcam |
| anti-Lgr5(212) | Mouse | Monoclonal | 1:100 | Origene |
| anti-Lgr5(2A2) | Mouse | Monoclonal | 1:100 | Origene |
| anti-Lysozyme | Rabbit | Polyclonal | 1:100 | Abcam |
| anti-Lysozyme | Mouse | Monoclonal | 1:100 | Abcam |
| anti-MBP | Mouse | Monoclonal | 1:50 | Biorbyt |
| anti-Mucin-2 | Rabbit | Polyclonal | 1:100 | Santa-cruz |
| anti-Mucin-2 | Mouse | Polyclonal | 1:100 | Abcam |
| anti-OLFM4 | Rabbit | Polyclonal | 1:100 | Abcam |
| anti-$PGE_2$ blocking | Mouse | Monoclonal | 50µM | Creative Biolabs |
| anti-pSTAT3 Tyr705 | Rabbit | Polyclonal | 1:50 | Cell signaling |
| anti-Siglec-8 | Rabbit | Polyclonal | 1:50 | Novus |
| | | | | |
| **Antibody** | **Species Origin** | | **Working concentration** | **Supplier** |
| anti-rabbit Alexa Fluor 488 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-rabbit Alexa Fluor 568 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-rabbit Alexa Fluor 647 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-mouse Alexa Fluor 488 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-mouse Alexa Fluor 568 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-mouse Alexa Fluor 647 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-goat Alexa Fluor 488 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-goat Alexa Fluor 568 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-goat Alexa Fluor 647 | Donkey | | 1:100/1:200 | Invitrogen |
| anti-rat Alexa Fluor 488 | Goat | | 1:200 | Invitrogen |

| | | | | |
|---|---|---|---|---|
| anti-Rabbit IgG (H+L) Alexa Fluor 647 AffiniPure Fab Fragment | Donkey | | 1:100 | Jackson Immunoresearch |

## 2.2 Experimental Approach

Before the identification of the intestinal stem cell marker Lgr5 by Clevers laboratory, previous studies on epithelial cells relied on cell culture of a monolayer of epithelial cells. The recent development and characterisation of small intestinal organoid and colonic crypt culture models provide a 3D model culture system, which contains all the cell types of the epithelium, and maintains the topological hierarchy of crypts observed *in vivo* (Sato et al., 2009; Jung et al., 2011; Sato et al., 2011; Skoczek et al., 2014; Parris and Williams, 2015). This forms an ideal platform through which the roles of paracrine and autocrine signalling on epithelial renewal can be investigated.

In this thesis, organoid culture experiments were undertaken alongside *in vivo* antibody studies using Lgr5EGFP mice to enable visualisation of stem cells within the epithelium. Immunofluorescent labelling was utilised in combination with RT-PCR to characterise expression of IL-6 and $PGE_2$ signalling pathway components at the protein and molecular level. A pharmacological approach was undertaken to determine downstream signalling events induced by $PGE_2$. Immunofluorescent labelling was also utilised to characterise the distribution of immune cells in the intestinal lamina propria. Furthermore, a reductionist co-culture system of eosinophils and human colonic crypts was developed in order to investigate the roles of eosinophils in human colonic crypt renewal.

## 2.3 Mouse experiments

### 2.3.1 Mice and in vivo mouse experiments

LGR5-EGFP-Ires-CreERT2 (Jackson Labs) or C57BL/6, aged 8-12 weeks were used. Generation and genotyping of the LGR5-EGFP-Ires-CreERT2 allele has been described previously (Barker et al., 2007). All animal experiments were conducted in accordance with the Home Office Animals Scientific procedures Act of 1986 with approval of the University of East Anglia Ethical review Committee, Norwich, United Kingdom and under Home Office project licence number 80/2545 (AG/AS). Blocking antibodies for IL-6 and IL-6 receptor or IgG controls (BioXcell) were administered to mice 3 times on alternate days by intraperitoneal injection at a concentration of 58 μg/g. Previous studies have utilised intraperitoneal injection of IL-6 neutralising or IL-6 receptor blocking antibodies at concentrations between 20μg-1mg/g for

3-6 doses (Debock et al., 2012; Berger et al., 2013; Kugler et al., 2013; Barber et al., 2014; Khmaladze et al., 2014; Benevides et al., 2015; Tsukamoto et al., 2015). Consideration of the antibody efficacies previously described led us to arrive at a concentration of 58µg/g of IL-6 neutralising or IL-6 receptor blocking antibody for use in this study. Animals were euthanized by Schedule One approved methods on day 6, and intestinal tissue processed immediately. In addition, small intestinal tissue from IL-6 knockout mice (Jackson Labs B6.129S2-Il6tmlKopf/J, a kind gift from Marcello Chieppa) was used to count the number of lysozyme positive cells per small intestinal crypt. Tissue samples were either utilised for isolation of organoids/crypts, or immediately fixed in 4% paraformaldehyde for 2h at 4˚C.

### 2.3.2 Mouse fixed tissue sections

Following fixation in 4% paraformaldehyde, tissue samples were washed in PBS and left overnight before embedding in OCT, and freezing in Isopentane on liquid nitrogen. Sections (8-20µm thick) were cut on a Cryostat and stored at -20˚C until required. Sections were rehydrated in PBS before processing for immunofluorescent labelling (see **section 2.3.5**).

### 2.3.3 Isolation and culture of mouse small intestinal organoids

Small intestinal crypts were isolated from the proximal small intestine of LGR5EGFP mice as previously described (AS) (Barker et al., 2008). Briefly, the mouse small intestine was opened longitudinally, washed with phosphate buffered saline (PBS), cut into 2-4 mm pieces and incubated with 1mM EDTA in PBS (pH7.4) for 30mins at 4°C. Crypts were liberated by serial rounds of pipetting in ice cold PBS and removal of the crypt enriched supernatant; the solution was then filtered through a 70µm cell strainer followed by centrifugation. Crypts (50-100 per coverslip) were embedded in a 200 µl droplet of growth factor reduced-Matrigel and seeded on No. 0 coverslips contained within a 12 well plate. After polymerisation at 37°C for 5-10 mins, crypts were flooded with 0.5 ml of mouse small intestinal culture medium (MSICM): advanced F12/DMEM containing B27, N2, n-acetylcysteine (1mM), HEPES (10mM), penicillin/ streptomycin (100 U/ml), Glutamax (2mM), epidermal growth factor (50 ng/ml), noggin (100 ng/ml) and R-spondin 1 (1 µg/ml). Small intestinal crypts grow in culture as budding organoids containing crypt-like and villus-like domains (**Figure 2.1**).

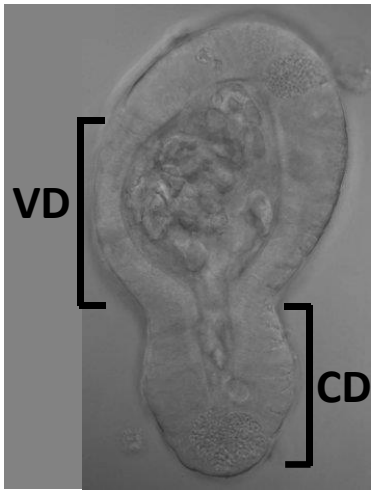


**Figure 2.1. Small intestinal crypts grow as budding organoids with crypt and pseudo-villus domains.**

Representative white light DIC image of a small intestinal organoid in culture, showing a crypt-like domain (CD) and pseudo-villus domains (VD).

### 2.3.4 IL-6 and STAT3 culture conditions

In IL-6 neutralising or IL-6 receptor blocking antibody experiments crypts were incubated for the entire 48 hour period in MSICM and appropriate antibody (100µg/ml) before addition of BrdU (1µM) for 17 hours.

Following 48 hours culture with MSICM crypts were stimulated with IL-6 at concentrations of 10ng/ml to 1µg/ml for 15 mins to 17 hours for pSTAT3 experiments; for proliferation experiments crypts were placed into 10% MSICM for 5 hours, after which BrdU (1µM) was added for 17 hours. For STAT3 inhibition studies, crypts were pre-incubated with STATTIC (20µM, as previously utilised for STAT3 inhibition in intestinal organoids by Lindemans et al. (2015)) or IWP2 (5µM, as previously described by Chen et al. (2009)) for 1 hour prior to addition of IL-6 (100ng/ml) for 17 hours.

For pSTAT3 activation studies, the IL-6 neutralising antibody was pre-incubated with IL-6 (100ng/ml) for 1h before addition to organoid culture for 1h. For receptor antibody studies, organoids were pre-incubated for 1h with the IL-6 receptor blocking antibody before addition of IL-6 (100ng/ml) for 1h. The same protocol was used for proliferation experiments, prior to the addition of IL-6 and BrdU (1µM) for 17h.

For chronic stimulation, crypts were incubated for 2-5 days with MSICM containing 100ng/ml IL-6 or STATTIC (20µM) before addition of BrdU (1µM) for 17h; media was changed every 2 days.

### 2.3.5 Immunofluorescent labelling

Following 2-5 days in culture, organoids were fixed in 4% paraformaldehyde for 1h at 4˚C. Following washing in PBS (or rehydrating for sections), samples were permeabilised with 13min $NH_4CL_2$, 5min 1% SDS, 30min 1% Triton X-100 and non-specific binding blocked with 10% donkey serum. Samples were incubated with primary antibody overnight before addition of appropriate fluorescently tagged secondary antibodies for 2h. A list of primary and secondary antibodies utilised in these studies are outlined in **Table 2.3**. For antibodies not previously characterised in the laboratory, negative controls were undertaken using IgG, secondary antibody incubation alone (i.e. no primary antibody added) and/or blocking peptide to primary antibody. No fluorescent labelling was observed in negative controls, confirming specify of antibodies utilised in the study. Example confocal images of negative staining are shown in **Figure 2.2**. After washing, samples were mounted with Vectashield containing Hoescht, Sytox blue or DAPI for visualisation of nuclei before imaging using epifluorescent (Nikon Ti) or confocal microscopy (Zeiss LSM META).

### 2.3.6 Isolation and culture of mouse colonic crypts

Using methodology previously described, mouse colonic crypts were isolated from the colon of LGR5EGFP mice (AS). Colonic tissue samples were collected in ice cold PBS, transported to the laboratory and incubated in Hepes-buffered saline (HBS), which was devoid of $Ca^{2+}$ and $Mg^{2+}$, and supplemented with EDTA (1mM) for 1h at room temperature. Crypts were liberated by vigorous shaking, crypt sedimentation and collection. Crypt were embedded in GF-reduced matrigel and 50-100 crypts seeded per coverslip on a 12WP. After polymerisation at 37°C for 5-10 mins, crypts were flooded with 0.5 ml of mouse colonic crypt culture medium (MCCCM): advanced F12/DMEM containing B27, N2, n-acetylcysteine (1mM), HEPES (10mM), penicillin/ streptomycin (100 U/ml), Glutamax (2mM), epidermal growth factor (50 ng/ml), noggin (100 ng/ml), R-spondin 1 (2 µg/ml) and Wnt3 (200ng/ml).

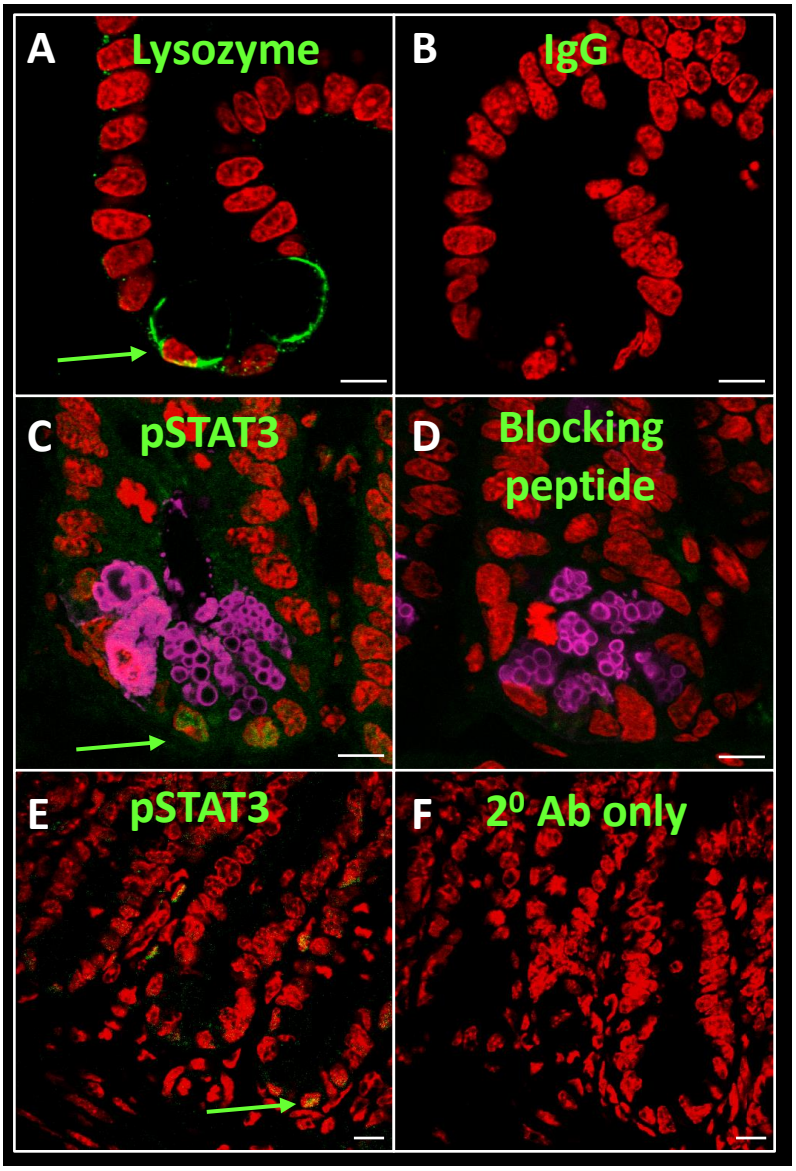


**Figure 2.2. Controls of immunofluorescent labelling specificity**

Representative confocal images of (**A**) lysozyme primary antibody (green) (**B**) IgG control (green) (**C**) pSTAT3 primary antibody (green) (**D**) Blocking peptide control, pSTAT3 antibody labelling in green (**E**) pSTAT3 primary antibody (green) (**F**) Secondary antibody only, no primary antibody added (green). Nuclei are marked with DAPI, red. Scale bars 10µm.

### 2.3.7 $PGE_2$ and EP Receptor activation and inhibition

$PGE_2$ or selective EP receptor agonists: EP1, EP2, EP3, EP4; or selective EP receptor antagonists: EP1, EP2, EP3, EP4, were dissolved in DMSO/methyl acetate/ethanol to give a 10mM concentration, and added at a final concentration of 1-20µM to the culture conditions (**Table 2.4**) on day 0-1 of isolation as described below. Appropriate controls (DMSO, methyl acetate or EtOH) were added to control crypts in the culture media. Colonic crypts were

incubated with selective EP antagonists, COX enzyme inhibitors or $PGE_2$ blocking antibody 2B2 (Mnich et al., 1995), for the entire culture period before addition of EdU (10μM) for 17h on day 1. For investigation into EP receptor activation and $PGE_2$-induced proliferation, following 24h of culture, colonic crypts were placed into growth factor free media for 5h, before incubation with selective EP agonists or $PGE_2$ and EdU for 17h. In order to determine the role of EP receptors in $PGE_2$-induced proliferation, following 24h in culture, colonic crypts were placed into growth factor free media for 5h, before pre-incubation with EP receptor antagonists for 2h, and subsequent addition of $PGE_2$ and EdU for 17h. Fixation with 4% paraformaldehyde occurred on day 2 after isolation.

**Table 2.4. List of chemical agonists and antagonists used to treat isolated organoids and crypts.**

Pharmacological agents were utilised to selectively inhibit and activate EP receptor subtypes, concentrations were optimised in order to minimise potential cross-reactivity (Funk et al., 1993; Kiriyama et al., 1997; Abramovitz et al., 2000; Juteau et al., 2001; Kabashima et al., 2002; Billot et al., 2003; Gil et al., 2008; Dey et al., 2009; af Forselles et al., 2011; Birrell et al., 2013).

| Name | Function | Working Concentration | Supplier | Potential Cross-reactivity |
|---|---|---|---|---|
| Iloprost | EP1 agonist | 10μM | Tocris | EP3R |
| Butaprost | EP2 agonist | 12μM | Cayman | None expected |
| Sulprostone | EP1/3 agonist | 10μM | Tocris | EP1R |
| TCS 2510 | EP4 agonist | 13μM | Tocris | None up to 14μM |
| SC 19220 | EP1 antagonist | 20μM | Tocris | None expected |
| PF04418948 | EP2 antagonist | 10μM | Tocris | None expected |
| L-798106 | EP3 antagonist | 1μM/20μM | Tocris | None expected |
| ONO-AE3-208 | EP4 antagonist | 10μM | Tocris | None expected |
| Aspirin | COX-1/2 inhibitor | 10μM | Sigma | |
| Valeryl Salicylate | COX-1 inhibitor | 10μM | Sigma | |
| NS-398 | COX-2 inhibitor | 20μM | Sigma | |
| STATTIC | STAT3 inhibitor | 20μM | Tocris | |
| IWP2 | WNT inhibitor | 5μM | Tocris | |

### 2.3.8 Immunofluorescent labelling using monovalent fab fragment antibody labelling

In order to immunolabel cryosections and isolated colonic crypts with two primary antibodies raised in the same species, a monovalent fab fragment secondary antibody protocol was developed. Monovalent fab fragments bind to a single antigen site on the primary antibody, and as such following incubation with an excess of monovalent antibody, the surface of immunoglobulins are covered and therefore no longer bind to any additional appropriate secondary antibody; i.e. a rabbit primary antibody bound to a monovalent fab fragment secondary would no longer bind an anti-rabbit secondary antibody (**Figure 2.3**). This was required for EP receptor labelling in conjunction with Mucin-2 labelling in cryosections and isolated crypts, as both antibodies are raised in rabbit.

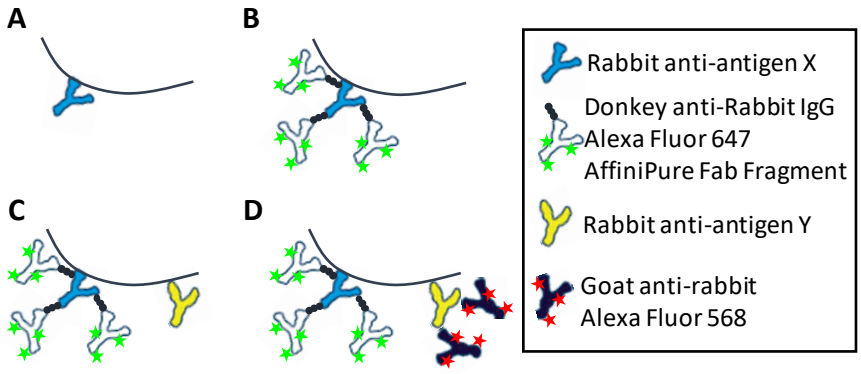


**Figure 2.3. Monovalent fab fragment antibody labelling**

(**A**) Samples are incubated with the first primary antibody overnight– rabbit anti-antigen X, and washed. (**B**) An excess of monovalent fab fragment bound to a donkey anti-rabbit IgG conjugated with Alexa Fluor 647 is added for 3h before washing. (**C**) Samples are incubated with the second primary antibody – rabbit anti-antigen Y and washed. (**D**) A goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 568 is added, before washing and visualisation.

In order to permeabilise samples, a similar protocol to that utilised for Immunofluorescent labelling of samples was used (**section 2.3.5**). Briefly, samples were incubated with 1% SDS and 0.5% Triton-X, blocked with 10% donkey serum and 1% bovine serum albumin for 2h and incubated with rabbit anti-Muc-2 or rabbit anti-EP1/2/3/4 primary antibodies diluted (1:100 or 1:50) in PBS overnight at 4°C. Samples were stained with donkey anti-rabbit monovalent fab fragment secondary antibody for 3h at 4°C, washed in PBS and blocked for a further 2h with 10% donkey serum and 1% BSA. Samples were incubated with rabbit anti-Muc-2 or rabbit anti-EP1/2/3/4 and goat anti-e-cadherin primary antibodies diluted in PBS overnight

at 4°C followed by the corresponding fluorescent-conjugated secondary antibodies for 2h. Antibody specificity was determined using primary and secondary antibody negative controls (**Figure 2.4**). Sections were mounted with Vectashield containing DAPI and imaged using a confocal microscope (Zeiss 510 META) and an X40 1.3 NA oil-immersion objective or an X63 1.4 0.75-mm WD oil-immersion objective.

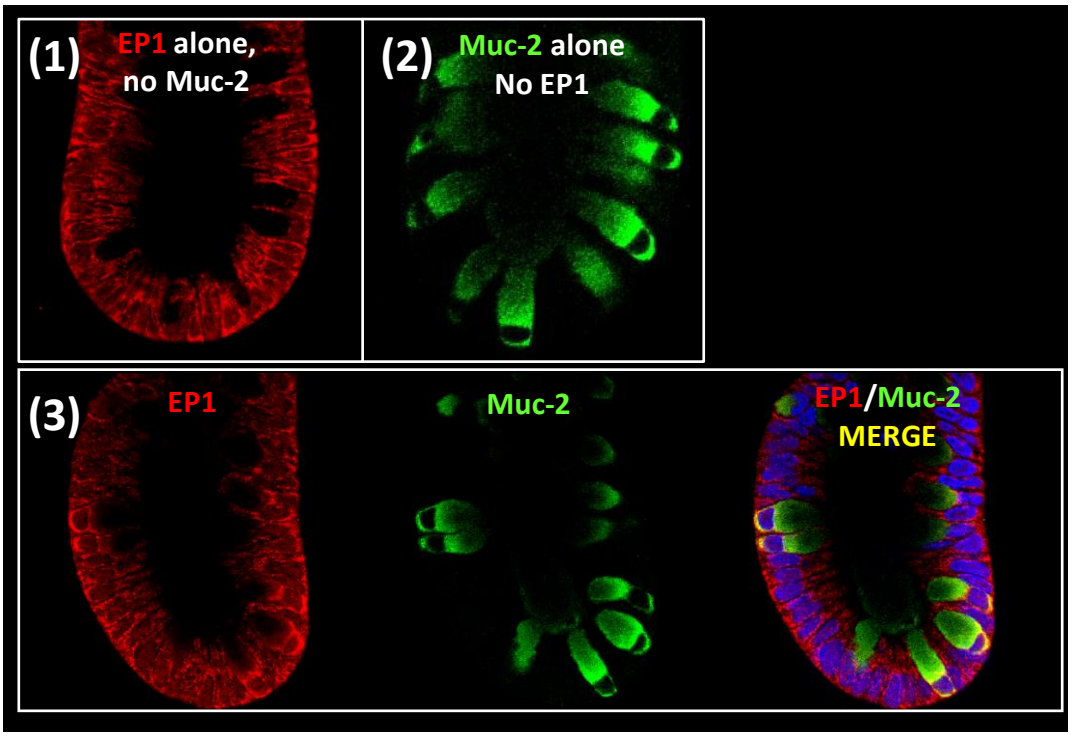


**Figure 2.4. Controls for fab fragment antibody labelling.**

(**1**) Rabbit anti-EP1 primary antibody with appropriate secondary antibody (red). (**2**) Rabbit anti-Muc-2 antibody with appropriate secondary antibody (green). (**3**) Combined labelling of two rabbit raised primary antibodies using the fab fragment labelling system. Left: rabbit anti-EP1 (red), mid: rabbit anti-muc-2 (green), right: rabbit anti-EP1 and rabbit anti-muc-2 merged image.

## 2.4 Human studies

### 2.4.1 Human Colorectal Tissue Samples

This study has been approved by the East of England National Research Ethics Committee (2013/2014 – 62 HT (ongoing approval)). Colorectal tissue samples were obtained with informed consent from patients undergoing sigmoid endoscopy, right-hemicolectomy or anterior resection procedures at the Norfolk and Norwich University Hospital. Histologically

normal mucosa samples were obtained from at least 10cm away from the tumour sites, and only used if there was no apparent intestinal pathology.

### 2.4.2 Human Fixed Tissue Sections

Human colonic mucosal biopsies were immediately fixed in 4% paraformaldehyde for 2h at 4˚C. Following fixation, tissue was washed in PBS and left overnight. Tissue was either: embedded in OCT and frozen on liquid nitrogen in Isopentane before cutting 8µm thick sections on a cryostat; or dehydrated with xylene and ethanol before paraffin embedding and sectioning (5µm thick) on a microtome.

### 2.4.3 Isolation and culture of human colonic crypts

Human colonic crypts were isolated by members of the Williams Laboratory using methodology previously described (Reynolds et al., 2007; Reynolds et al., 2014; Parris and Williams, 2015). Briefly, fresh intestinal tissue samples were collected in ice cold PBS, transported to the laboratory and incubated in HBS, which was devoid of $Ca^{2+}$ and $Mg^{2+}$, and supplemented with EDTA (1mM) for 1h at room temperature. Crypts were liberated by vigorous shaking, crypt sedimentation and collection. Crypts were embedded in growth-factor reduced matrigel and a 20µl droplet was seeded on no.0 glass coverslips contained within a 12 well plate. After polymerisation at 37˚C for 5-10min, crypts were flooded with human colonic crypt culture medium (hCCCM): advanced F12/DMEM containing B27, N2, n-acetylcysteine (1mM), hepes (10mM), pen/strep (100U/ml) L-Glutamine (2mM), Wnt-3A (100ng/ml), IGF-1 (50ng/ml), Noggin (100ng/ml) or Gremlin-1 (200ng/ml), RSPO-1 (500ng/ml), and the ALK 4/5/7 inhibitor A83-01-01 (0.5µM). Isolated crypts were cultured for 0-4 days in hCCCM, which was modified further to the stated experimental conditions.

### 2.4.4 Immunohistochemistry of human colonic crypts

Human colonic crypts were fixed in 4% paraformaldehyde for 1h at 4˚C. Crypts were permeabilised with 13min $NH_4Cl_2$, 5min 1% SDS and 30min 1% Triton X-100. Non-specific binding was blocked using 10% donkey serum, 1% BSA for 2h, before addition of primary antibodies overnight at 4˚C (**Table 2.3**). Crypts were incubated for 2h with appropriate Alexa fluor-conjugated secondary antibodies before mounting with Vectashield-Hoescht. Immunolabelling was visualised using laser confocal microscopy (Zeiss LSM), or epifluorescent microscopy (Nikon). Antibody specificity was determined using primary antibody negative controls.

## 2.5 EdU labelling of isolated crypts

Crypts were fixed in 4% paraformaldehyde for 1h (human) or 35min (mouse) at room temperature. Crypts were permeabilised with 13min $NH_4Cl_2$, 5min 1% SDS and 30min 1% Triton X-100. The Click-IT reaction was prepared according to the manufacturers instructions. Crypts were incubated with the Click-IT reaction cocktail for 35min in dark. The reaction was stopped with 3% BSA, and non-specific binding blocked using 10% donkey serum, 1% BSA for 2h. Samples were incubated with primary antibodies overnight at 4˚C. Crypts were incubated for 2h with appropriate Alexa fluor-conjugated secondary antibodies (Invitrogen): before mounting with Vectashield-Hoescht. Antibodies used in these studies are listed in (**Table 2.3**). Fluorescent labelling was visualised using epifluorescent (Nikon Ti) or laser confocal microscopy (Zeiss LSM).

## 2.6 Immune cell-crypt co-culture models

A monocyte-crypt co-culture model has previously been developed by Skoczek et al. (2014) for the study of the effects of monocytes on colonic crypt proliferation. Monocytes derived from the bone marrow, or THP-1 cells were added into co-culture with isolated mouse or human colonic crypts (Skoczek et al., 2014). In this thesis, this technique was adapted to add HL-60-eosinophils or human tissue resident eosinophils isolated from the colonic mucosa into co-culture with isolated human colonic crypts.

The promyelocytic leukemic cell line HL-60 Clone 15 cells have proved a useful tool for eosinophil research. Stimulation of HL-60 cells with butyric acid induces differentiation towards a stable eosinophil phenotype (Fischkoff, 1988). Butyric acid differentiated eosinophils (HL-60-eosinophils) have since been utilised for the study of eosinophil chemotaxis, where similar results have been obtained using HL-60-eosinophils and primary eosinophils isolated from the blood of healthy donors (Fu et al., 2016). HL-60-eosinophils were utilised in co-culture studies with isolated human colonic crypts in these studies.

### 2.6.1 HL-60-eosinophil-crypt co-culture

HL-60 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated FBS, 100U/ml penicillin and 100U/ml streptomycin in a humidified incubator with 5% $CO_2$ at 37˚C. Cells were differentiated towards an eosinophil phenotype as described previously (Fischkoff et al., 1986) by suspending $5x10^6$ cells/ml in RPMI 1640 media containing 10% FBS, 100U/ml penicillin/streptomycin and 0.5mM butyric acid. The media was changed every 2 days, cells

were used for experiments on D5 following addition of butyric acid. Eosinophil phenotype was confirmed using H&E staining to confirm the presence of a bi-lobed nucleus and cytoplasmic acidic granule content, and the expression of eosinophil-specific markers Siglec-8, CCR3 and MBP (**Figure 2.5**). HL-60 cells differentiated towards an eosinophil phenotype are referred to as HL-60/eos in this thesis.

For HL-60-eosinophil-crypt co-culture studies, following 5 days of culture, HL-60-eosinophils were resuspended at different concentrations (5-15x$10^4$) in growth factor reduced matrigel containing isolated human colonic crypts (20-50 crypts per well) and cultured for 2 days in human colonic crypt culture medium (hCCCM), as described in **Section 2.4.3**. For proliferation studies, following 2 days in culture, EdU (10µM) or BrdU (1µM) was added to hCCCM for 4h before fixation in 4% paraformaldehyde. EdU labelling was visualised as described in **Section 2.5**. For visualisation of BrdU labelling, the protocol for Immunofluorescent labelling (as described in **Section 2.3.5**) was undertaken, using a primary antibody for BrdU.

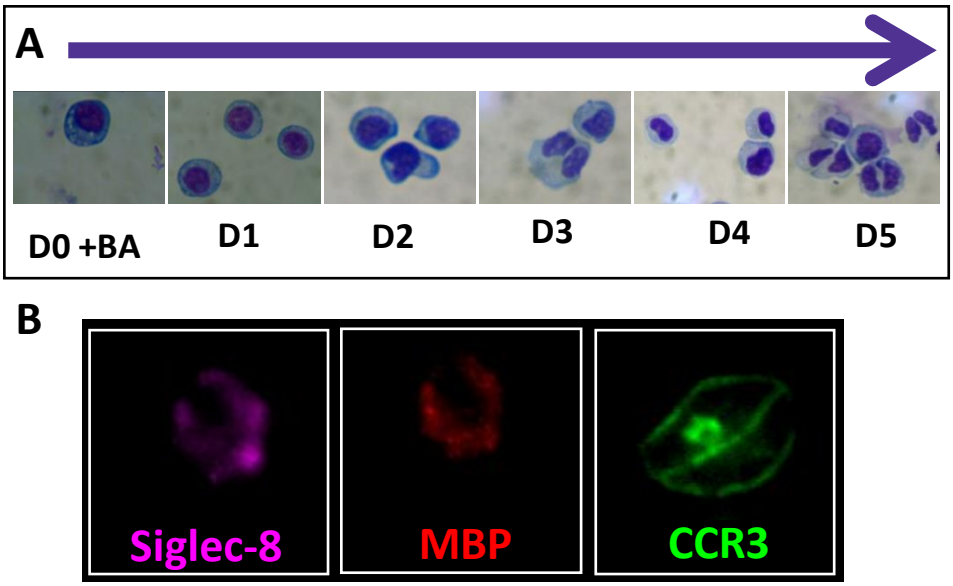


**Figure 2.5. HL-60 cells differentiate towards an eosinophil phenotype.**

(**A**) H&E staining of Butyric acid treated HL-60 clone 15 cells. Butyric acid was added to HL-60 cells (Day 0). Following 5 days in culture, cells are characterised by a bi-lobed nucleus and presence of acidic granules. (**B**) Immunofluorescent labelling of day 5 HL-60-eosinophils showing expression of markers characteristic of eosinophils; Siglec-8 (pink), MBP (red), CCR3 (green).

### 2.6.2 Human tissue eosinophil isolation and purification

Eosinophils isolated from the blood of healthy donors have previously been used in co-culture studies with T84 epithelial cells (Jawien et al., 2002; Furuta et al., 2005). Cell density separation using Ficoll-Paque or a Percoll gradient, followed by Magnetic-Activated Cell Sorting (MACS) using anti-CD16 beads to remove neutrophils are the purification methods of choice (Sedgwick et al., 1996; Jawien et al., 2002; Farahi et al., 2007; Bates et al., 2010; Akuthota et al., 2012). Immune cells have previously been isolated from the small intestine of mice for the study of the effects of eosinophils on plasma cells *in vitro*. Chu et al. (2014) used an EDTA-mediated removal of the epithelium, before a collagenase digestion to disassociate immune cells within the lamina propria followed by flow cytometric cell sorting using antibodies specific for markers for eosinophils (B220/CD3, CD45, CD11b, Fr-1 and Siglec-F), B cells and T cells (Chu et al., 2011). These techniques were adapted in this thesis in order to develop a novel protocol for the isolation and purification of eosinophils from the lamina propria of human colonic tissue.

Human tissue resident immune cells were isolated from the lamina propria or human colorectal tissue samples obtained from patients undergoing either a right hemicolectomy or anterior resection at the NNUH. Where the diagnosis was cancer, histologically normal mucosa samples were obtained from at least 10cm away from the tumour sites, and only used if there was no apparent intestinal pathology. Tissue was also obtained from patients undergoing resection due to IBD (Crohn's disease or UC) or Diverticular disease. Intestinal tissue samples were collected in ice cold PBS and transported to the laboratory.

#### 2.6.2.1 Removal of epithelium

Full thickness fresh intestinal tissue samples were stripped of underlying smooth muscle and submucosal layers to leave only the mucosa and cut into approximately 3mm$^2$ pieces (**Figure 2.6**). Approximately 1-1.5g of tissue samples were placed into a 50ml falcon tube containing 10ml Hepes-buffered saline (HBS): NaCl (140mM), KCl (5mM), Hepes (10mM), d-glucose (5.5mM), $Na_2HPO_4$ (1mM); which was devoid of $Ca^{2+}$ and $Mg^{2+}$, supplemented with EDTA (1mM) for 15 minutes at 37˚C. The falcon tube was inverted, and samples transferred to a new 50ml falcon tube containing HBS devoid of $Ca^{2+}$ and $Mg^{2+}$, supplemented with EDTA for 15mins. This was repeated for a total of 6 x 15 minute incubations, which was sufficient to remove the majority of crypts from the mucosa. Tissue was then washed 3x with HBS devoid

of $Ca^{2+}$ and $Mg^{2+}$ to ensure maximal removal of crypts. Sufficient removal of the epithelium was confirmed by light microscopy.

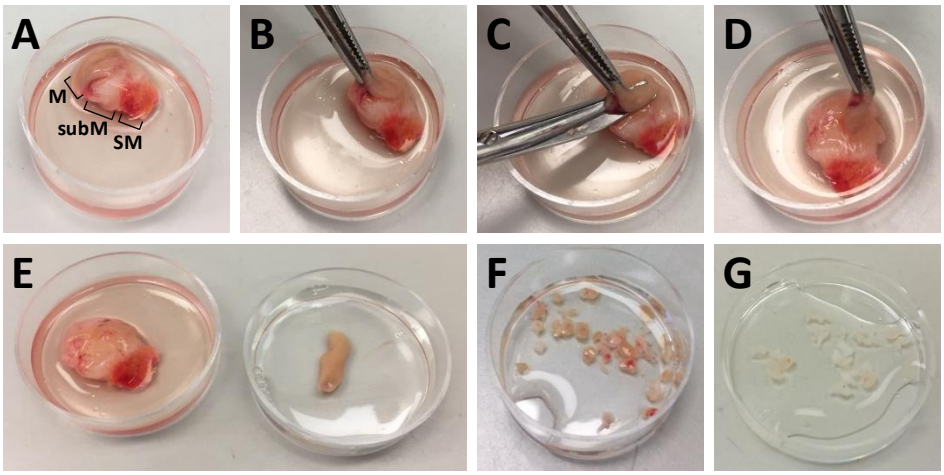


**Figure 2.6. Preparation of colonic tissue for enzymatic isolation of mucosal immune cells.**

(**A**) Full thickness human colon sample is obtained, M=mucosa, subM=submucosa, SM=smooth muscle. (**B**) The mucosal layer containing the epithelium and immune cells is held with forceps, (**C**) The underlying submucosa and smooth muscle is removed by cutting with scissors, (**D**) the mucosa is removed with forceps. (**E**) Submucosa and smooth muscle are removed – LEFT, mucosal sample is washed in HBS –RIGHT. (**F**) Mucosal sample is cut into approximately $2mm^2$ pieces prior to EDTA treatment to remove epithelium. (**G**) Following EDTA treatment, tissue samples are washed in HBS to ensure maximal removal of crypts, before addition to a falcon tube containing enzymatic digestion buffer.

#### 2.6.2.2 Digestion of lamina propria

Samples consisting of lamina propria were placed into RPMI media supplemented with 10% FBS, containing 2.5mg/ml Collagenase A, and 0.5mg/ml DNase I for 2h on a shaker at 37˚C. Tissue samples were filtered through a 20µm filter and washed 3x in ice cold PBS. The average yield was $9.0^6$ cells/mg, of which approximately 33% were eosinophils before purification was attempted. These numbers are however only an estimate as the donor/preparation variation was high; the cell yield was 1.8-$22.7^6$, of which 15.2-64.5% were eosinophils. Cells were resuspended in 2ml RPMI media containing 10% FBS before separation using Percoll gradient separation.

### 2.6.2.3 Percoll gradient separation

Cells suspended in RPMI media were underlain with 45% Percoll, and subsequent 70% Percoll layers, and centrifuged at 280g for 13 minutes with no brake (**Figure 2.7**). Cells were obtained from the 45%/ 70% Percoll interface using a sterile Pasteur pipette and washed 3x with ice cold PBS before resuspending in MACS buffer: PBS containing 1mM EDTA. Protocol for Percoll gradient separation was adapted from protocol used for separation of granulocytes in blood (Farahi et al., 2007).

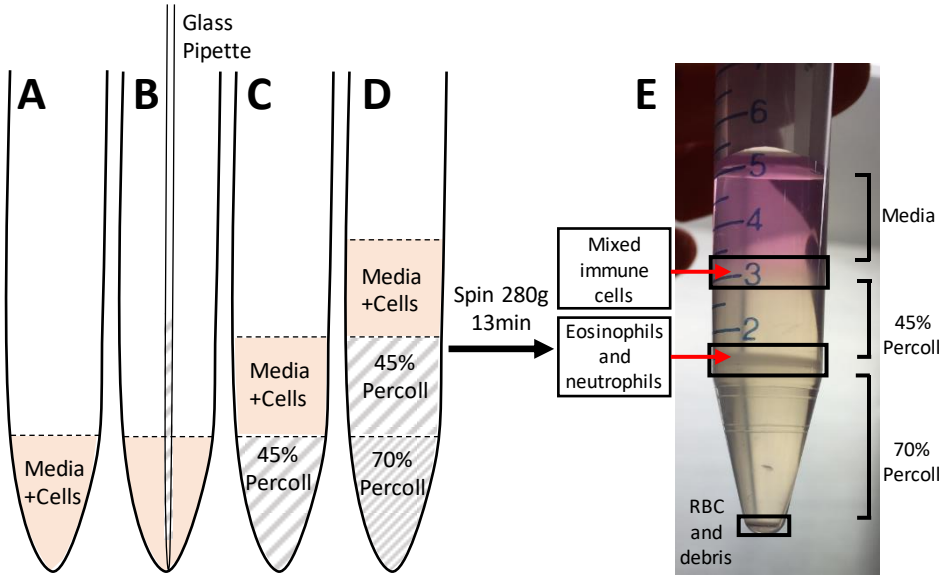


**Figure 2.7. Protocol for Percoll gradient separation of cells.**

(**A**) Mixed immune cell population is resuspended in 2ml of RPMI media. (**B**) A glass pipette containing 45% Percoll is carefully placed into the bottom of the falcon tube. (**C**) Release 45% Percoll to underlay the cell suspension. (**D**) Repeat stage (**B**) this time containing 70% Percoll and carefully release under the 45% Percoll. (**E**) Following centrifugation at 280g for 13min, a pure population of eosinophils and neutrophils is present at the interface between 45% and 70% Percoll.

### 2.6.2.4 MACS separation

Negative selection of eosinophils using a Quadromacs cell separator (Miltenyi Biotec, SF lab) and a commercially available kit for eosinophil purification (Miltenyi Biotec) was undertaken in order to purify the eosinophil population further following Percoll separation. The kit was previously optimised for the purification of eosinophils from blood and contains beads for markers of neutrophils, macrophages, dendritic cells, B cells, NK cells and red blood cells (see **Table 2.5**). All these cell types have been described to be resident in the human colonic

lamina propria. However, the relative level of contamination of these cell types in the lamina propria is less well defined, and additional CD14 beads were required in order to obtain a population of eosinophils with a higher purity. The kit was used in accordance with the manufacturer's instructions, with an additional 10µl of anti-CD14 beads added.

Positive selection utilising anti-CD15 beads conjugated to biotin was also attempted, however high levels of neutrophil contamination were observed and therefore negative selection was deemed superior for eosinophil purification in the case of human tissue resident eosinophil purification.

**Table 2.5. MACS negative selection for purification of human eosinophils**

| Marker | Cell Type |
|---|---|
| CD2 | T cells |
| CD14 | Macrophages, neutrophils, dendritic cells |
| CD16 | NK cells, macrophages, neutrophils |
| CD19 | B cells |
| CD56 | NK cells, neurons |
| CD123 (IL-3R) | B cells |
| CD235a (Glycophorin A) | Red blood cells |

#### 2.6.2.5 Macrophage adhesion

Due to macrophages being the dominant contaminating cell type following MACS separation, and the inherent adhesive property of macrophages, a macrophage adhesion step was introduced in order to try to improve the purity of eosinophil population. This step was attempted either before or after MACS separation. Cells were placed into a T25 flask and left in a 37˚C/ 5%$CO^2$ humidified incubator for 4 hours. Following this step, cells in suspension were removed and eosinophil purity determined using H+E staining, however no increase in the purity of eosinophils was observed following the macrophage adhesion step (N=3).

### 2.6.2.6 Eosinophil Characterisation

Purity of eosinophils was assessed at each stage of isolation and purification utilising Hematoxylin & Eosin (H&E) staining (**Figure 2.8A** and **B**). Eosinophils are characterised by a bi-lobed nucleus and the presence of acidic granules which stain pink following H&E staining (**Figure 2.8C**).

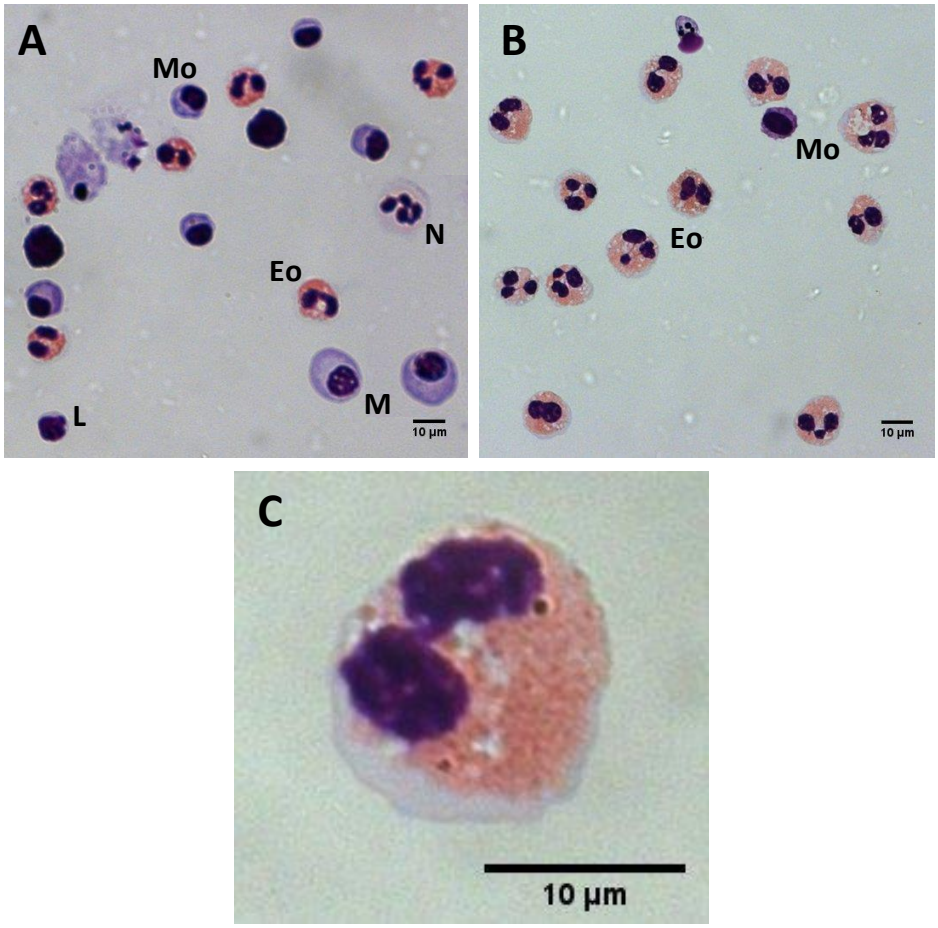


**Figure 2.8. Characterisation of immune cells using H&E staining**

(**A**) H&E staining of mixed immune cell population containing 35% eosinophils (Eo). Contaminating cells include neutrophils (N), macrophages (M), monocytes (mo) and lymphocytes (L). (**B**) H&E staining of purified eosinophil population following Percoll gradient separation and negative selection using MACS, population is 85% eosinophils (Eo), contaminating cells include monocytes (Mo). (**C**) H&E image of eosinophil showing characteristic bilobed nucleus and pink granules.

## 2.7 PCR

RNA from freshly isolated colonic or small intestinal crypts, tissue biopsy, or isolated immune cells was isolated using the Isolate II RNA mini kit (Bioline) according to manufacturers instructions. Total RNA yield was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific) and purity assessed by the ratio of absorbance at 260 and 280nm. cDNA was generated from 0.5µg of RNA using the RT2 First Strand cDNA synthesis kit (Qiagen).

End-point PCR reactions were performed in a final volume of 25 µl, comprising 200 nm forward and reverse primers, 200 µM dNTP, 0.5 U Taq polymerase, PCR buffer (Roche), 2.5 mM $MgCl_2$ and 0.5 µl cDNA using a Tprofessional TRIO thermal cycler (Biometra) with the following thermal profile: 1 cycle at 94°C for 3 mins, 30 cycles at 94°C for 25 s, 58°C for 30 s, and 72°C for 50 s and 1 cycle at 72°C for 5 mins. The Primer sequences used are listed in **Table 2.6**. The following primers were kindly provided by SF lab: CCR1, CCR3, CCR7, CCR9, CCR10, CCRL2, CCL2, CCL5.

**Table 2.6. Conventional PCR primers utilised in the study.**

| Gene | Accession | Primer Sequence (F) | Primer Sequence (R) | Product size (bp) |
|---|---|---|---|---|
| IL-6 | NM_031168 | GCTACCAAACTGGATA TAATCAGGA | CCAGGTAGCTATGGT ACTCCAGAA | 86 |
| WNT3a | NM_033131.3 | CTGCTCAGCTGCGCCC CCTTCTTT | TTCAGCGGCCTCCCC ATTCATTCC | 404 |
| COX 1 | NM_008969 | CCTCTTTCCAGGAGCTC ACA | TCGATGTCACCGTAC AGCTC | 96 |
| COX 2 | NM_011198 | GCCGTACACATCATTTG AAGAA | TGTCACTGTAGAGGG CTTTCAA | 70 |
| EP1 R | NM_013641 | CCTCGTCTGCCTCATCC ATC | CCACCAACACCAGCA GGG | 165 |
| EP2 R | NM_008964 | GCCATTATGACCATCAC CTTCG | CTTTAGGGAAGAGG TTTCATCCAT | 402 |
| EP3 R | NM_011196 | ATCATGTGTGTGCTGT CCGT | GCAGCAGATAAACC CAGGGA | 438 |
| EP4 R | NM_001136079 | CTCATCTGCTCCATTCC GCT | GGATGGGGTTCACA GAAGCA | 351 |
| EP3α | D10204.1 | ATGGGGATCATGTGT GTGCTG | ACCTGGTAGTCTGAA GTCTGGA | 261 |
| EP3β | NM 011196.2 | TGGGGATCATGTGTGT GCTG | TCAGGTTGTTCATCA TCTGGCA | 349 |
| EP3γ | D17406.1 | ATGGGGATCATGTGT GTGCTG | GGCCCTGGCTGTACT ACTTC | 311 |
| 15-PGDH | NM_000860.5 | TTGGAAGACTGGACAT TTTGG | CCTTCACCTCCATTTT GCTT | 145 |
| CCR1 | NM_001295.2 | GCCTACGAGAGTGGAA GCTG | TTCCGGAACCTCTCAC CAAC | 358 |
| CCR3 | NM_001837.3 | TTGTCCATGCTGTGTTT GCC | AAAAATGAGCCGGAT GGCCT | 323 |
| CCR7 | NM_001838.3 | CAAGTCCTGGGTCTTC GGTG | CCAGGACCACCCCAT TGTAG | 497 |
| CCR9 | NM_006641.3 | GGCAATTGCTGACCTC CTCT | CGAAGGGAAGGAAG AACCCC | 413 |
| CCR10 | NM_016602.2 | TTGCTACAAGGCCGAT GTCC | TGGAAGGAGGCCGA GTAGAG | 285 |
| CCRL2 | NM_003965.4 | TCTTCCTTCTGATGTGG GCG | TTCCCTCGATGTGCCT TGTG | 272 |
| CCL2 | NM_002982.3 | GATGCAATCAATGCCC CAGTC | CTTCGGAGTTTGGGT TTGCT | 219 |
| CCL5 | NM_002985.2 | GCATCTGCCTCCCCATA TTCC | AGAGTTGATGTACTC CCGAACC | 201 |

## 2.8 Microscopy

Following Immunohistochemistry, labelling was visualised using fluorescence microscopy which allows for detection of proteins labelled with fluorescent dyes. Proteins of interest are illuminated utilising light of specific wavelengths. When light hits the specimen, the fluorescent protein of interest is excited and emits fluorescence of a longer wavelength, which is detected and visualised. In this study, immunolabelling was visualised using either a laser scanning confocal (Zeiss 510 META) or epifluorescence (Nikon Ti) microscope.

Epifluorescence microscopy uses a high-intensity light source, which emits broad spectrum light which is limited to a narrow range of wavelengths using excitation filters. Emission filters allow specific fluorescence to be detected by the detectors. Epifluorescence microscopy is a relatively fast imaging technique which is useful when high resolution images are not required. In this thesis, a Nikon Ti epifluorescent microscopy was utilised to image cells labelled with fluorescently tagged EdU or BrdU in isolated intestinal crypts and organoids. A x40 1.1 NA oil objective was utilised to capture images.

In order to obtain high resolution images of Immunofluorescent labelling, laser scanning confocal microscopy was utilised to capture images. Confocal microscopy uses the addition of a confocal pinhole in order to eliminate out of focus light and point illumination, whereby only one point of the sample is illuminated by a laser at any one time. For confocal microscopy, a X40 1.3 NA oil objective of X63 1.4NS 0.75mm WD oil immersion objective was used to capture images. Image stacks were taken at 1-3µm intervals which allowed for selection of previse focal planes. In this study, laser scanning confocal microscopy was utilised to characterise the expression of proteins within specific cell types, including EP receptor labelling of colonic crypts and sections, and pSTAT3 expression of the small intestine.

### 2.8.1 Image Analysis

For all experiments, a minimum of between 20 and 100 crypts were counted per condition and each experiment was performed at least three separate times.

For proliferation experiment analysis, ImageJ software was used to count the total number of DAPI labelled cell nuclei in the crypt equatorial plane, and the number of cells that were positive for BrdU was then expressed as a percentage of the total nuclei number. For analysis of EdU positive nuclei in human colonic crypts, the crypts were split into four regions along

the crypt-axis, each containing an equal number of nuclei and designated base, supra-base, mid and top. In some cases, the base and supra-base regions were combined to give three functional regions, base, mid and top (**Figure 2.9**).

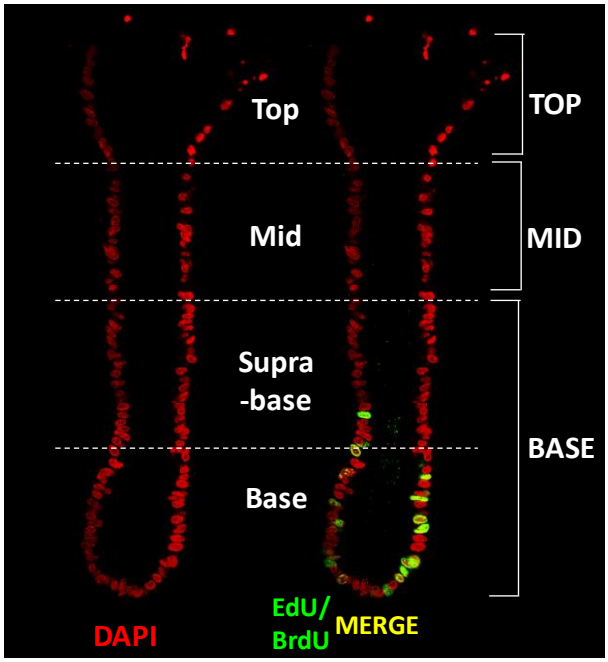


**Figure 2.9. Analysis of human colonic crypt growth experiments.**
Human colonic crypts were split into 3 functional regions along the crypt axis, base, supra-base, mid and top. The number of nuclei (red) incorporating EdU/BrdU (green) were quantified in each functional region, proliferating cells are shown in yellow. In some cases, crypts were split into 3 regions, base, mid and top.

Quantification of organoid survival was determined utilising epifluorescent microscopy. On day 1 of culture, before the addition of experimental groups, organoids were imaged using a timelapse system at 37˚C with 5% humidified carbon dioxide. Regions of interest were saved in order to enable the imaging of the same organoids after incubation with experimental groups but before fixation with paraformaldehyde. Survival was determined through analysis of organoid morphology.

In order to quantify the intensity of Immunofluorescent labelling of proteins, ImageJ software was utilised to draw appropriate regions of interest and measure the average fluorescence intensity within the region. For semi-quantitative analysis of pSTAT3 activation, regions of interest were drawn around the nuclei of Paneth cells (as determined by expression of UEA-1 and/or lysozyme immunofluorescence), and the average fluorescence intensity of pSTAT3 labelling within the region calculated (**Figure 2.10A-C**). Data were normalised to unstimulated control organoids and background corrected. For the analysis of

WNT3 protein expression in small intestinal organoids stimulated with IL-6 or untreated controls, organoids were split into three hierarchical regions, base, mid and top, containing an equal number of nuclei. Regions of interest were drawn below the nuclei and containing the surrounding basal membrane. The average intensity per unit area was background corrected and calculated in control organoids, and IL-6-treated organoids (**Figure 2.10D**).

For analysis of stem cells and Paneth cell number *in vivo*, Lgr5EGFP stem cells, lysozyme or UEA-1 positive Paneth cells were counted by taking a z-stack across the equatorial plane of the crypt and counting the number of nuclei in the stack that were positive for each marker. To determine the crypt length and villus height, at least 25 well orientated crypts and villi were measured using the segmented line tool (Fiji 1.50e) from three animals in each treatment group.

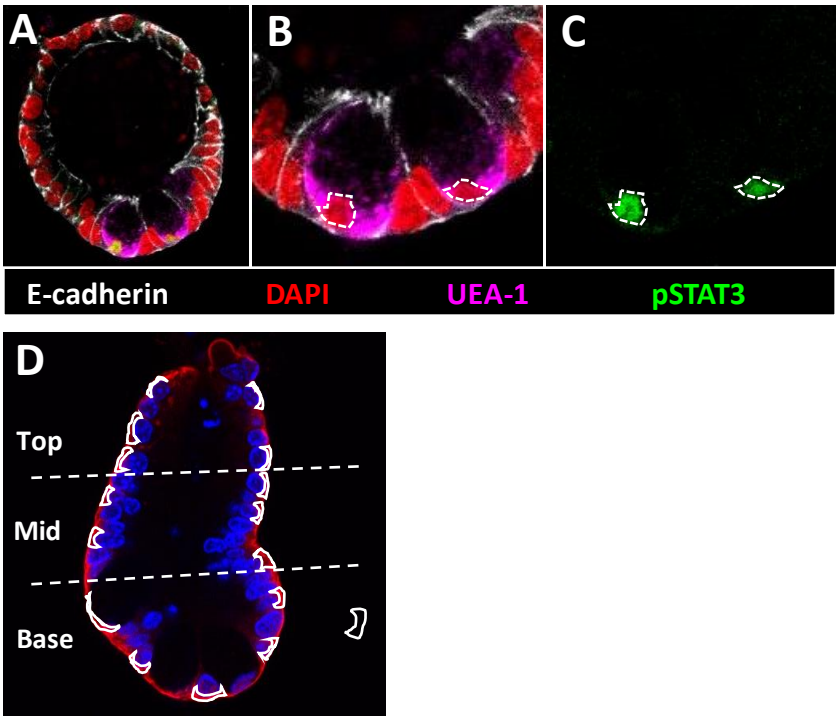


**Figure 2.10. Intensity analysis of Immunofluorescent labelling.**
(**A**) Representative image of small intestinal organoid stimulated with IL-6; nuclei (DAPI-red), UEA-1 (pink), pSTAT3 (green), E-cadherin (white). (**B**) Regions of interest (white dashed) were drawn around the nuclei (DAPI-red) of UEA-1 (pink) positive cells. (**C**) Fluorescence intensity of pSTAT3 (green) was measured in ROI (white dashed). Fluorescence intensity was normalised to average fluorescence observed in ROI drawn in nuclei of UEA-1 positive nuclei of unstimulated organoids. (**D**) Representative image of small intestinal organoid immunolabelled for WNT3 (red). Organoids were split into hierarchical regions, base, mid and top, and regions of interest (white) drawn below the nuclei (blue) and including the basal membrane. Intensity was background corrected.

In order to determine the spatial characteristics of immune cell distribution *in vivo*, colonic crypts were divided into three equal regions, base, mid and top, and the number of immune cells positive for markers of immune cells e.g. Siglec-8, CCR3, MBP, F4/80 or CD14 present in the surrounding lamina propria of crypts were quantified (**Figure 2.11A**). Following 24h in co-culture with isolated human colonic crypts, the spatial distribution of immune cells along the crypt-axis was quantified *in vitro*. Isolated human colonic crypts were divided into three equal regions, base, mid and top, and the number of immune cells present in a 2-cell thick radius surrounding crypts was quantified (**Figure 2.11B**).

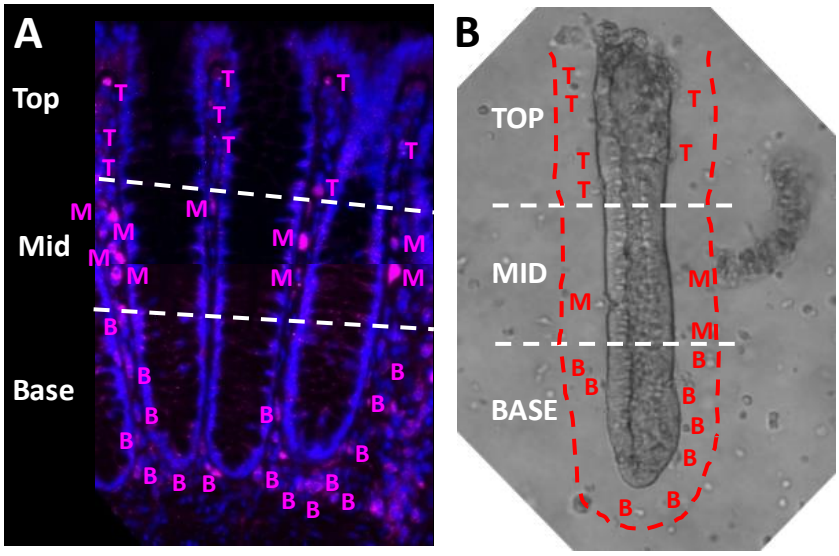


**Figure 2.11. Image analysis for spatial distribution of immune cells along the crypt-axis.**

(**A**) Fixed colonic crypt sections were divided into three equal regions, base mid and top (white dashed lines). The number of immunofluorescently labelled immune cells in the lamina propria surrounding crypts in each region was quantified (pink, B=base, M=mid, T=top) *in vivo*. (**B**) The spatial distribution of immune cells *in vitro* was quantified using white light microscopy. Isolated human colonic crypts were divided into three equal regions, base, mid and top (white). Following 24h in co-culture, the number of immune cells present within a 2-cell thick radius surrounding the crypt (red dashed line) was quantified (red, B=base, M=mid, T=top).

## 2.9 Statistics

Experiments were performed at least three separate times. Data are expressed as mean +/- SEM; N= total number of subjects (i.e. mice/patients), n= number of crypts/organoids derived from those 'N' subjects. Differences between two groups were determined by a paired or unpaired students' t-test; comparisons between two or more groups were determined one-way analysis of variance (ANOVA) with post-hoc Tukey analysis. For each test, a P value of less than 0.05 was considered significant.

For gradient analysis of EP receptor fluorescence intensity, multilevel modelling was carried out on the data to avoid any pseudo-replication i.e. avoid inflating the number of independent samples in the data (Peugh, 2010). The R statistics package was used for the analysis (with the ''lme'' package). Where multilevel modelling indicated that there was a significant ($P<0.05$) difference in the ''crypt area'' variable (levels: base, supra-base, mid and top), pairwise comparison of each level was carried out to assess where the significant difference arose (JD).

# 3 Chapter 3: Autocrine Interleukin-6 Drives Small Intestinal Epithelial Homeostasis

## 3.1 Introduction

The intestinal epithelium is one of the most rapidly renewing tissues in the body. Dynamic renewal in the small intestinal epithelium is achieved by way of Lgr5+ stem cells residing at the base of intestinal crypts (Barker et al., 2007). Stem cells are maintained in the stem cell niche by Paneth cells, which secrete factors including WNT and DLL4 (Sato et al., 2011). Other growth factors and cytokines as well as immune cells have previously been shown to modulate epithelial stem cell driven tissue renewal during homeostasis (Reynolds et al., 2014; Skoczek et al., 2014). The mechanism by which these pathways are regulated in the intestinal epithelium through autocrine signalling is not yet fully understood.

Seminal work in the *Drosophila* midgut demonstrated that following acute stress such as enteric infection, enterocytes are stimulated to produce Upd3, an IL-6-like cytokine, which activates JAK/STAT signalling in intestinal stem cells to promote cell division and therefore regeneration (Jiang et al., 2009; Osman et al., 2012). In the mammalian gut, STAT3 and IL-6 are known to play a role in regeneration of the intestinal epithelium following injury (Pickert et al., 2009; Kuhn et al., 2014) and to promote the survival of epithelial cells (Grivennikov et al., 2009; Jin et al., 2010) during inflammation and IBDs (Bollrath et al., 2009; Lee et al., 2012). Mice with an IEC-specific depletion of STAT3 were highly susceptible to the induction of experimental colitis, and showed a significant defect in colonic epithelial regeneration (Pickert et al., 2009). In addition, following bowel injury induced by biopsy or bacterial triggered colitis, inhibition of IL-6 decreased epithelial proliferation and resulted in impaired wound healing (Kuhn et al., 2014). Use of an IL-6-/- mouse demonstrated that whilst there was no changes in the basal rate of proliferation in the colon of IL-6KO mice, a significant decrease in proliferation was observed following DSS exposure compared to wild type suggesting that IL-6 is required for proliferation following induction of inflammation. IL-6 was shown to promote tumour growth by enhancing proliferation of tumour initiating cells, however a role for IL-6 in the survival of normal intestinal tissue was also identified (Grivennikov et al., 2009). Furthermore, Jin et al. (2010) observed that IL-6 reduced enterocyte apoptosis in the small intestine by activation of anti-apoptotic proteins (Jin, Zimmers et al. 2010).

Interleukin-6 is a pleiotropic cytokine involved in a plethora of cellular and immune responses in health and disease (Mihara et al., 2012; Garbers et al., 2015; Hunter and Jones, 2015). IL-6 has traditionally been associated with pro-inflammatory responses including B cell activation and immune cell recruitment in response to pathogenic challenge, however IL-6 is also emerging as an important cytokine in the regulation of immune cells during homeostasis. In the intestinal mucosa, secretion of IL-6 by eosinophils has also been shown to be vital for the maintenance of plasma cells in the lamina propria, which in turn is crucial for the maintenance of long-term immunity (Chu et al., 2014).

Classical IL-6 signalling is traditionally associated with homeostasis (Scheller et al., 2011) and occurs through binding of IL-6 to the membrane-bound IL-6R, which then complexes to the ubiquitously expressed gp130 receptor. This results in activation of downstream signalling components including JAK and STAT3 (Garbers et al., 2015). Several mouse models have identified a role for IL-6 classic signalling in the activation of anti-inflammatory pathways on target cells, including apoptosis of neutrophils which is key to the resolution of inflammation (Kaplanski et al., 2003). Furthermore, IL-6 classic signalling has been shown to be essential for the regeneration of intestinal epithelial cells following DSS-induced tissue injury (Kuhn et al., 2014).

Cells which do not express the IL-6R are still able to respond to IL-6 *via* trans-signalling. Soluble IL-6R (sIL-6R) is shed from the cell membrane through proteolytic cleavage, and binds to IL-6. The IL-6/sIL-6R complex can then activate IL-6 signalling in any cell expressing the gp130 protein, which is constitutively expressed by the intestinal epithelium (Scheller et al., 2011). The trans-signalling pathway has been associated with inflammatory disorders including Crohn's disease and rheumatoid arthritis, and cancer (Jones et al., 2005; Scheller et al., 2011). IL-6 trans-signalling has a pivotal role in the switch from innate to adaptive immunity; following acute neutrophil infiltration, proteolytic cleavage of the IL-6 receptor drives trans-signalling in resident tissue cells, which then leads to switching from neutrophil to monocyte recruitment to the inflamed area. IL-6 is also required for the recruitment of T cells, and trans-signalling on T-cells leads to inhibition of $T_{reg}$ cell differentiation, and promotion of differentiation into $T_{H17}$ cells, which have been implicated in autoimmune tissue injury.

Autocrine IL-6 signalling has been implicated in pro-inflammatory responses in a number of systems. In lung cancers with an associated EGFR mutation, constitutive activation of STAT3 was shown to be required for tumour cell growth through autocrine IL-6 signalling (Gao et

al., 2007). HER2 overexpressing breast adenocarcinomas express high levels of IL-6 which contribute to the induction of proliferation and HER2-mediated oncogenesis through activation of STAT3 (Hartman et al., 2011). Furthermore, in experimental inflammatory conditions, mouse biliary epithelial cells secrete and respond to IL-6 through upregulation and activation of IL6R/STAT3 signalling in an autocrine manner (Yokomuro et al., 2000). However, the roles of IL-6 autocrine signalling in homeostasis remain unclear.

The aim of this programme of work was to determine the roles of classical IL-6 signalling in the modulation of small intestinal crypt homeostasis and renewal. This work demonstrates a previously undefined role for autocrine IL-6 signalling in the maintenance of the crypt stem cell niche, *via* differential expression of the IL-6 receptor and pSTAT3 activation in Paneth cells, with involvement of the WNT signalling pathway.

## 3.2 Results

In order to investigate the role of IL-6 signalling during homeostasis, a variety of approaches including mouse *in vitro* organoid culture and *in vivo* experiments were undertaken. The effects of IL-6 and inhibition of the STAT3 signalling pathway on proliferation, stem cell number and budding were determined using *in vitro* organoid culture. The status of STAT3 signalling within the small intestine was characterised in *in vitro* organoid culture, as well as *in vivo* utilising tissue from control and IL-6 knockout mice. Furthermore, the effects of perturbation of the IL-6 signalling pathway *in vivo* using antibodies to neutralise IL-6 or block the IL-6 receptor, on stem cell number, Paneth cell number and tissue morphology were investigated.

### 3.2.1 IL-6 stimulates proliferation of small intestinal organoids *via* a STAT3-mediated signalling pathway

In order to determine the effects of exogenous IL-6 on the proliferation of small intestinal organoids, IL-6 (10-1000ng/ml) and BrdU (1μM) was added to mouse small intestinal organoids on Day 1 following isolation, for 17 hours as described in **Section 2.3.4**, and the percentage of nuclei incorporating BrdU was visualised using fluorescence microscopy (**Section 2.3.5**). Representative fluorescence images of BrdU incorporation of mouse small intestinal organoids following IL-6 stimulation are shown in **Figure 3.1A**. A significant increase in incorporation of BrdU was observed following addition of 10 and 100ng/ml IL-6. A bell-shaped response curve was exhibited at higher concentrations (1000ng/ml), with no increase in BrdU incorporation compared to control (**Figure 3.1B**). Further experiments utilised 100ng/ml IL-6 for stimulation due to the consistent significant increase in organoid proliferation. STATTIC is a small molecule which has been shown to selectively inhibit STAT3 activation, dimerization and nuclear translocation of STAT3 (Schust et al., 2006). Previous studies have utilised 20μM STATTIC for the inhibition of STAT3 in intestinal organoids (Lindemans et al., 2015). Incubation of organoids with STATTIC (20μM) and IL-6 (100ng/ml) showed a significant reduction in BrdU incorporation compared to IL-6 alone (**Figure 3.1C**). Organoid survival (determined as described in **Section 2.8.1**) following STATTIC treatment in the presence or absence of IL-6, and IL-6 alone were comparable to control (**Figure 3.2A**). However, a small but significant increase in the percentage of caspase-3 positive cells (a marker of apoptosis) per organoid was observed following incubation with STATTIC

compared to control; the percentage of caspase-3 positive cells per crypt in organoids treated with IL-6 alone was comparable to control (**Figure 3.2B**).

Whilst 17 hour treatment of organoids with STATTIC had no effect on basal proliferation (see **Figure 3.1C**), this was in contrast to the effects seen following treatment of organoids with STATTIC for 48h from the point of isolation, where a significant decrease in BrdU incorporation was observed compared to untreated organoids (**Figure 3.3A**). Chronic stimulation of organoids with IL-6 induced a significant increase in organoid proliferation following 5 days of IL-6 treatment (**Figure 3.3B**).

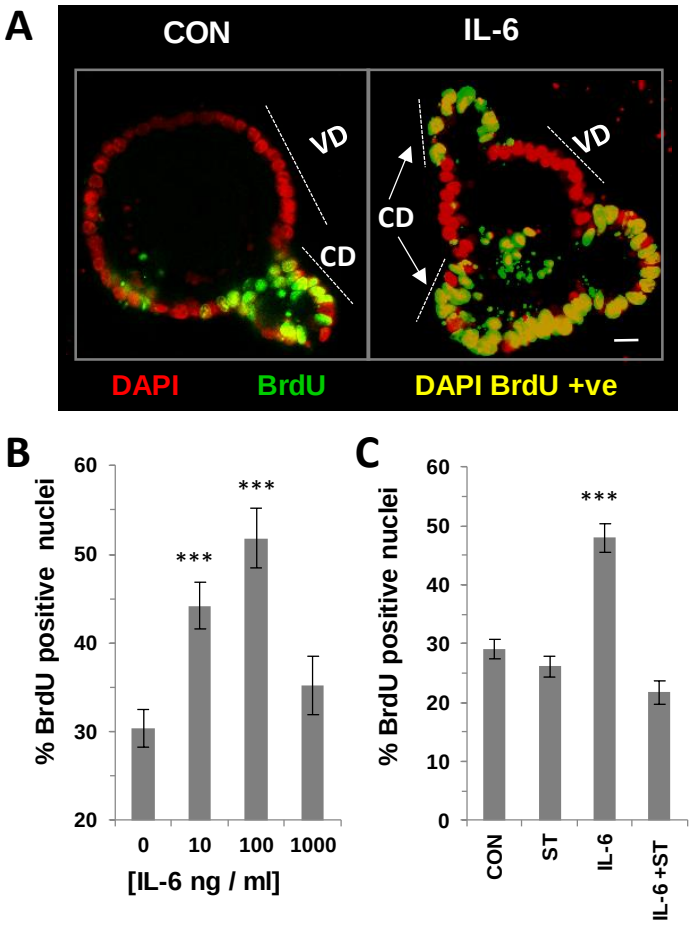


**Figure 3.1. IL-6 stimulates proliferation of small intestinal organoids via a STAT3-mediated signalling pathway.**
(**A**) Representative confocal images of BrdU incorporation (green) into the nuclei (DAPI; red) of small intestinal organoids cultured in the absence or presence of IL-6 (100 ng / ml). Co-labelling of DAPI and BrdU positive cells shown in yellow, scale bar 10µm. CD indicates crypt domain, VD indicates villi domains (**B**) Histogram showing the percentage of BrdU positive nuclei in mouse small intestinal organoids following 24 hours IL-6 (10-1000 ng / ml) stimulation compared to control (N=3, n≥30, ***P<0.001). (**C**) Histogram showing percentage of BrdU positive nuclei in mouse small intestinal organoids following 24 hour IL-6 stimulation (100 ng / ml) in the presence or absence of STATTIC (20µM) and compared to control (N=3, n≥118 ***P<0.001). (**A**) and (**B**) obtained by AS.

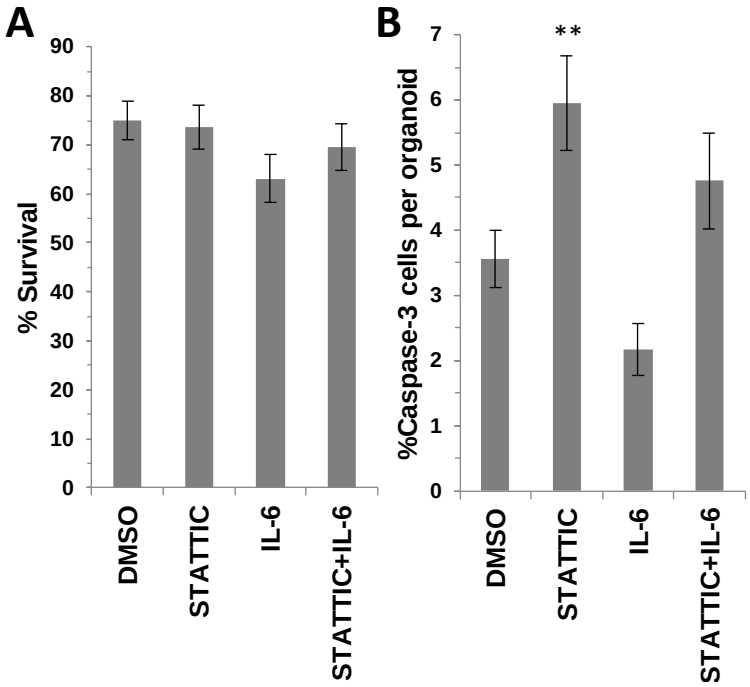


**Figure 3.2. Inhibition of STAT3 induces apoptosis of small intestinal organoids.**

(**A**) Histogram showing organoid survival following stimulation with IL-6 (100ng/ml) in the presence or absence of STATTIC (20μM), and compared to DMSO control (N=3, n≥118). (**B**) Histogram showing a significant increase in percentage of caspase-3 positive cells in organoids treated with STATTIC compared to control organoids (N=3, n≥118, **P<0.01).

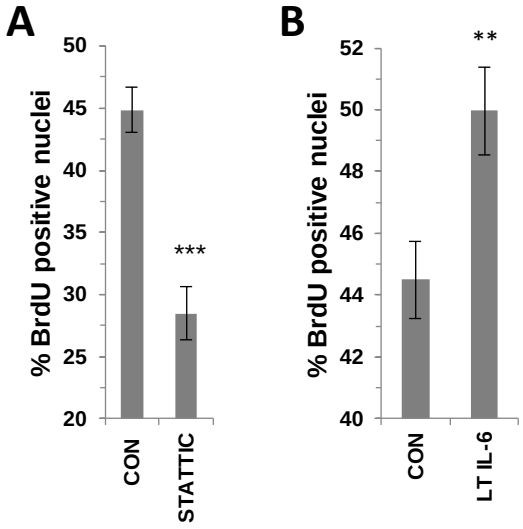


**Figure 3.3. Chronic exposure of small intestinal organoids to STATTIC or IL-6 modulates proliferation in vitro.**

(**A**) Histogram showing a significant reduction in the percentage of BrdU positive nuclei in small intestinal organoids following 48 hour culture with STATTIC (20μM) in full growth factor media compared to control organoids (N=3, n≥124, ***P<0.001) AS. **B**) Histogram showing a significant increase in the percentage of BrdU positive nuclei in small intestinal organoids following stimulation with IL-6 (100ng/ml) for 5 days (N=4, n≥94 **P<0.01).

### 3.2.2 IL-6 induces nuclear activation of pSTAT3 in Paneth cells of the small intestine *via* differential expression of the IL-6 receptor

Next, the effects of IL-6 on pSTAT3 activation in the small intestinal epithelium were investigated using immunolabelling and visualisation of pSTAT3 activation in Day 1 mouse small intestinal organoids by confocal microscopy as described in **Section 2.3.5**. When no IL-6 was added, pSTAT3 activation was not commonly observed in organoids (**Figure 3.4A**, left panel, white arrows). Following 1 hour of stimulation with IL-6, pSTAT3 was observed in the nucleus of UEA-1 and lysozyme positive cells, which was abrogated by a blocking peptide to pSTAT3 (**Figure 3.4A,** mid and right panels). All lysozyme positive cells also expressed UEA-1, and displayed morphology characteristic of Paneth cells (**Figure 3.4A** white light images, top row). As shown utilising a projection of images taken across multiple Z planes, following 1h IL-6 stimulation, all UEA-1 positive cells in a crypt domain were positive for nuclear pSTAT3 (**Figure 3.4B**). No fluorescent labelling was observed in IgG controls for the Lysozyme antibody (**Figure 3.4C**). Whilst the majority of untreated control organoids expressed no pSTAT3 (as represented in **Figure 3.4A**), nuclear pSTAT3 fluorescence significantly above baseline was observed in UEA-1 positive cells of 9 out of 113 control organoids counted.

In order to assess the temporal effects of IL-6 stimulation on pSTAT3 activation, organoids were exposed to IL-6 (100ng/ml or 1000ng/ml) for 30 minutes to 6 hours before fixation and immunolabelling for pSTAT3. Maximal activation of nuclear pSTAT3 in UEA-1 positive cells occurred 30 minutes following stimulation with IL-6, and persisted for 1 and 3 hours after stimulation (**Figure 3.5A**). Semi-quantitative analysis showed that the level of nuclear pSTAT3 activation remained significantly higher than unstimulated controls for 30 minutes, 1 hour and 3 hours following stimulation with IL-6. After 6 hours of exposure to IL-6 there was no significant increase in pSTAT3 fluorescence in UEA-1 positive cells compared to control organoids. Furthermore, there was no significant difference in the level of pSTAT3 activation when organoids were stimulated with either 100 ng/ml or 1000ng/ml IL-6 (**Figure 3.5B**).

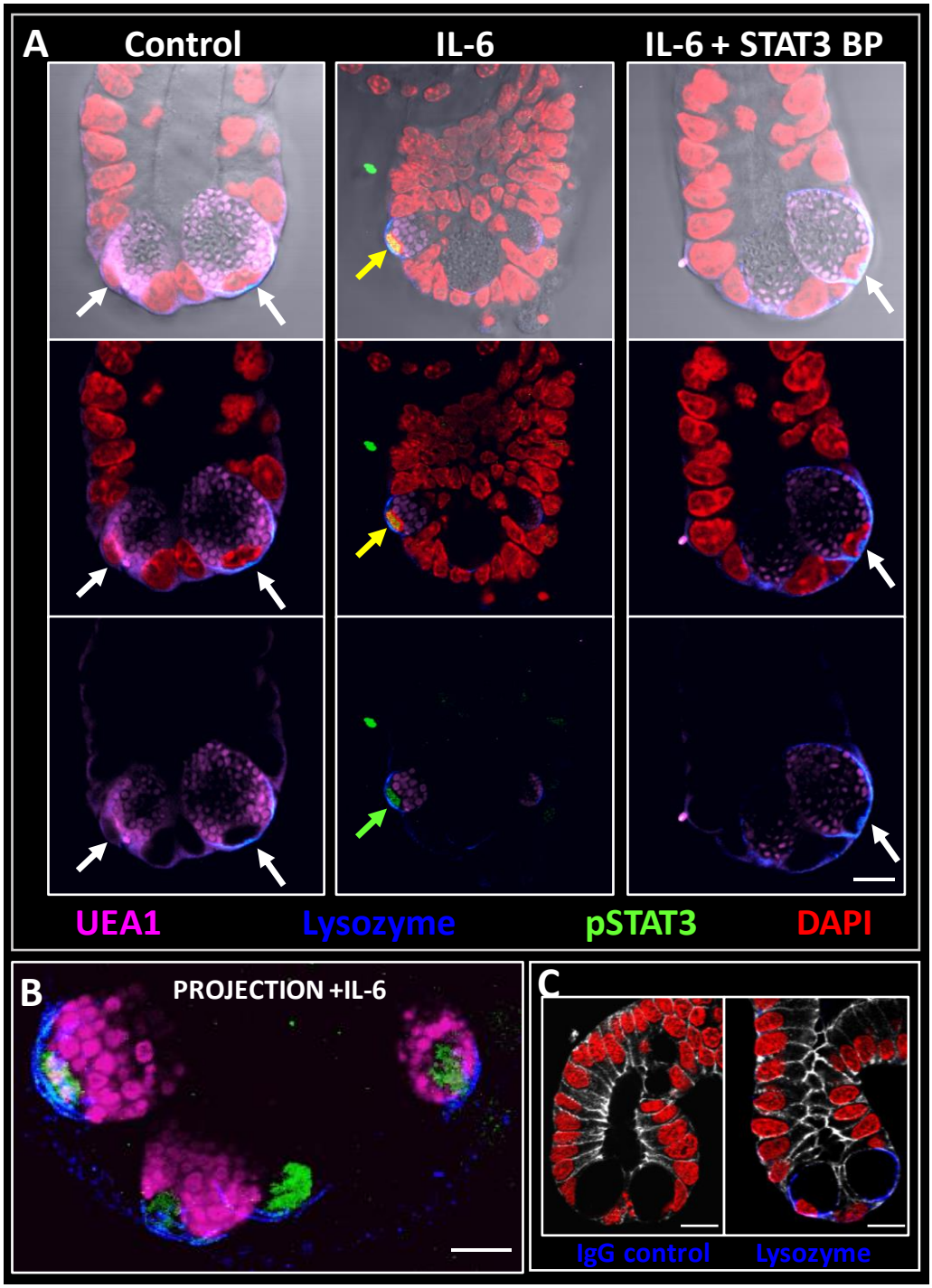


**Figure 3.4. IL-6 induces nuclear activation of pSTAT3 in Paneth cells of the small intestine.**

(**A**) Representative confocal images showing pSTAT3 (green) immunofluorescent labelling in the nuclei (DAPI- red) of UEA-1 (pink), lysozyme (blue) cells in organoids following 1 hour IL-6 (100 ng/ml) stimulation in the presence or absence of a pSTAT3 blocking peptide. (**B**) Confocal image projection pSTAT3 in the nuclei (DAPI-red) of all UEA-1(pink) lysozyme (blue) cells following 1h IL-6 (100 ng/ml) stimulation. (**C**) Representative confocal image showing absence of labelling with IgG control for lysozyme antibody (blue), nuclei (DAPI-red), E-cadherin (white). Scale bar 10µm, images are representative of N>3 independent experiments, n≥30.

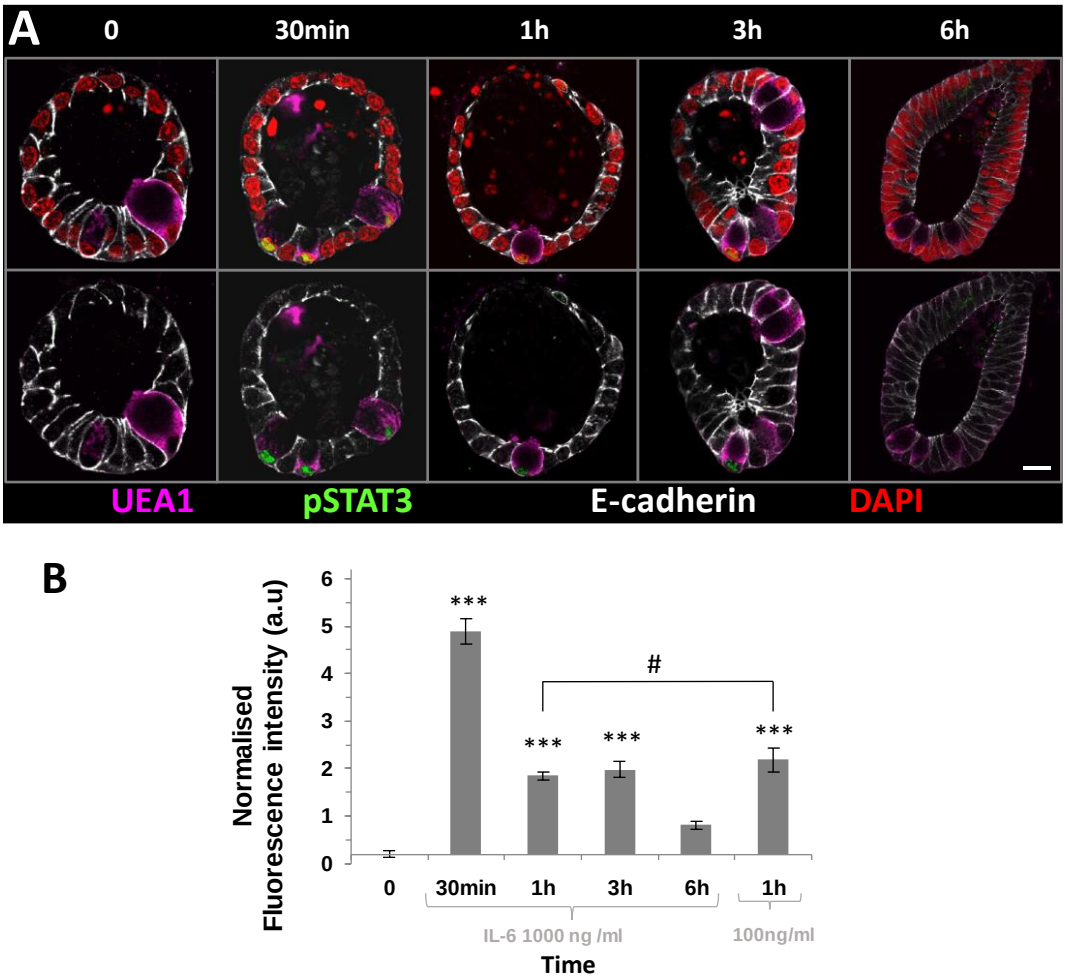


**Figure 3.5. Nuclear activation of pSTAT3 in Paneth cells occurs 30 minutes after stimulation with IL-6.**

(**A**) Representative confocal images of pSTAT3 (green) in the nuclei (DAPI - red) of UEA-1 (pink) positive cells in organoids stimulated with IL-6 (1000 ng/ml) for 0-6 hours, E-cadherin - white. (**B**) Histogram showing the time course of IL-6 induced pSTAT3 activation in UEA-1 positive cells from 0 to 6 hours post-stimulation using 1000 or 100 ng/ml. Data expressed as fluorescence intensity (a.u) and normalised to control UEA-1 positive nuclei (N=3, n≥104 ***P<0.001, #P>0.05 ns). Scale bar 10µm.

As pSTAT3 activation is known to be a downstream effect of IL-6 receptor activation, the expression of the IL-6 receptor within small intestinal organoids and *in vivo* in intestinal tissue sections was characterised using fluorescent microscopy. The localisation of the IL-6 receptor in organoids was restricted to the basal membrane of lysozyme positive cells of the small intestine *in vitro* (**Figure 3.6A**). There was also evidence of the IL-6 receptor being secreted from lysozyme positive cells, into the lumen of small intestinal organoids *in vitro* (**Figure 3.6B**). When the IL-6 receptor was immunofluorescently labelled *in vivo*, a similar distribution to the *in vitro* organoid labelling was observed; IL-6 receptor expression was observed on the basal membrane of lysozyme positive cells, with no IL-6 receptor expression observed in Lgr5EGFP stem cells (**Figure 3.6C).** IL-6 receptor expression was also observed on the membrane of intracellular granules and on the membrane at the apical pole of lysozyme positive cells (**Figure 3.6D**).

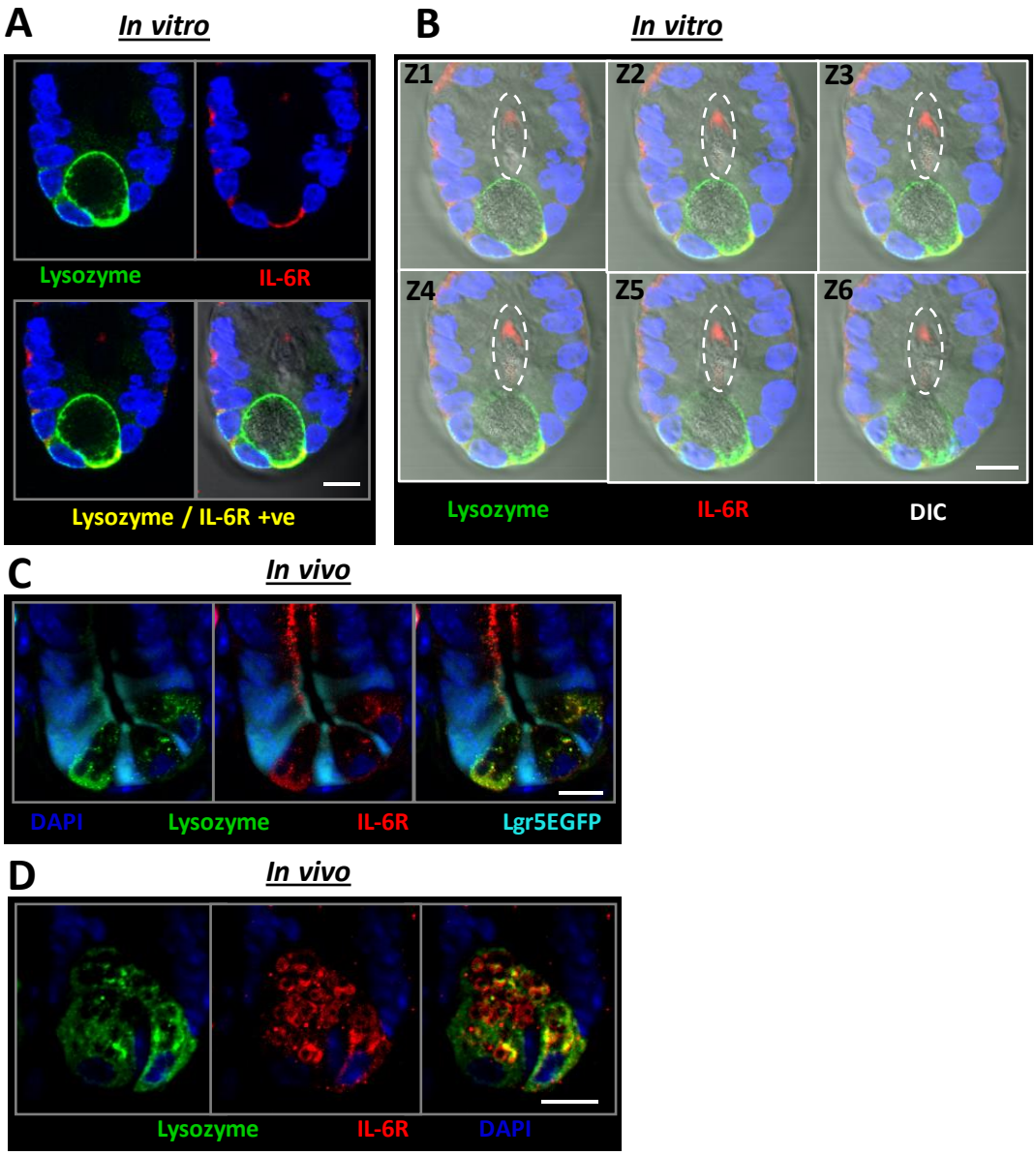


**Figure 3.6. Expression of the IL-6 receptor is restricted to Paneth cells.**

(**A**) Representative confocal images showing IL-6 receptor (red) expression in lysozyme (green) positive cells in cultured small intestinal organoids (representative image from one focal plane). (**B**) Consecutive z-stack confocal images showing IL-6 receptor (red) expression in the lumen of small intestinal organoids, white dashed oval. (**C**) Representative confocal images of small intestine taken from Lgr5EGFP (cyan) mice showing IL-6 receptor localisation (red) on the basal membrane of lysozyme positive cells (green). (**D**) Representative confocal images of IL-6 receptor (red) on intracellular granules and on the apical membrane of lysozyme positive cells (green). Confocal imaging data is representative of N=3 independent experiments, n≥10. Scale bar 10 µm.

Cells which do not express the IL-6R are still able to respond to IL-6 through trans-signalling, whereby soluble IL-6R binds IL-6 and can then activate signalling in any cell expressing gp130, which is constitutively expressed by the epithelium (Scheller et al., 2011). We therefore also confirmed that in our system, mouse small intestinal organoids show ubiquitous expression of the gp130 protein (**Figure 3.7**). The localisation of the gp130 protein was observed on the basal membrane of small intestinal organoids, as well as the baso-lateral membrane in all cells of the small intestinal organoids (**Figure 3.7A**). Expression of gp130 was observed on Lysozyme positive cells, on the basolateral membrane and in the intracellular space (**Figure 3.7B**, yellow arrows).

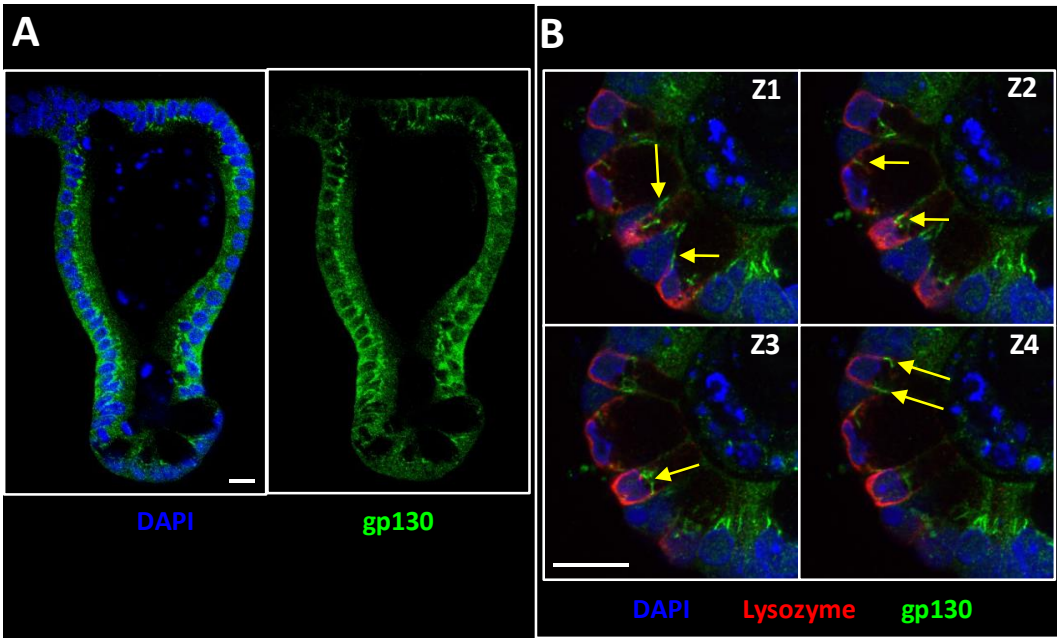


**Figure 3.7. Gp130 is ubiquitously expressed by small intestinal organoids.**

(**A**) Representative confocal image showing gp130 (green) expression in small intestinal organoids, nuclei (DAPI-blue), image representative of a single focal plane. (**B**) Representative confocal image showing gp130 (green) expression in Lysozyme (red) positive cells of small intestinal organoids. Images were taken across consecutive z-planes of 1µm thick. Confocal imaging data is representative of N>3 independent experiments, n≥20. Scale bar 10µm.

The ability to modulate the IL-6 STAT3 signalling axis in mouse small intestinal organoids utilising an IL-6 receptor blocking antibody or IL-6 neutralising antibody in *in vitro* culture is demonstrated in **Figures 3.8** and **3.9**. Day 1 mouse small intestinal organoids were pre-incubated with an IL-6 receptor blocking antibody or IgG control before stimulation with IL-6 and subsequent immunofluorescence labelling of pSTAT3 (as described in **Section 2.3.4**). As observed previously, stimulation of organoids with IL-6 (in the presence of IgG control), induced pSTAT3 activation in UEA-1 positive cells (**Figure 3.8A**). Treatment of organoids with an IL-6 receptor blocking antibody and IL-6 abrogated the IL-6-induced pSTAT3 activation in UEA-1 positive cells (**Figure 3.8A**). Semi-quantitative analysis of pSTAT3 fluorescence intensity in the nuclei of UEA-1 positive cells (described in detail in **Section 2.8.1, Figure 2.9**) demonstrated that treatment of organoids with an IL-6 receptor blocking antibody (100µM) and IL-6 (1000ng/ml) significantly reduced pSTAT3 fluorescence in Paneth cells compared to IgG plus IL-6 (**Figure 3.8B**). This suggests that a concentration of 100µM IL-6 receptor blocking antibody is sufficient to inhibit IL-6 induced activation of pSTAT3 *in vitro*. A similar experimental approach was taken utilising the IL-6 neutralising antibody, whereby media containing IL-6 was pre-incubated with an IL-6 neutralising antibody or IgG control before addition to Day 1 mouse small intestinal organoids and subsequent immunofluorescent labelling of pSTAT3 and UEA-1. When organoids were stimulated with media containing IL-6 pre-incubated with an IL-6 neutralising antibody, the IL-6-induced pSTAT3 activation in UEA-1 positive cells was abrogated (**Figure 3.9A**). Semi-quantitative analysis of pSTAT3 fluorescence intensity in the nuclei of UEA-1 positive cells showed a significant reduction in pSTAT3 intensity following treatment with an IL-6 neutralising antibody (100µM) with IL-6 (1000ng/ml) compared to IgG plus IL-6 (**Figure 3.9B**). Similarly, this suggests that 100µM IL-6 neutralising antibody is sufficient to inhibit STAT3 activation *in vitro*.

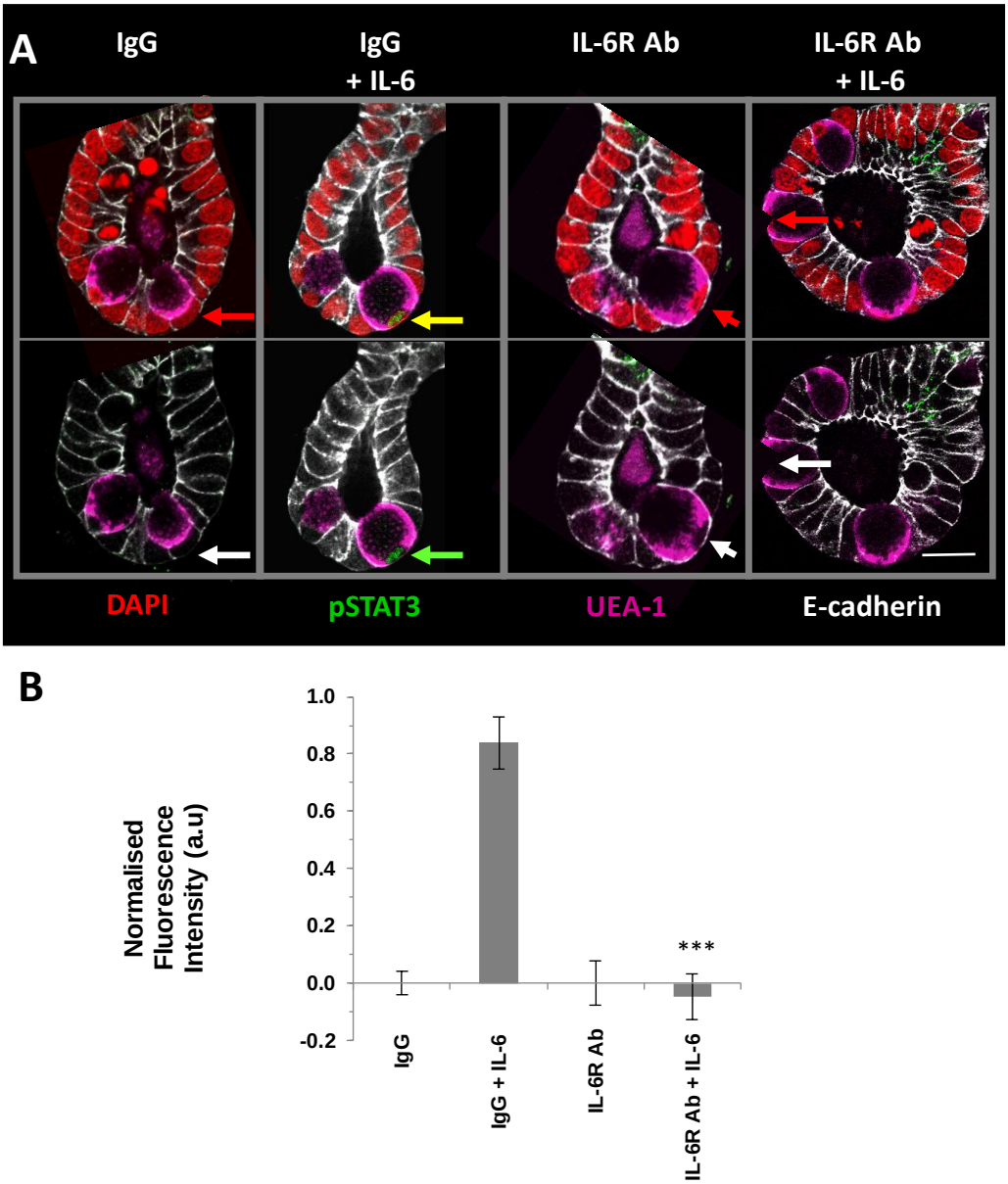


**Figure 3.8. Blocking the IL-6 receptor abrogates IL-6-induced pSTAT3 activation in Paneth cells.**

(**A**) Representative confocal images showing abrogation of IL-6 induced pSTAT3 (green) labelling in the nucleus (DAPI-red) of UEA-1 (pink) positive cells in organoids treated with an IL-6 receptor blocking antibody compared to IL-6 + IgG treated organoids. Red (top) and white (bottom) arrows denote lack of pSTAT3; yellow (top) and green (bottom) arrows denote pSTAT3 labelling. (**B**) Histogram showing a significant reduction in IL-6 (1000ng/ml) induced pSTAT3 fluorescence intensity (a.u.) in organoids treated with an IL-6 receptor blocking antibody (100µM) compared to IgG+IL-6 treated organoids. Fluorescence intensity was normalised to control (N=2, n≥32, ***P<0.001). Scale bars 10µm.

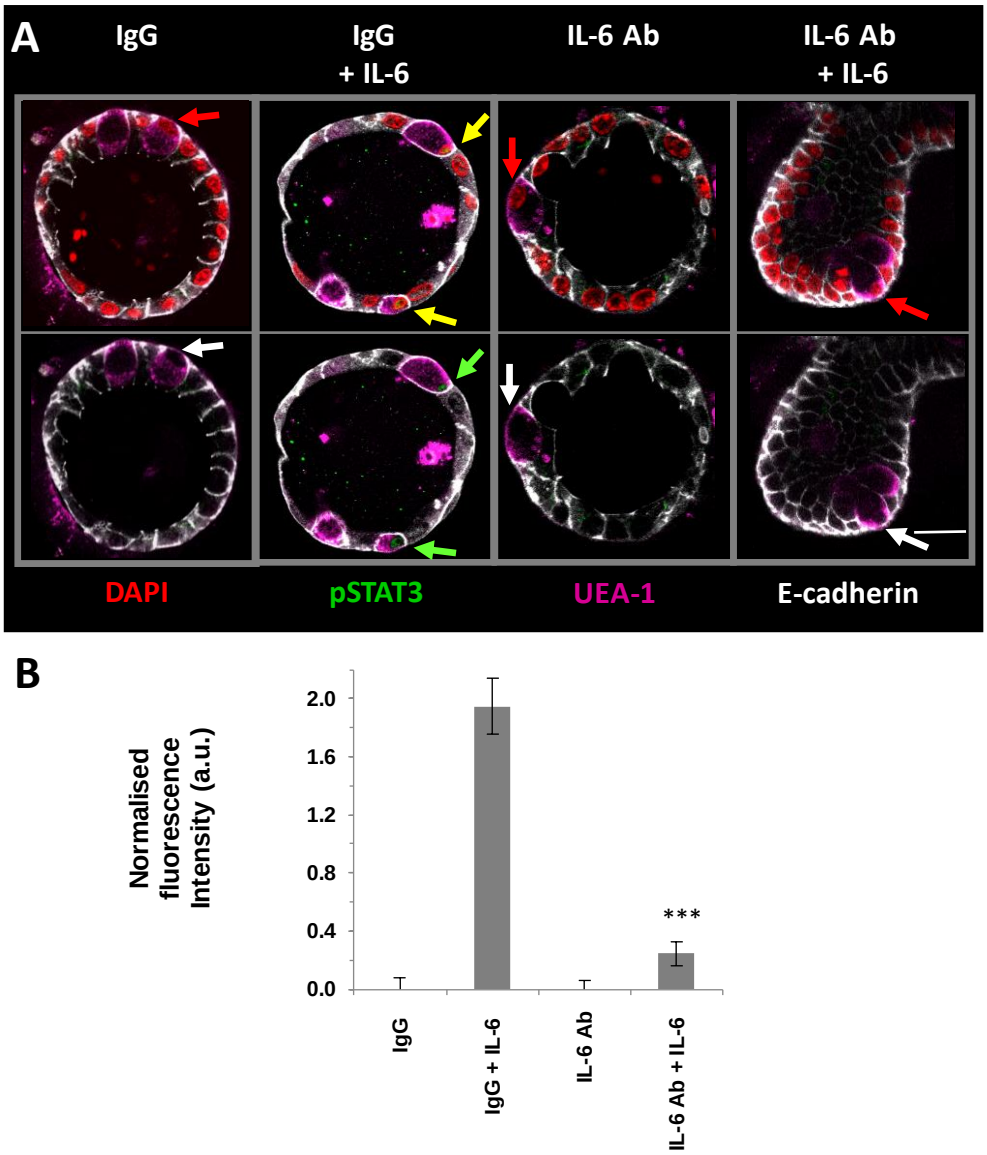


**Figure 3.9. Neutralising IL-6 abrogates IL-6-induced pSTAT3 activation in Paneth cells.**

(**A**) Representative confocal images showing abrogation of IL-6 induced pSTAT3 (green) labelling in the nucleus (DAPI-red) of UEA-1 (pink) positive cells in organoids treated with an IL-6 neutralising antibody compared to IL-6 + IgG treated organoids. Red (top) and white (bottom) arrows denote lack of pSTAT3; yellow (top) and green (bottom) arrows denote pSTAT3 labelling. (**B**) Histogram showing a significant reduction in IL-6 (1000ng/ml) induced pSTAT3 fluorescence intensity (a.u.) in organoids treated with an IL-6 neutralising antibody (100µM) compared to IgG+IL-6 treated organoids. Fluorescence intensity was normalised to control (N=2, n≥44, ***P<0.001). Scale bars 10µm.

### 3.2.3 Autocrine IL-6 signalling in the small intestinal epithelium modulates crypt cell proliferation *in vitro*

In order to determine whether autocrine IL-6 signalling played a role in crypt homeostasis, the expression of IL-6 mRNA and protein in small intestinal tissue was characterised. Firstly, conventional PCR was utilised to demonstrate the expression of IL-6 mRNA in small intestinal organoids *in vivo* in freshly isolated (0h) and in *in vitro* cultured small intestinal organoids (48h) (**Figure 3.10A**). Using Immunofluorescent labelling of the IL-6 protein, it was demonstrated that IL-6, was localised inside epithelial cells towards the basal pole of cells, and in extracellular discrete pools surrounding the basal membrane of organoids (see white arrows, (**Figure 3.10B**). When autocrine IL-6 signalling was blocked using an IL-6 receptor blocking antibody (**Figure 3.10C**) or an IL-6 neutralising antibody (**Figure 3.10D**), a significant decrease in the percentage of crypt nuclei incorporating BrdU was observed compared to the respective IgG control antibodies.

The ability of these antibodies to functionally block IL-6 signalling in small intestinal organoids was assessed by incubating organoids with either the IL-6 receptor blocking antibody or the IL-6 neutralising antibody in combination with IL-6. In these experiments, a significant reduction in the percentage of organoid nuclei incorporating BrdU was observed in organoids treated with either the IL-6 receptor blocking antibody plus IL-6 or the IL-6 neutralising antibody plus IL-6, compared to the respective IgG control plus IL-6 (**Figure 3.11A** and **3.11B** respectively).

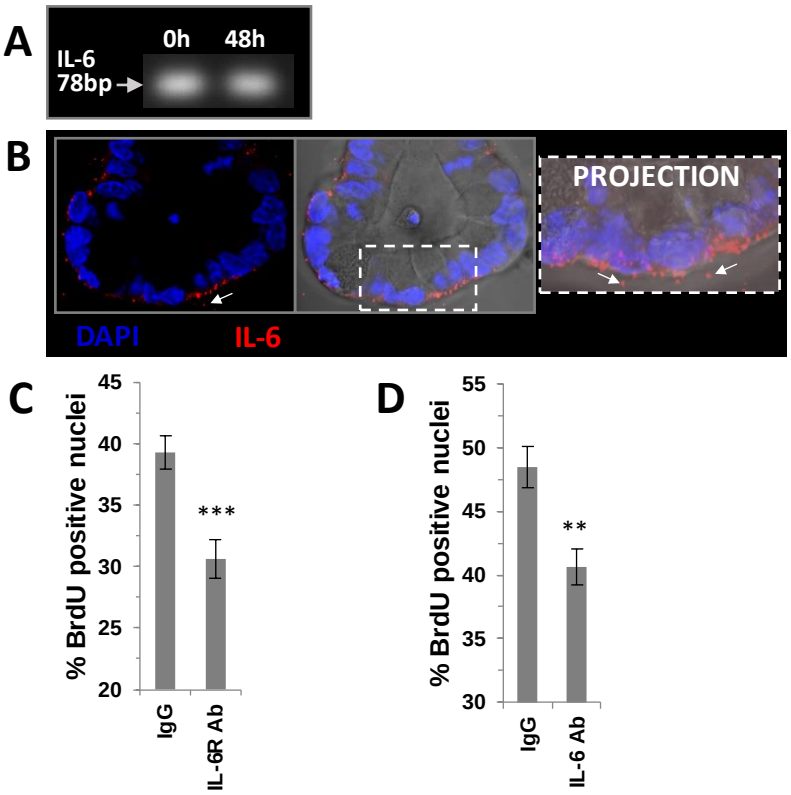


**Figure 3.10. IL-6 signalling regulates small intestinal organoid proliferation.**

(**A**) PCR gel showing IL-6 transcript expression in *in vivo* / freshly isolated (0 h) and cultured (48 h) small intestinal crypts; PCR results obtained AG (**B**) Representative confocal image showing immunofluorescent labelling of IL-6 (red), nuclei (DAPI-blue) of small intestinal organoids and associated projection image *in vitro*. White arrows indicate extracellular pools of IL-6. Scale bar 10 µm. (**C**) Histogram showing percentage BrdU positive nuclei in mouse small intestinal organoids in the presence of an IL-6 receptor blocking antibody (100µM, N=3, n≥152, **P<0.01) and (**D**) an IL-6 neutralising antibody (100µM, N=3, n≥157, ***P<0.001) compared to respective IgG control.

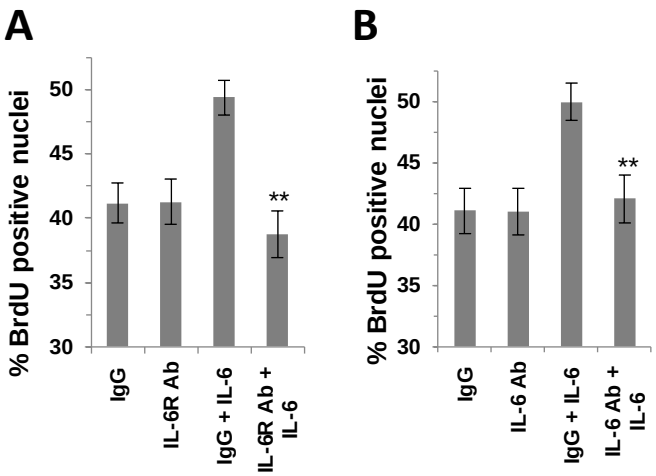


**Figure 3.11. IL-6 receptor blocking or IL-6 neutralising antibodies abrogate the IL-6-induced increase in organoid proliferation.**

Histograms showing percentage positive BrdU nuclei in mouse small intestinal organoids in the presence of IL-6 (100ng/ml) with (**A**) an IL-6 receptor blocking antibody, or (**B**) an IL-6 neutralising antibody (100µM, N=3, n≥83, **P<0.01).

### 3.2.4 Lack of gut phenotype in IL6KO mice

In order to understand the effects of perturbation of the IL-6 signalling pathway *in vivo*, small intestinal tissue was obtained from IL-6KO mice. No difference was observed in the average crypt length, the number of nuclei per crypt or the number of lysozyme positive cells per crypt in IL-6KO tissue compared to WT tissue (**Figure 3.12**). Next, the level of pSTAT3 expressed within the small intestinal epithelium was determined using immunolabelling. Representative confocal images of low level pSTAT3 expression in UEA-1 positive cells in WT and IL-6KO tissue are shown in **Figure 3.13A**, high levels of expression was seen in a small population of crypts (**Figure 3.13B**). Zoomed projections of low and high pSTAT3 labelling are displayed in **Figure 3.13C**. The specificity of the pSTAT3 antibody labelling was confirmed using a blocking peptide to pSTAT3 which abrogated the pSTAT3 signal in UEA-1 positive cells (**Figure 3.13D**). Quantitative analysis of the number of UEA-1 cells expressing pSTAT3 at the base of small intestinal crypts *in vivo* revealed no difference in the number of UEA-1 positive cells expressing pSTAT3 in IL-6KO tissue compared to WT (**Figure 3.1E**). As the levels of pSTAT3 expression observed by the epithelium was generally low, the level of pSTAT3 fluorescence was categorised into none, low, medium and high expression, with the aim of detecting subtle differences between IL-6KO tissue and control. Semi-quantitative analysis of pSTAT3 fluorescence intensity confirmed that expression of pSTAT3 in UEA-1 positive cells at the base of crypts was generally low, with a small percentage of cells expressing high levels of pSTAT3. No differences were observed between IL-6KO and control tissue (**Table 3.1**).

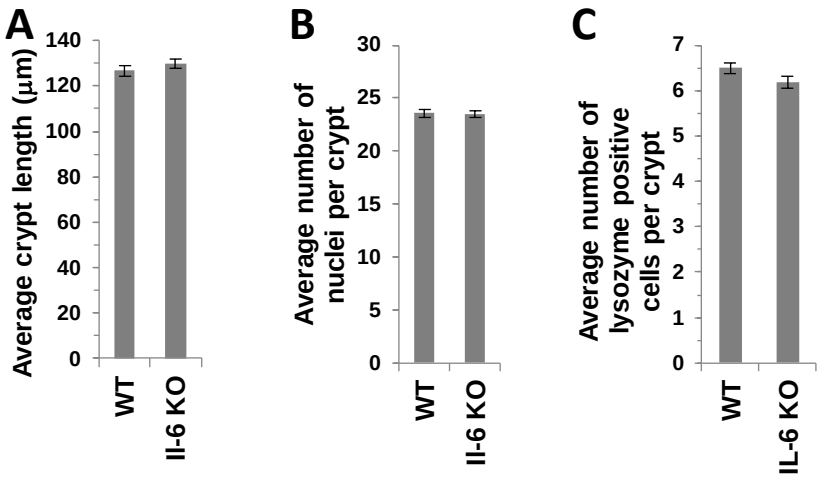


**Figure 3.12. Crypt morphology of IL-6KO tissue is the same as WT.**

Histogram showing (**A**) the average crypt length (**B**) the average number of crypt nuclei and (**C**) the average number of lysozyme positive cells per crypt in the small intestine of IL-6 knockout mice and WT (N=3, n≥20)

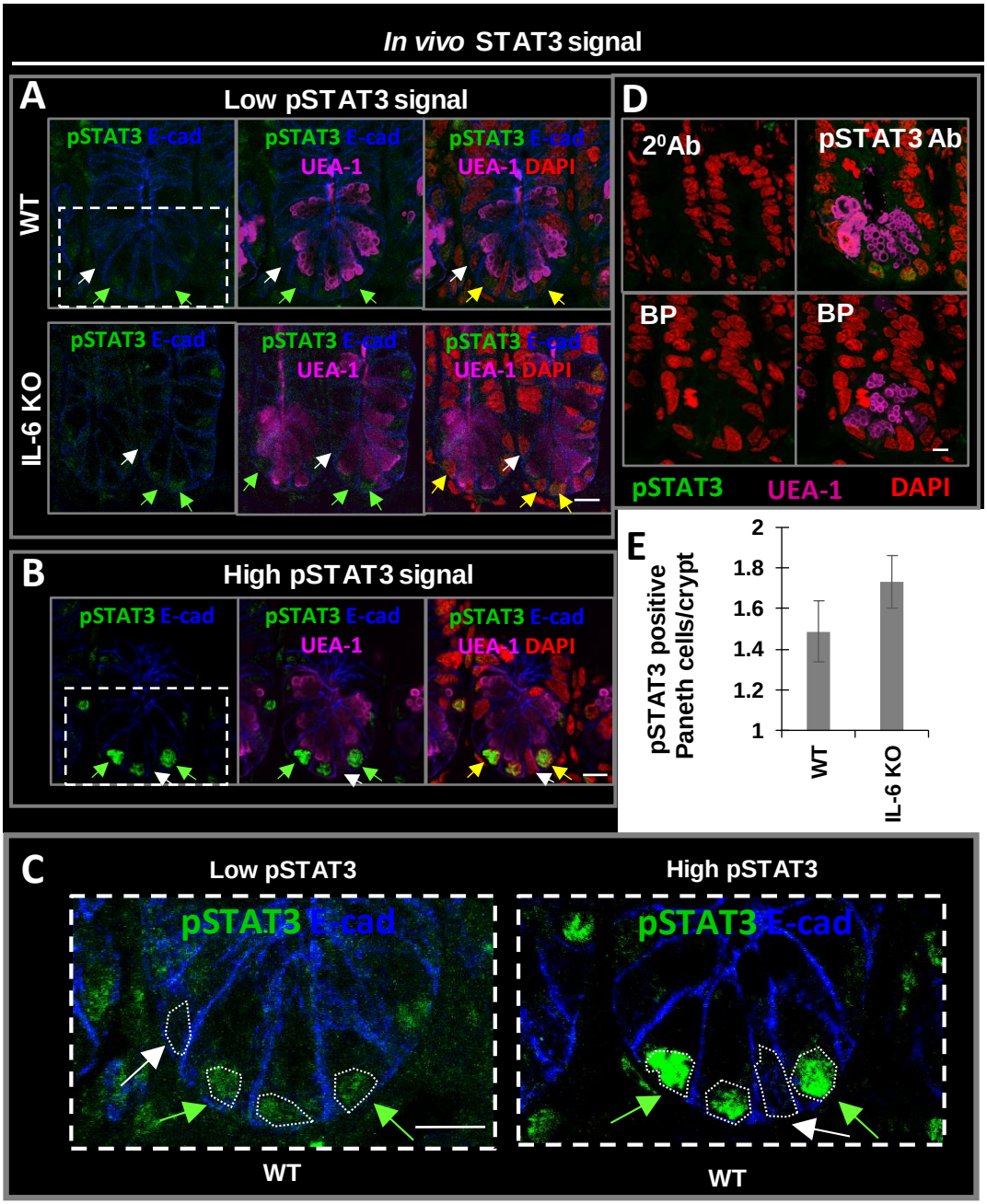


**Figure 3.13. Levels of pSTAT3 activation in IL-6KO tissue is the same as WT.**

Representative confocal images of *in vivo* WT and IL-6KO small intestine: (**A**) Representative confocal images demonstrating low level pSTAT3 (green) labelling in UEA-1 (pink) positive cells of both WT and IL-6 KO mice. Images are projections of several focal planes formed using image analysis software; (**B**) Representative confocal image of high pSTAT3 (green) fluorescent labelling observed in the mouse small intestine. (**C**) Representative confocal images of low pSTAT3 (green, LEFT from (**A**)), and high pSTAT3 (green, RIGHT from (**B**)) labelling in WT small intestine; brightness and contrast have been increased on images, white dashed region denotes nuclei. (**D**) A blocking peptide (BP) to pSTAT3 pre-incubated with the pSTAT3 antibody abrogated the pSTAT3 labelling in the nucleus of UEA-1 (pink) positive cells compared to tissue treated with pSTAT3 (green) alone. No nuclear labelling was observed utilizing the secondary antibody ($2^0$Ab) alone; Scale bar 10µm. (**E**) Histogram showing the

average number of pSTAT3 positive Paneth cells per crypt in WT compared to IL6KO (N=3, n≥136). Confocal imaging data is representative of N=3 independent experiments.

**Table 3.1. Level of pSTAT3 activation was the same in IL-6KO tissue and WT.**

The percentage of UEA-1 positive cells at the base of small intestinal crypts *in vivo* expressing No, Weak (+), medium (++) and high (+++) pSTAT3 activation in IL-6KO and WT tissue (N=3, n≥136). Images were captured using the same acquisition settings, fluorescence intensity of pSTAT3 was assessed and assigned 0, +, ++ or +++.

| | No pSTAT3 | pSTAT3 + | pSTAT3 ++ | pSTAT3 +++ |
|---|---|---|---|---|
| **Control** | 42.5 | 39.4 | 7.1 | 11.0 |
| **IL6 KO** | 29.8 | 55.3 | 7.1 | 7.8 |

### 3.2.5 IL-6 signalling modulates Lgr5EGFP stem cell number *in vitro* and *in vivo*

Intestinal stem cells drive the renewal of the intestinal epithelium; therefore, the effects of modifying the IL-6 signalling axis on crypt stem cells both *in vitro* and *in vivo* were investigated. Following 17h stimulation with IL-6, a significant increase in the percentage of Lgr5EGFP stem cells (**Figure 3.14A**) and the number of new buds formed per organoid (**Figure 3.14B**), another indicator of stem cell number, was observed *in vitro*. Blocking IL-6 classical signalling by treatment of organoids with an IL-6 receptor blocking antibody *in vitro* also significantly reduced the number of new buds formed per organoid compared to IgG control (**Figure 3.14C**). No significant difference was observed in the number of new buds formed per organoid following treatment with an IL-6 neutralising antibody compared to IgG control (**Figure 3.14D**).

In order to investigate the effects of perturbation of the IL-6 signalling pathway on stem cell number *in vivo*, an *in vivo* antibody approach was undertaken, whereby Lgr5EGFP mice were injected intraperitoneally with either an IL-6 receptor blocking or IL-6 neutralising antibody (or respective IgG control) over a period of 5 days (as described in detail in **Section 2.3.1**). Treatment of Lgr5EGFP mice with the IL-6 receptor blocking antibody induced a significant reduction in the number of Lgr5EGFP stem cells per crypt, the number of crypt nuclei, crypt length and villus height compared to IgG treated mice (**Figure 3.15A-E** respectively). Similar findings were observed when mice were treated with an IL-6 neutralising antibody, where a significant reduction in the number of Lgr5EGFP stem cells per crypt, the number of crypt nuclei and villus height were observed compared to IgG control (**Figure 3.16A-C** and **F**). No significant difference in the crypt length was observed in IL6 neutralising antibody treated mice compared to IgG control (**Figure 3.16D**).

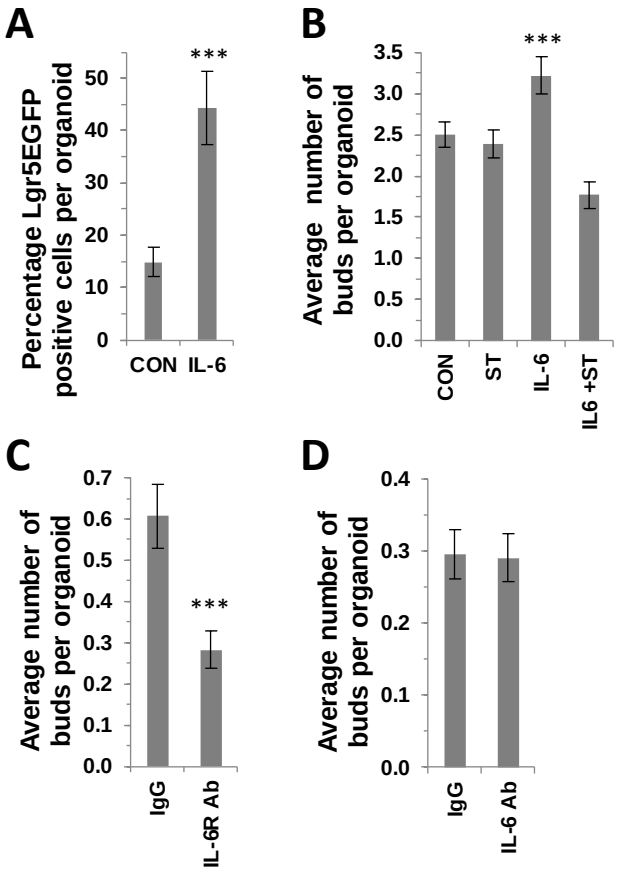


**Figure 3.14. IL-6 signalling modulates Lgr5EGFP stem cell number in vitro.**

(**A**) Histogram showing the effect of IL-6 (100 ng/ml) treatment on the percentage of Lgr5EGFP positive cells per crypt organoid compared to control (N=3, n≥30, ***P<0.001). (**B**) Histogram showing the average number of buds per crypt organoid following treatment with IL-6 (100 ng/ml) (N=3, n≥30, ***P<0.001) or STATTIC (20 µM). (**C**) Histogram showing the average number of buds per organoid in vitro when cultured in the presence of an IL-6 receptor blocking antibody (100µM, N=3, n≥152, ***P<0.001) compared to IgG control. (**D**) Histogram showing the average number of buds per organoid in vitro when cultured in the presence of an IL-6 neutralising antibody (100µM, N=5, n≥271, ns) compared to IgG control.

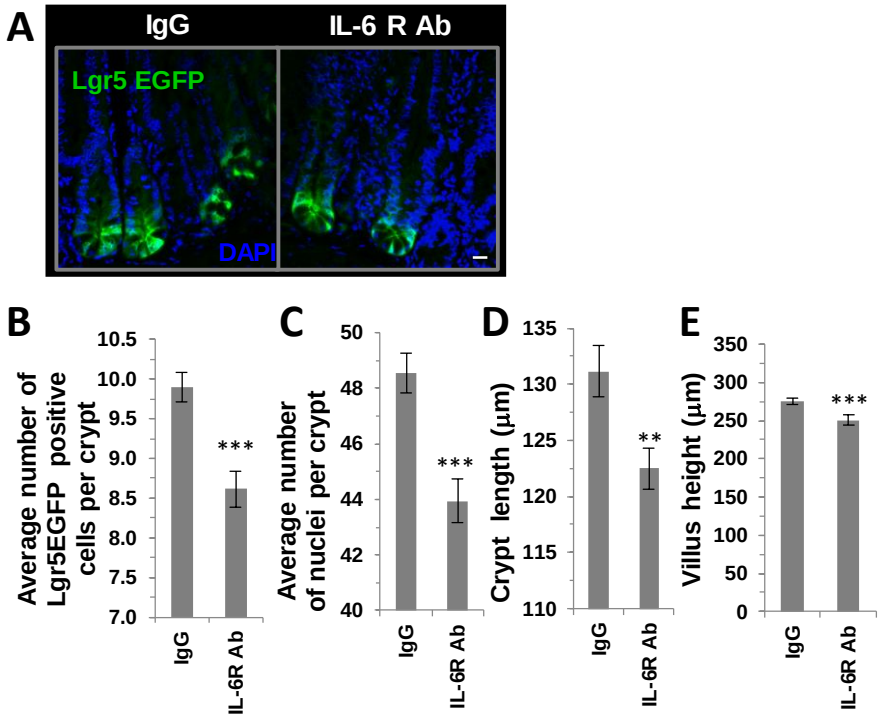


**Figure 3.15. Perturbing IL-6 signalling using an IL-6 receptor blocking antibody decreases Lgr5EGFP stem cells in vivo.**

(**A**) Representative confocal images showing a decrease in Lgr5EGFP (green) stem cells in small intestinal crypts following treatment with IL-6 receptor blocking antibody or IgG control, scale bar 10µm. (**B**) Histogram showing the average number of Lgr5EGFP positive stem cells per crypt (**C**) the average number of nuclei per crypt, (**D**) the crypt length and (**E**) the villus height in the small intestine of mice treated with a IL-6 receptor blocking antibody compared to respective IgG controls (N=3, n≥83, ***P<0.001; **P<0.01). Based on a single optical section on the equatorial plane along crypt-axis.

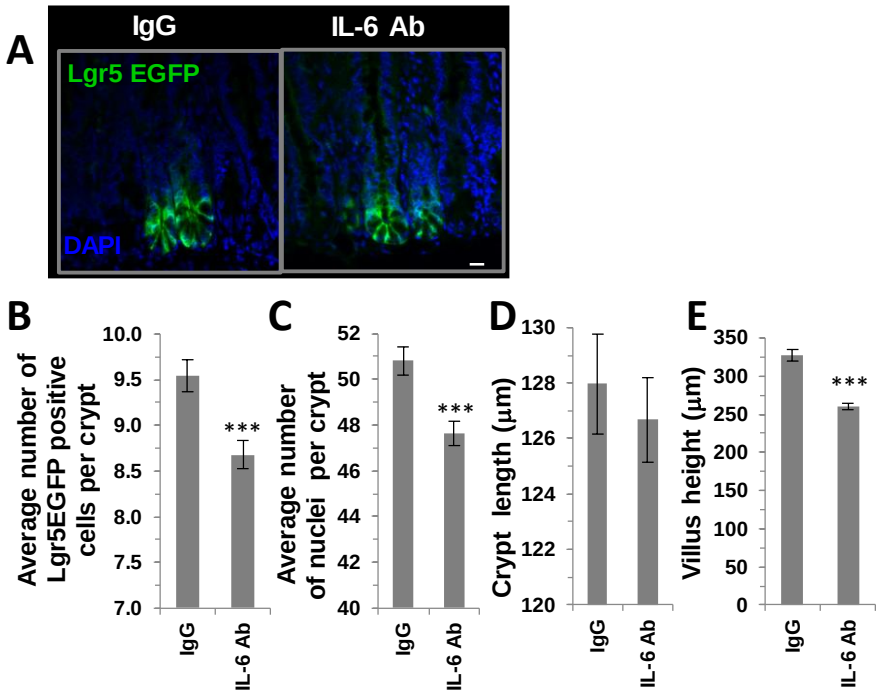


**Figure 3.16. Perturbing IL-6 signalling using an IL-6 neutralising antibody decreases Lgr5EGFP stem cells in vivo.**

(**A**) Representative confocal images showing a decrease in Lgr5EGFP (green) stem cells in small intestinal crypts following treatment with IL-6 neutralising antibody or IgG control, scale bar 10µm. (**B**) Histogram showing the average number of Lgr5EGFP positive stem cells per crypt (**C**) the average number of nuclei per crypt, (**D**) the crypt length and (**E**) the villus height in the small intestine of mice treated with a IL-6 neutralising antibody compared to respective IgG controls (N=3, n≥108, ***P<0.001; **P<0.01). Based on a single optical section on the equatorial plane along crypt-axis.

### 3.2.6 Perturbation of the IL-6 signalling axis modulates Paneth cell number *in vivo*

Paneth cells have previously been shown to be required for the maintenance of the stem cell niche (Sato et al., 2011), therefore the number of Paneth cells per small intestinal crypt following treatment with an IL-6 receptor blocking or IL-6 neutralising antibody was compared to IgG treated tissue. Use of the IL-6 receptor blocking antibody significantly reduced the number of in Lysozyme and UEA-1 positive cells per crypt compared to control (**Figure 3.17A-C**). Similarly, treatment of organoids with a neutralising antibody to IL-6 also significantly decreased the number of Lysozyme and UEA-1 positive cells per crypt compared to IgG control (**Figure 3.17D-F**). In order to determine whether cell death and proliferation was affected following antibody treatment, Immunofluorescent labelling of caspase-3, a marker of apoptosis, or Ki67, a marker of proliferation, was undertaken. A similar level of caspase-3 labelling was observed in both antibody treated mice compared to the respective IgG controls, whereby very low levels of caspase-3 were observed in *in vivo* crypts and villi. Explant sections cultured in conditions previously described to induce apoptosis (Sobolewski lab, unpublished) were utilised as a positive control for caspase-3 labelling (**Figure 3.18A**). A similar level of Ki67 labelling, was observed in the transit amplifying region of small intestinal crypts *in vivo* in antibody treated mice compared to the respective IgG controls (**Figure 3.18B**). This indicates that cell proliferation and apoptosis is not affected in antibody compared to IgG treated mice as has previously been described using IL-6/IL-6R knockout mice (Kuhn et al., 2014).

The decrease in Paneth cell number following *in vivo* treatment with IL-6 neutralising or IL-6 receptor blocking antibody, and data demonstrating that Paneth cells are the site of pSTAT3 activation *in vitro* begs the question of whether the pSTAT3 signal is abrogated in tissue obtained from mice treated with IL-6 receptor blocking or IL-6 neutralising antibody *in vivo*. Therefore, the level of pSTAT3 activation in antibody treated mice compared to IgG controls was determined. In one IgG control mouse, pSTAT3 was consistently detected in the nuclei of lysozyme positive cells (**Figure 3.19A LEFT**). However, pSTAT3 was below the levels of detection when the fluorescent labelling of pSTAT3 was repeated in two additional IgG treated mice. No pSTAT3 was detected in the nuclei of lysozyme cells from mice treated with an IL-6 receptor blocking antibody or an IL-6 neutralising antibody (**Figure 3.19A MID/RIGHT**). No pSTAT3 labelling was observed in the nuclei of Lgr5EGFP stem cells (**Figure 3.19B**).

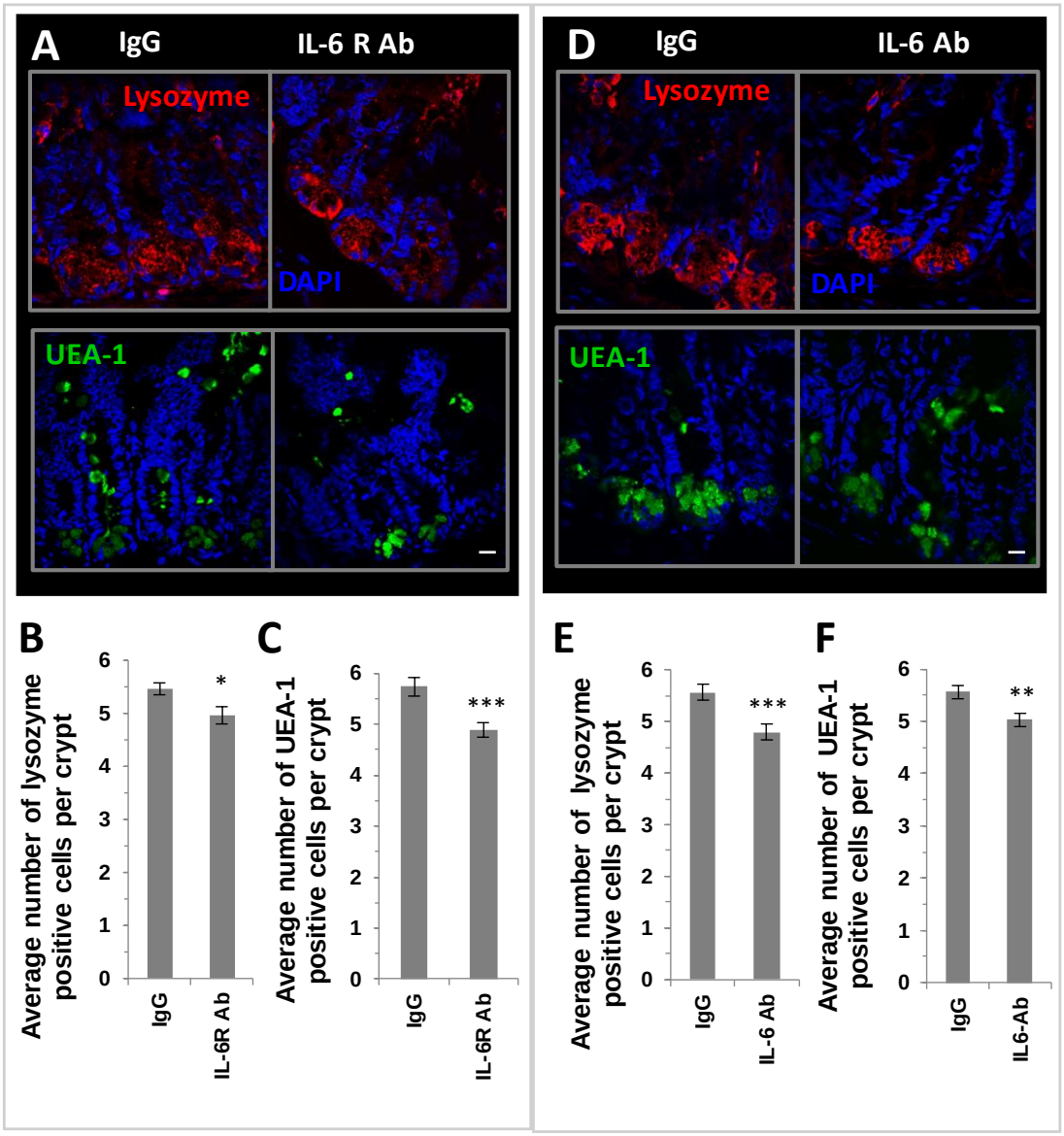


**Figure 3.17. IL-6 alters Paneth cell status in vivo.**

(**A**) Representative confocal images showing a reduction in Lysozyme (red) and UEA-1 (green) positive cells in small intestinal crypts following treatment with an IL-6 receptor blocking antibody compared to IgG control, nuclei (DAPI-blue). Scale bars 10µm. Histograms showing the average number of Lysozyme (**B**) and UEA-1 (**C**) positive cells per crypt following treatment with an IL-6 receptor blocking antibody and IgG control (N=3, n≥75, *P<0.05, ***P<0.001). (**D**) Representative confocal images showing a reduction in Lysozyme (red) and UEA-1 (green) positive cells in small intestinal crypts following treatment with an IL-6 neutralising antibody compared to IgG control, (DAPI-blue), scale bar 10µm. Histograms showing the average number of Lysozyme (**E**) and UEA-1 (**F**) positive cells per crypt following treatment with an IL-6 neutralising antibody and IgG control (N=3, n≥106, *P<0.05, ***P<0.001).

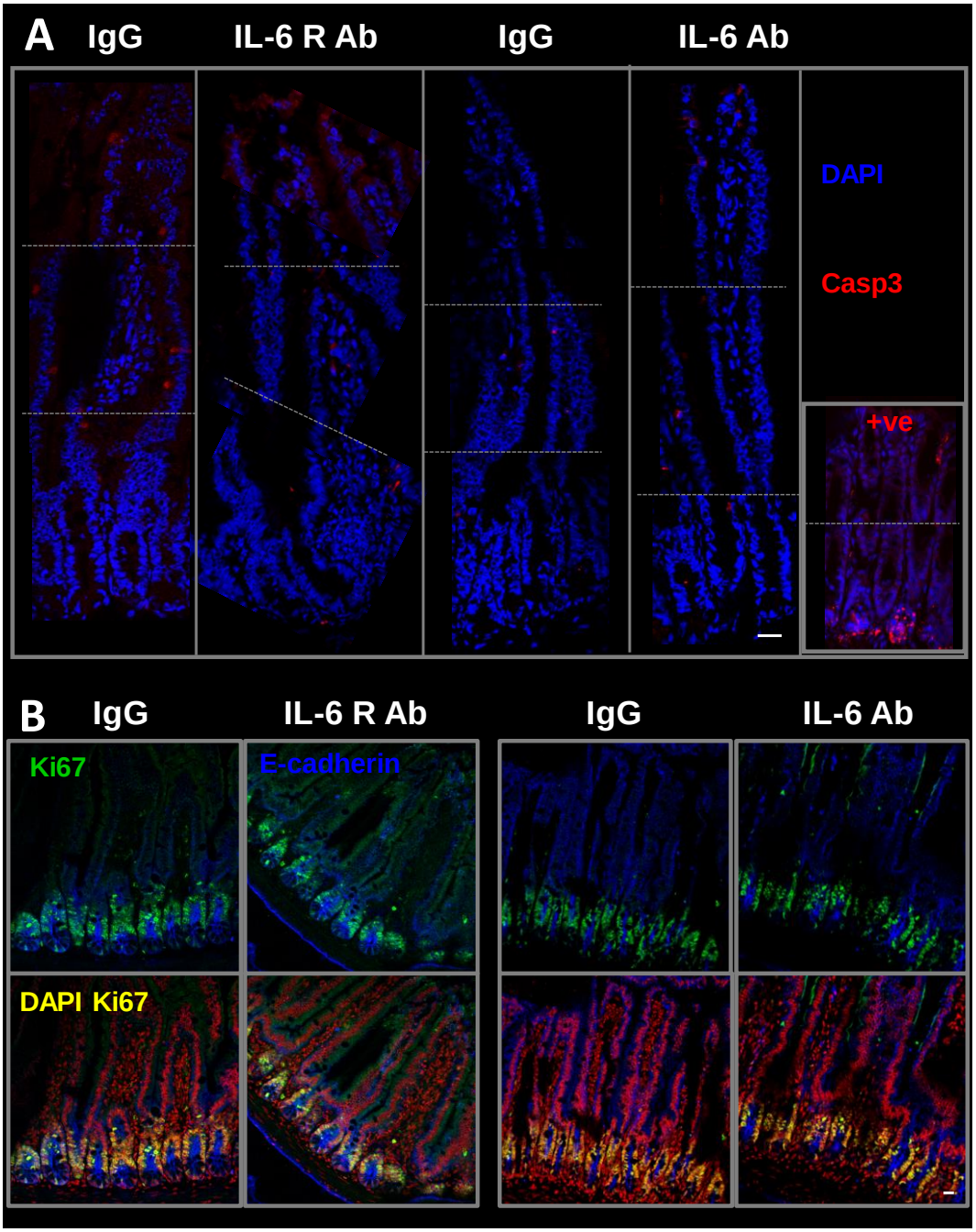


**Figure 3.18. Perturbation of the IL-6 signalling axis does not induce cell death in small intestinal tissue.**

(**A**) Representative confocal images of caspase-3 (red) labelling in small intestine crypts following treatment with an IL-6 receptor blocking antibody or IL-6 neutralising antibody compared IgG control, nuclei (DAPI-blue). (+ve indicates positive control for caspase-3 antibody) (**B**) Representative confocal images of Ki67 (green) positive nuclei (DAPI-red) in small intestinal crypts from mice treated with the IL-6 receptor blocking antibody or IL-6 neutralising antibody compared to IgG control, E-cadherin (blue). Confocal imaging data is representative of N=3 independent experiments, n≥30. Scale bars 10μm

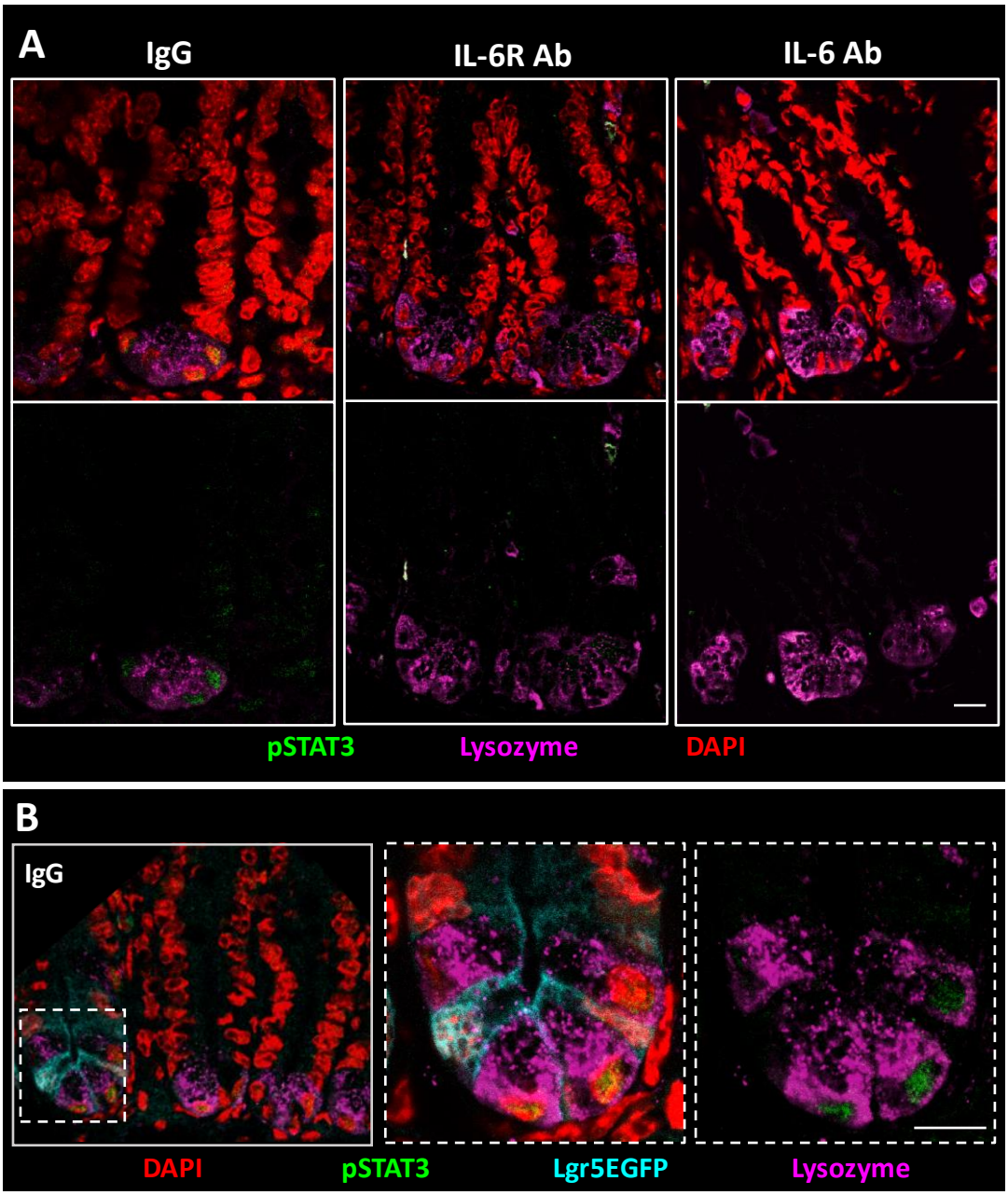


**Figure 3.19. Low levels of pSTAT3 activation are observed in vivo, no pSTAT3 was detectable following in vivo treatment with an IL-6 receptor blocking antibody or IL-6 neutralising antibody.**

(**A**) Representative confocal image of pSTAT3 (green) in Lysozyme (pink) positive cells in small intestine of mice treated with IgG (left, N=1), an IL-6 receptor blocking antibody (mid, N=3), or an IL-6 neutralising antibody (right, N=3), nuclei (DAPI-red). (**B**) Representative confocal image showing pSTAT3 (green) activation in Lysozyme (pink) positive cells, Lgr5EGFP (cyan), scale bars 10µm, images are representative of N=1, n≥20.

### 3.2.7 IL-22 modulates crypt cell proliferation and pSTAT3 activation *in vitro*

A number of other factors can activate the STAT3 signalling cascade (Nagalakshmi et al., 2004; Pickert et al., 2009; Lindemans et al., 2015). STAT3 activation following DSS-induced experimental colitis in mice was shown to be dependent on IL-22, rather than IL-6 (Pickert et al., 2009). Therefore, the effects of IL-22 on small intestinal organoid proliferation and pSTAT3 activation were investigated. Addition of IL-22 (100ng/ml) to small intestinal organoids for 1 hour induced global pSTAT3 activation in the nuclei of all cells within the organoids (**Figure 3.20A**). Furthermore, addition of 0.1, 1, 10, 100 or 1000ng/ml IL-22 to small intestinal organoids for 24 hours significantly increased BrdU incorporation (**Figure 3.20B**). Treatment with 10ng/ml IL-22 also showed a significant increase in the number of buds per small intestinal organoid (**Figure 3.20C**).

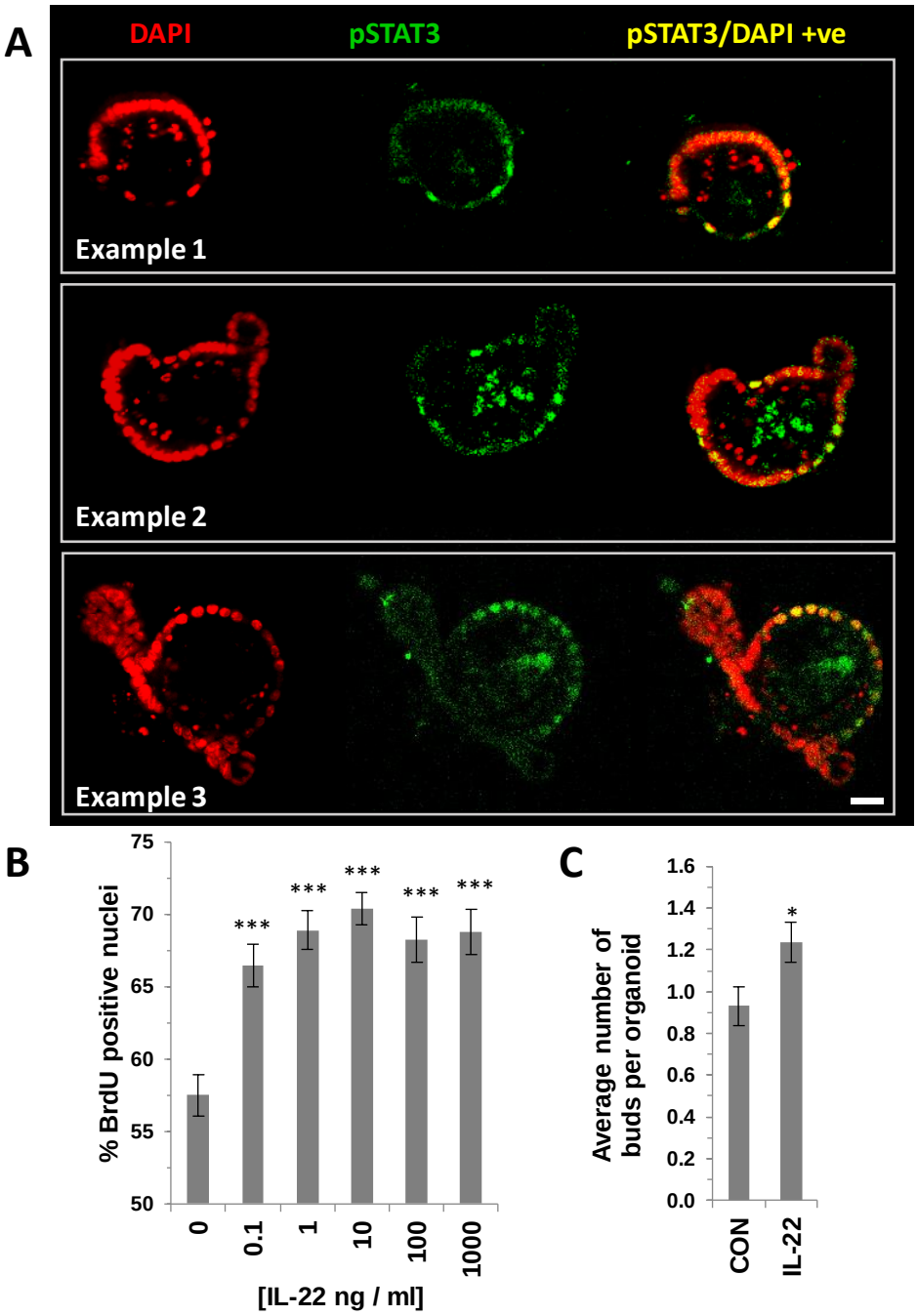


**Figure 3.20. IL-22 induces global pSTAT3 activation, BrdU incorporation and budding in small intestinal organoids.**

(**A**) Representative confocal images of small intestinal organoids following 1 hour IL-22 (100ng/ml) stimulation, showing pSTAT3 (green) Immunofluorescent labelling in the nuclei (DAPI-red). Scale bar 50µm. Immunofluorescent images obtained by AS (**B**) Histogram showing the percentage of BrdU positive nuclei in small intestinal organoids following 24 hour stimulation with IL-22 (0.1-1000ng/ml) compared to control (N=3, n≥54, ***P<0.001). (**C**) Histogram showing the average number of buds per small intestinal organoid following 24 hour IL-22 (10ng/ml) treatment (N=3, n≥54 *P<0.05).

### 3.2.8 IL-6 induces crypt cell proliferation through a WNT signalling pathway

Paneth cells are known to be a major source of WNTs and the WNT signalling pathway is a major regulator of epithelial renewal (Sato et al., 2011). Therefore, the possibility of the effects of IL-6 on epithelial renewal/ proliferation and stem cell number acting *via* the WNT pathway were investigated. The small molecule inhibitor IWP2 inhibits porcupine, which is responsible for the secretion of WNT protein, therefore inhibiting WNT signal activation (Kadowaki et al., 1996). A concentration of 5μM IWP2 was previously shown to be sufficient to inhibit WNT signalling *in vitro* (Chen et al., 2009). When organoids were treated with IWP2 (5μM) and IL-6 (100ng/ml), the IL-6-induced increase in proliferation was abrogated compared to IL-6 treated organoids (**Figure 3.21A**). The average number of buds per organoid was also significantly decreased in IWP2 and IL-6 compared to IL-6 alone (**Figure 3.21B**). However, the percentage of caspase 3 positive cells and survival of organoids when treated with IWP2 with and without IL-6 were comparable to control (**Figure 3.21C** and **D**).

The effects seen on organoids following treatment with IWP2 begs the question of whether components of the WNT signalling pathway are expressed by the small intestine before and after stimulation with IL-6. Immunofluorescent labelling revealed that the expression of WNT3 was localised on the basal membrane of all cells within small intestinal organoids and was comparable in organoids treated with and without IL-6 (**Figure 3.22A**). Semi-quantitative analysis of WNT3 immunofluorescent labelling on the basal membrane of small intestinal crypts (as described in **Section 2.8.1**) showed that the level of WNT3 was unchanged in IL-6 treated organoids compared to control in all regions of the crypt (**Figure 3.22B**).

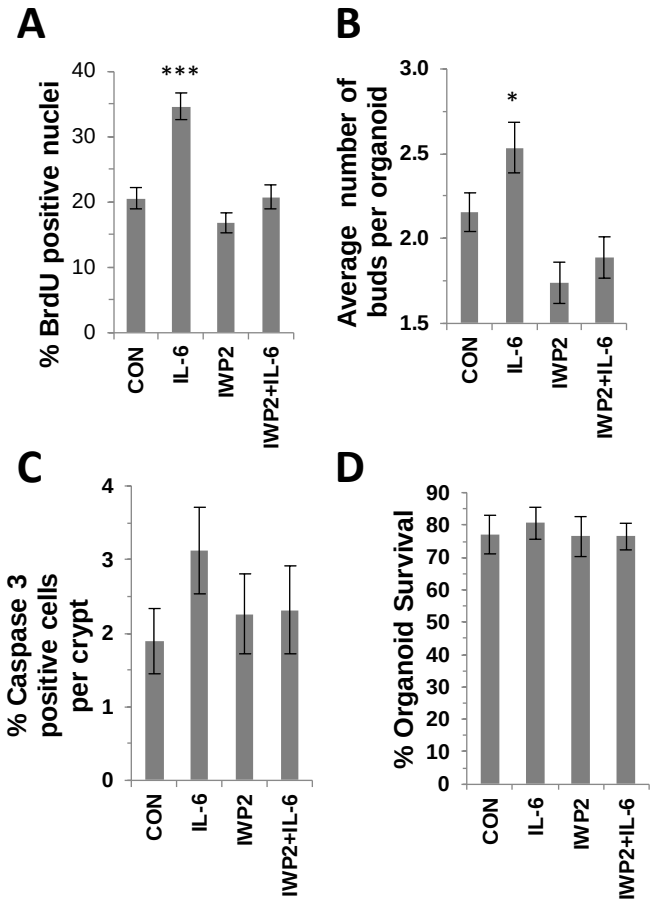


**Figure 3.21. WNT inhibition abrogates IL-6-induced proliferation and budding of small intestinal organoids.**

Histograms showing the effect of IWP2 (5μM) on (**A**) the percentage of BrdU positive crypt nuclei (N=3, n≥118, ***P<0.001), (**B**) the average number of buds per crypt organoid (N=3, n≥118, *P<0.05), (**C**) the percentage of caspase-3 positive cells per crypt and (**D**) the average organoid survival, following IL-6 (100ng/ml) stimulation. Data are represented as mean +/- SEM (N=3, n≥118).

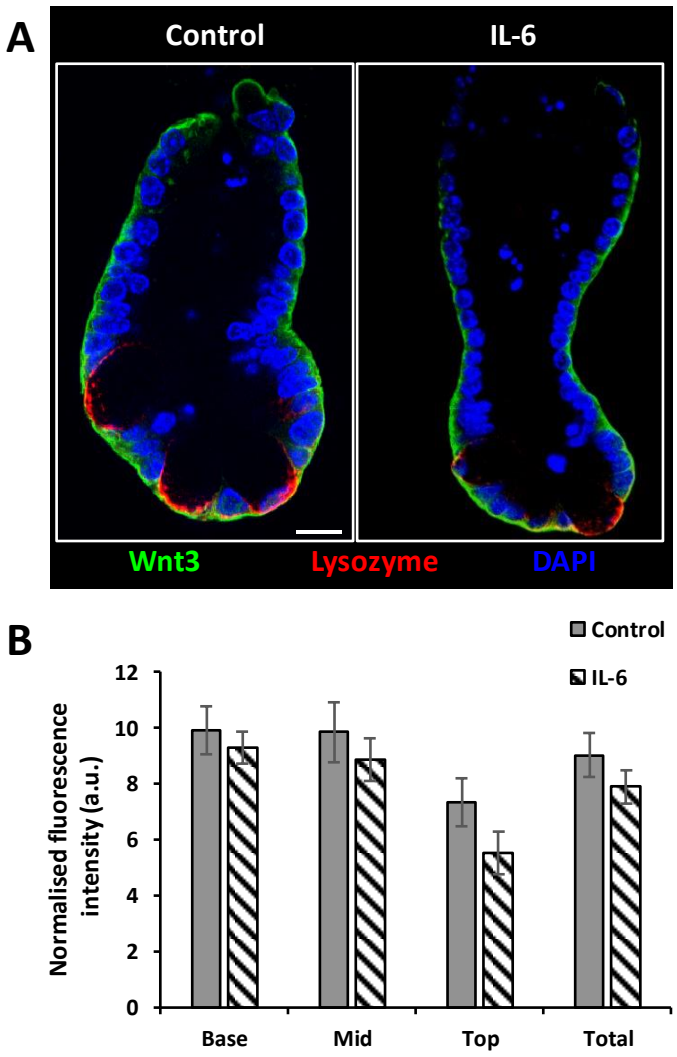


**Figure 3.22. WNT3 expression is unchanged following stimulation with IL-6.**

(**A**) Representative confocal images of WNT3 (green) expression in small intestinal organoids following stimulation with IL-6 (100ng/ml) or untreated control. Lysozyme (red), nuclei (DAPI-blue). Scale bar 10µm. (**B**) Histogram showing that fluorescence intensity along crypt-axis (a.u.) of WNT3 is unchanged following treatment with IL-6 compared to control (N=3, n≥20, ns). Confocal imaging data is representative of N=3 independent experiments.

## 3.3 Discussion

This work demonstrates a previously unrecognised role for autocrine IL-6 signalling during homeostasis in the small intestine (**Figure 3.23**). Stimulation of organoids with IL-6 increased *in vitro* proliferation and stem cell number *via* the STAT3 signalling pathway. IL-6 activated pSTAT3 in the nuclei of Paneth cells through differential expression of the IL-6 receptor located on the basal membrane of Paneth cells. IL-6 was also shown to be expressed in the intestinal epithelium at the mRNA and protein level. Blocking the IL-6 receptor or neutralising IL-6 with antibodies decreased organoid proliferation and crypt budding *in vitro* and also reduced stem cell number and Paneth cell number *in vivo*. The IL-6-induced increase in proliferation and budding was abrogated with IWP2, a pan WNT inhibitor. These data suggest a role of autocrine IL-6 signalling and the WNT signalling pathway in the regulation of small intestinal homeostasis.

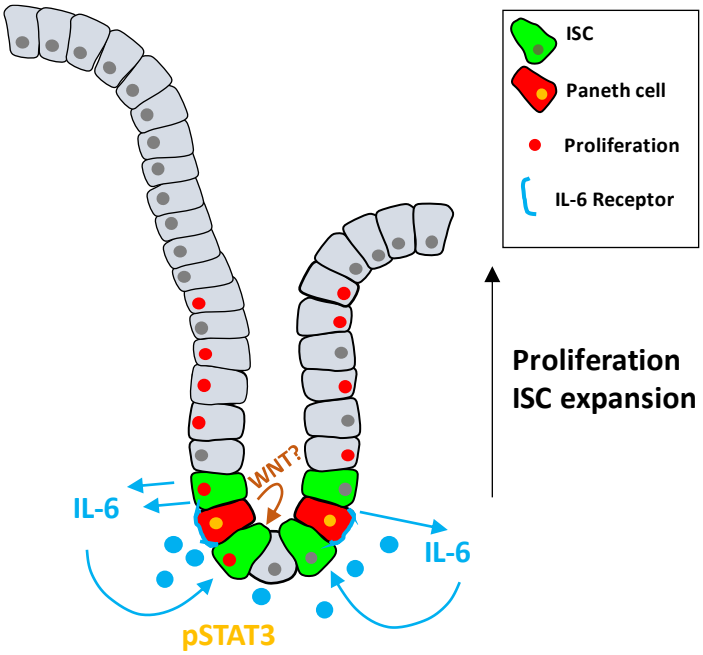


**Figure 3.23. Autocrine IL-6 induces small intestinal crypt cell proliferation.**

IL-6 induces pSTAT3 activation in Paneth cells, which express the IL-6 receptor. This induces small intestinal crypt cell proliferation and ISC expansion through a WNT dependent pathway.

### 3.3.1 Classical IL-6 signalling modulates small intestinal homeostasis *via* differential activation of pSTAT3 in Paneth cells

These findings demonstrate that IL-6 mediates effects on organoids *in vitro* through the STAT3 pathway. Crypt cell proliferation in response to IL-6 exhibited a bell-shaped concentration response curve, which has previously been described (van Dam et al., 1993). STAT3 mediated IL-6 signalling has previously been shown to protect healthy intestinal epithelial cells from apoptosis in DSS-induced colitis (Grivennikov et al., 2009), and epithelial STAT3 signalling was shown to be key for small intestinal stem cell survival (Matthews et al., 2011). Interestingly, inhibition of STAT3 in our system also caused a significant increase in the percentage of caspase-3 positive cells per crypt indicating that more cells in the crypt were undergoing apoptosis. However, the percentage of cells undergoing apoptosis in these organoids was still low (6.0% ± 0.7) compared to the overall decrease in IL-6-induced proliferation observed following treatment with STATTIC (26.2% ± 2.0). Notably, organoid survival following treatment with STATTIC in the presence or absence of IL-6 was comparable to control. These studies therefore indicate that the major effects of IL-6/STAT3 signalling on the small intestinal epithelium is the induction of proliferation. Epithelial STAT3 signalling has also been shown to be important for crypt regeneration during intestinal mucosal wound healing (Jiang et al., 2009; Pickert et al., 2009). Using full growth factor media and chronic STAT3 inhibition with STATTIC it was demonstrated that epithelial STAT3 was important for crypt regeneration even in the absence of exogenous IL-6.

An emerging role for IL-6 in the maintenance of homeostasis is beginning to be recognised (Giraud et al., 2007). The finding that the IL-6 receptor is differentially expressed on Paneth cells, which was shown to be the site of pSTAT3 activation following IL-6 stimulation, suggests that IL-6 plays a role in maintaining homeostasis *via* the classical IL-6 signalling pathway. Interestingly, expression of the IL-6 receptor was also observed in the lumen of small intestinal organoids in culture, suggesting that the IL-6 receptor can be secreted by Paneth cells in order to potentially bind IL-6 and induce *trans*-signalling. We demonstrate ubiquitous labelling of gp130; in our system if soluble IL-6 receptor was binding IL-6 and activating signalling *via* gp130 we would expect to see global pSTAT3 activation in all cells of the crypt. Interestingly, Aden et al. (2016) observed no pSTAT3 activation in organoids following treatment with IL-6; however, they did observe pSTAT3 activation following addition of hIL-6 (hyper-IL-6, a fusion protein of IL-6 linked to sIL-6R). The approach taken by Aden et al. (2016) was to investigate inducible pSTAT3 utilising a western blot on protein

lysate; it is possible that the levels of pSTAT3, which were observed only in Paneth cells in this study, a relatively small proportion of cells within the organoid, are not detectable using this method. However, activation of trans-IL-6 signalling utilising IL-6 fused to IL-6R (hIL-6) induced pSTAT3 activation in all cells within the organoid expressing gp130, which is therefore easily detectable by Western blot (Aden et al., 2016). The findings presented by Aden et al. (2016) also suggest that IL-6 classical signalling is not important for the regenerative response to mucosal injury induced by DSS, assessed using a mouse with an IEC specific deletion of the IL-6R. However, the effects of IL-6 receptor deletion in intestinal organoid renewal were not determined (Aden et al., 2016). Therefore, it is possible that classical IL-6 signalling mediates STAT3 signalling and proliferation during steady state renewal of crypts, and following tissue injury, IL-6 trans-signalling is activated in order to induce epithelial repair.

We also detected pSTAT3 fluorescence significantly above baseline occasionally in unstimulated organoids. However, following treatment with IL-6, activation of pSTAT3 was observed in all UEA-1 positive Paneth cells at the base of organoids. This suggests that the STAT3 signalling pathway is activated transiently and dynamically during homeostasis and that this signalling is synchronised to occur simultaneously across all crypts in artificial experimental conditions. In order to enable spatial and temporal visualisation of gene expression, bacterial artificial chromosome (BAC) technology has recently been developed (Schwank et al., 2013). In the future, we hope to apply BAC technology to our small intestinal organoid culture, in order to add a fluorescent probe to pSTAT3. Transfection of fluorescently tagged pSTAT3 gene into organoid culture would allow visualisation of the spatial and temporal dynamics of pSTAT3 activation in real time during steady state renewal and in response to cytokine addition.

Several publications have eluded to the fact that the intestinal epithelium expresses IL-6 (Drastich et al., 2011), however the location of IL-6 expression in organoids has not yet been characterised. In this study, initial findings suggested that both freshly isolated (*in vivo)* and cultured (*in vitro)* small intestinal organoids express mRNA and IL-6 protein. The location of the IL-6 protein was restricted to the basal membrane at the base of small intestinal organoids, and in discrete pools in the surrounding matrix. Interestingly, Farin et al. (2016) recently confirmed that WNTs are tethered to the membrane of Paneth cells residing at the base of intestinal crypts, where they deliver WNT ligand to adjacent stem cells (Farin et al., 2016). In this chapter, we show for the first time that IL-6 acts through the WNT signalling

pathway in order to modulate intestinal crypt cell proliferation. Further studies are required to fully elucidate the functional effects of this interaction.

We were not able to detect IL-6 in the conditioned media from organoid culture using a commercially available ELISA (data not shown); we speculate that this is due to the low amount of secreted IL-6 from 20-50 organoids into a volume of 120µl volume of matrigel and media. We also noted that the IL-6 was located in pools close to the basal pole of the crypts but not at sites where there were no crypts present. Previous groups demonstrating detectable levels of IL-6 in culture supernatants using ELISA were using large tissue samples (Coeffier et al., 2002; Drastich et al., 2011) or high numbers of cells (Parikh et al., 1997). In order to detect secreted IL-6 in the culture supernatant, it could be speculated that high numbers of organoids would be required in a smaller volume of media. It is also likely that there would be high levels of contaminating factors in the culture supernatants and therefore the IL-6 protein secreted would need to be concentrated in order to detect using an ELISA. The detection of low levels of protein is now more easily investigated using mass spectroscopy, which may be a more relevant technique to detect secreted IL-6 protein in our system.

### 3.3.2 IL-6 signalling modulates stem cell and Paneth cell number *in vivo*

Perturbation of the IL-6 signalling axis utilising IL-6 or IL-6 receptor knockout mice has previously described a role for IL-6 in inflammation rather than homeostasis (Grivennikov et al., 2009; Jin et al., 2010; Lee et al., 2012; Kuhn et al., 2014). In line with previous studies, we show that small intestinal tissue obtained from IL-6KO mice was similar to tissue obtained from wild type mice. However, in this study we also used a blocking/ neutralising antibody approach to demonstrate a previously unrecognised role for autocrine IL-6 signalling in the maintenance of stem cell and Paneth cell number *in vivo*. It is possible that the lack of gut phenotype observed in IL-6KO mice may be explained by redundancy effects known to occur in knockout mouse models, and highlights the importance of interrogating this pathway using a variety of methods, as has been shown in other studies (Sommer et al., 2014). Future studies aim to use genetic manipulation techniques, such as Cre-recombinase-inducible retroviral vectors or CRISPR/Cas9, to induce a loss-of-function of IL-6/IL-6R in organoids and investigate whether redundancy mechanisms are in place (Koo et al., 2011; Driehuis and Clevers, 2017). The transient nature of IL-6/STAT3 signalling was demonstrated in this system, as activation of pSTAT3 in Paneth cells was observed consistently in one IgG treated mouse, but levels of pSTAT3 activation were below the limits of detection in two further IgG

treated mice. Further experiments are required in order to fully determine the effects of perturbation of the IL-6 signalling pathway on pSTAT3 activation *in vivo*.

### 3.3.3 STAT3 activation by IL-22

The STAT3 signalling pathway is also activated by a number other cytokines including IL-22 (Nagalakshmi et al., 2004; Lindemans et al., 2015). Indeed, STAT3 activation following DSS-induced experimental colitis in mice was shown to be dependent on IL-22, rather than IL-6 (Pickert et al., 2009). We concur with Lindemans et al. (2015) and demonstrate that IL-22 induces an increase in proliferation in both small intestine and colonic organoids. The expression of the IL-22 receptor has been shown to be widely distributed, on enterocytes in the transit amplifying progenitor crypt region as well as on intestinal stem cells at the base of crypts (Lindemans et al., 2015), in contrast to the restricted expression of the IL-6 receptor exclusively by Paneth cells. Interestingly, addition of IL-22 to small intestinal organoids induced global activation of pSTAT3 in all nuclei within intestinal organoids. Similar labelling was previously observed in dysplastic epithelial cells, which also exhibit global nuclei pSTAT3 labelling, which was associated with increased levels of soluble IL-6 receptor suggesting that IL-6 trans-signalling induces widespread pSTAT3 activation in cancer (Becker et al., 2004). The relative contribution of different cytokines in regulating the STAT3 pathway in gut homeostasis and inflammation, in the small and large intestine remains the focus of future work.

### 3.3.4 IL-6 induces crypt proliferation through a WNT dependent signalling pathway

WNT signalling is the master regulator of intestinal tissue renewal. We demonstrate that changes in organoid proliferation and budding following IL-6 stimulation are abrogated by treatment with a WNT inhibitor, IWP2. This indicates that these effects are dependent on the WNT signalling pathway, activation of which is required for the maintenance of the stem cell niche (Sato et al., 2011). To our knowledge this is the first time that the mechanism of IL-6-mediated intestinal stem cell proliferation has been shown to be through the WNT signalling pathway. Previously, IL-6 has been shown to upregulate WNT5A signalling through non-canonical WNT signalling mediator ROR2 in human adipose tissue-derived mesenchymal stem cells to induce calcification through a STAT3-dependent pathway (Fukuyo et al., 2014). Similarly, in malignant melanoma IL-6 was shown to upregulate WNT5A and induce melanoma cell migration and invasion (Linnskog et al., 2014). In these studies, an IL-6/sIL-6R

complex was utilised in order to activate IL-6 trans-signalling which induced non-canonical WNT signalling to drive inflammation and cancer. Interestingly, WNT5A has also previously been shown to play a major role in crypt regeneration in order to re-establish intestinal homeostasis in response to intestinal tissue injury, through the activation of TGF-β (Miyoshi et al., 2012). A role for IL-6 in the negative regulation of canonical WNT signalling in primary human synovial fibroblasts and osteoclasts has previously been described. Osteogenic WNT signalling is important for joint remodelling and therefore IL-6 is thought to play a role in the pathogenesis and progression of osteoporosis (Malysheva et al., 2016). In this system, we speculate that IL-6 classical signalling is required to maintain homeostasis, therefore it is possible that an association with a different WNT, and possibly a different branch of pathway (i.e. canonical/non-canonical PCP/non-canonical calcium) can be activated by IL-6 during homeostasis compared to inflammation.

Previous studies have identified that the most predominant WNT in the intestinal epithelium, WNT3 is expressed by Paneth cells at the base of intestinal crypts. WNT3 is tethered to the membrane of Paneth cells by Frizzled receptors, and delivered to adjacent Lgr5+ stem cells. Following cell division, WNT ligand is distributed to daughter cells where the signal is diluted (Farin et al., 2016). In agreement with these studies, the expression of WNT3 was restricted to the basal membrane of intestinal crypts, with more WNT3 present at the base of crypts compared to mid and top. We speculated that WNT3 expression may be altered in IL-6 treated tissue, however the expression of WNT3 was comparable in IL-6 treated organoids compared to control. Future work aims to identify whether IL-6 mediates intestinal stem cell driven renewal through canonical or non-canonical WNT signalling, and to identify which WNT signalling pathway components are modulated by IL-6 stimulation of organoids.

### 3.3.5 Modulation of the IL-6 signalling pathway for treatment of diseases

Although Paneth cells are not an absolute requirement for cell survival, proliferation and stem cell activity (Durand et al., 2012; Kim et al., 2012), previous work has shown that they provide important regenerative factors for modulation of the stem cell niche (Sato et al., 2011; Farin et al., 2016). This study has identified a novel role for IL-6 signalling in regulating Paneth cell number *in vivo*. Paneth cell metaplasia in the colon is a feature of IBD (Tanaka et al., 2001; Simmonds et al., 2014), in addition to elevated levels of IL-6 in the serum and tissues (Mudter and Neurath, 2007; Waldner and Neurath, 2014). We therefore speculate that alterations in the IL-6 signalling axis may be a contributing factor to this phenotype.

In the clinic, patients with rheumatoid arthritis have been treated with the IL-6 antibody tocilizumab have shown severe adverse side effects including perforation of the lower bowel (Emery et al., 2008; Gout et al., 2011), however the mechanism through which this occurs is unknown. Current antibody treatments do not distinguish between IL-6 classic and IL-6 trans signalling. It is therefore possible that side effects observed following IL-6 antibody treatment are due to inhibition of classic IL-6 signalling which is important for the maintenance of homeostasis. Pro-inflammatory responses from IL-6 signalling have previously been associated with IL-6 trans signalling (Rose-John, 2012); IL-6 trans signalling was shown to play a major role in the development of colitis-associated premalignant cancer in murine models (Matsumoto et al., 2010). More targeted treatments for inflammatory disorders that retain classic IL-6 signalling and therefore homeostatic crypt renewal, but inhibit trans IL-6 signalling through specific cell types appears to be the ideal scenario for the future (Schaper and Rose-John, 2015).

## 3.4 Conclusions

The results discussed in this chapter demonstrate a previously unrecognised role for IL-6 signalling in the maintenance of small intestinal homeostasis. Understanding the mechanisms behind IL-6 modulation of intestinal renewal is vital for the potential interrogation of this pathway for the treatments of diseases. Indeed, the possibility of inhibiting IL-6 trans-signalling, whilst retaining the IL-6 classical signalling required for homeostasis could hold promise for the future development of treatments for IBD and cancer.

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Similarly to IL-6, another factor with a diverse range of functions which has also been linked to inflammatory diseases and cancer is Prostaglandin-$E_2$. However, the enzyme responsible for $PGE_2$ synthesis is constitutively present in the gastrointestinal tract during health, and $PGE_2$ has many roles associated with homeostasis including fluid secretion, renewal and the amelioration of inflammation. This highlights the need for investigation into the roles of $PGE_2$ in intestinal homeostasis. The next chapter investigates the pathway through which $PGE_2$ exerts effects on intestinal renewal.

# 4 Chapter 4. $PGE_2$ regulates proliferation of the colonic epithelium

## 4.1 Introduction

Prostaglandin-$E_2$ is an endogenous signalling molecule with a diverse range of functions in health and disease. High levels of $PGE_2$ have been associated with exacerbation of the inflammatory response, disrupted barrier function and promotion of tumour growth (Wang and Dubois, 2006). A role for $PGE_2$ in the maintenance of tissue renewal and repair following injury is beginning to be recognised, however the mechanism through which intestinal homeostasis is maintained by $PGE_2$ signalling is not yet fully understood.

$PGE_2$ is synthesised *via* the arachidonic acid pathway by COX enzymes (Smith et al., 2000). $PGE_2$ mediates actions by binding to one of four G-protein-coupled receptors EP1-4, which have distinct signalling transduction pathways. EP1 receptor activation is coupled to intracellular calcium mobilisation through $G_{\alpha q}$; EP2 and EP4 receptors are coupled to adenylate cyclase stimulation *via* $G_s$; and EP3 receptor is coupled to activation of adenylate cyclase through $G_{\alpha s}$ and inhibition of adenylate cyclase and intracellular calcium mobilisation through $G_i$. The generation of mouse models deficient in each EP receptor subtype, and the development of EP receptor selective agonists and antagonists provides a valuable tool to investigate the physiological role of each EP receptor subtype.

Prostaglandin-$E_2$ has been shown to play a homeostatic role in the crypt regenerative response following intestinal injury induced by DSS or biopsy wounding. Following DSS-induced epithelial injury, innate sensing of microbes by myofibroblasts residing in the intestinal lamina propria induced the expression of COX-2, which synthesises $PGE_2$ and induced a compensatory proliferation response in intestinal crypts through activation of the EP4 receptor subtype (Roulis et al., 2014). In a model of biopsy-induced mucosal wounding in mice that were deficient in the EP4 receptor subtype, multiple deficiencies in tissue repair including incomplete wound covering with wound associated epithelial (WAE) cells were observed (Manieri et al., 2012; Manieri et al., 2015). WAE cell differentiation was shown to be induced by EP4 receptor expression in mesenchymal cells in the lamina propria induced WAE cell differentiation (Manieri et al., 2012), later shown to be mediated through direct inhibition of GSK3β and nuclear β-catenin activation (Miyoshi et al., 2017).

$PGE_2$ signalling through the EP1, EP2 and EP3 receptor subtypes have also been shown to induce epithelial regeneration in DSS-induced colitis. Induction of $Ca^{2+}$ response in the rat ileum using Oxytocin (OT) ameliorated inflammation through a COX-2-dependent release of $PGE_2$ release (Chen et al., 2015). This response was inhibited following inhibition of the EP1, EP2 and EP3 receptors with the selective $PGE_2$ antagonist, AH6809, (Abramovitz et al., 2000), suggesting that $PGE_2$ activates these EP receptor subtypes to induce intestinal regeneration (Chen et al., 2015). Furthermore, $PGE_2$ signalling through the EP4 receptor subtype has been described to downregulate the immune response to reduce mucosal damage and severe colitis (Kabashima et al., 2002). These data suggest that $PGE_2$ plays a homeostatic role in crypt regeneration and amelioration of inflammation following intestinal injury. However, the role of $PGE_2$ in epithelial regeneration during steady-state renewal during health remains to be determined.

Increased mucosal levels of $PGE_2$ have also been shown to induce intestinal inflammation through disruption of barrier function, which is associated with IBD (McGuckin et al., 2009; Rodriguez-Lagunas et al., 2010). $PGE_2$ signalling was shown to increase paracellular permeability of Caco-2 cells through EP1 and EP4 receptor signalling (Rodriguez-Lagunas et al., 2010). EP4 receptor signalling has also been implicated in inflammation in a model of inducible colitis in mice, where naïve T cells are transferred into mice deficient in recombinase-activation gene 2. Transfer of T cells deficient in the EP4 receptor subtype exhibited less severe colitis than transfer of wild type T cells, indicating that EP4 receptor signalling plays a pathogenic role in this model of colitis (Yao et al., 2013). Further studies are required to investigate the role of EP4 receptor signalling in health and inflammation.

The expression of COX enzymes for $PGE_2$ synthesis are upregulated in colorectal cancer and are associated with unfavourable survival (Wang and Dubois, 2010). Inhibition of COX enzyme activity using NSAIDs has been shown to have potent anti-tumorigenic properties in initial clinical studies of patients with FAP (Bresalier et al., 2005; Solomon et al., 2005; Arber et al., 2006; Rostom et al., 2007). NSAIDs inhibit both endogenous COX-1 and inducible COX-2 enzymes (Wang and Dubois, 2010; Gerbe et al., 2011); therefore it is possible that side effects observed following NSAID treatment may be due to inhibition of endogenous $PGE_2$ activity required to induce tissue renewal required to maintain homeostasis. The effects of selective COX-1 or COX-2 inhibition on tumorigenesis or steady-state tissue renewal have not yet been defined.

A number of previous studies have investigated the role of differential EP receptor signalling in tumorigenesis. Signalling through the EP1, EP2 and EP4 receptors have been shown to promote intestinal polyposis and induce the growth and metastasis of tumours through the activation of a number of signalling pathways required for cell invasion and migration (reviewed in O'Callaghan and Houston (2015)). In contrast, activation of the EP3 receptor has been shown to reduce tumorigenesis *in vivo* and cell proliferation in tumour cell lines (Macias-Perez et al., 2008). Whilst the roles of EP receptor subtype activation in tumorigenesis have been relatively well characterised, the relative contributions of EP receptor activation by $PGE_2$ in maintaining colonic crypt cell proliferation during homeostasis remains to be elucidated.

Recently, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) has been identified as an enzyme which acts as a negative regulator of prostaglandin levels and activity *in vivo* (Myung et al., 2006). The healthy colonic mucosa expresses high levels of 15-PGDH, however 15-PGDH is lost in colorectal cancers and FAP. Recent studies suggest that inhibition of 15-PGDH accelerates hematopoietic recovery in mice following bone marrow transplant, and promotes tissue regeneration in mouse models of colitis and liver injury (Zhang et al., 2015). The role of inhibition of 15-PGDH in colonic crypt renewal during homeostasis has not yet been examined.

This chapter aims to determine the role of autocrine and paracrine $PGE_2$ signalling in the maintenance of mouse colonic crypt homeostasis. The relative contribution of each of the EP receptor subtypes, EP1-4, in colonic crypt renewal was also investigated. This chapter demonstrates a previously unrecognised role for autocrine $PGE_2$ signalling in colonic crypt cell proliferation, through activation of EP1 and EP3 receptor subtypes.

## 4.2 Results

The effects of $PGE_2$ signalling on mouse and human colonic crypts were investigated using the isolated colonic crypt 3D culture model. Expression of the components of the $PGE_2$ signalling pathway by mouse colonic crypts was characterised at the mRNA and protein level. A pharmacological approach was undertaken in order to inhibit or activate EP receptor subtypes in the colonic epithelium *in vitro*; the effects on crypt cell proliferation as shown by incorporation of EdU, cell survival and budding were determined.

### 4.2.1 Characterisation of $PGE_2$ signalling pathway components in the mouse colonic epithelium

Whilst the addition of exogenous $PGE_2$ is widely accepted to stimulate epithelial cell proliferation, the role of autocrine $PGE_2$ signalling in the maintenance of colonic crypt homeostasis remains largely unknown. In order to determine whether the mouse colonic epithelium is able to synthesis autologous $PGE_2$, the expression of COX-1 and COX-2 enzymes were characterised. Firstly, conventional PCR was utilised to demonstrate expression of COX-1 and COX-2 mRNA in freshly isolated mouse colonic crypts (**Figure 4.1A**). Immunofluorescent labelling of colonic crypts *in vivo* utilising antibodies to COX-1 and COX-2 demonstrated that discrete cells within colonic crypts express COX-1 and COX-2 *in vivo* (**Figure 4.1B TOP**). Quantitative analysis of the number of cells within colonic crypts expressing COX-1 and COX-2 cells *in vivo* revealed that the numbers of COX-1 and COX-2 expressing cells in mouse colonic crypts were comparable (**Figure 4.1B BOTTOM**). Next, the expression of COX-1 and COX-2 enzymes was determined in our *in vitro* isolated crypt culture system. The expression of COX-1 and COX-2 enzymes *in vitro* in isolated mouse colonic crypts was similar to that observed *in vivo* (**Figure 4.1C TOP**). Furthermore, when the number of COX-1 and COX-2 positive cells were quantified *in vitro*, we observed no significant difference between the number of cells expressing COX-1 and COX-2 enzymes (**Figure 4.1C BOTTOM**) as observed *in vivo*. We therefore conclude that colonic crypts are able to synthesis $PGE_2$ both *in vivo* and in our *in vitro* crypt culture system.

$PGE_2$ is known to signal through four EP receptor subtypes; EP1, EP2, EP3 and EP4 receptors. Therefore, the expression of each EP receptor subtype by the mouse colonic epithelium was investigated. Firstly, conventional PCR was utilised to demonstrate the expression of EP1, EP2, EP3 and EP4 mRNA in freshly isolated mouse colonic crypts (**Figure 4.2A**). Three different splice variants have been described for the EP3 receptor subtype (Namba et al.,

1993); we therefore confirmed expression of mRNA for EP3α, EP3β and EP3γ variants (**Figure 4.2B**).

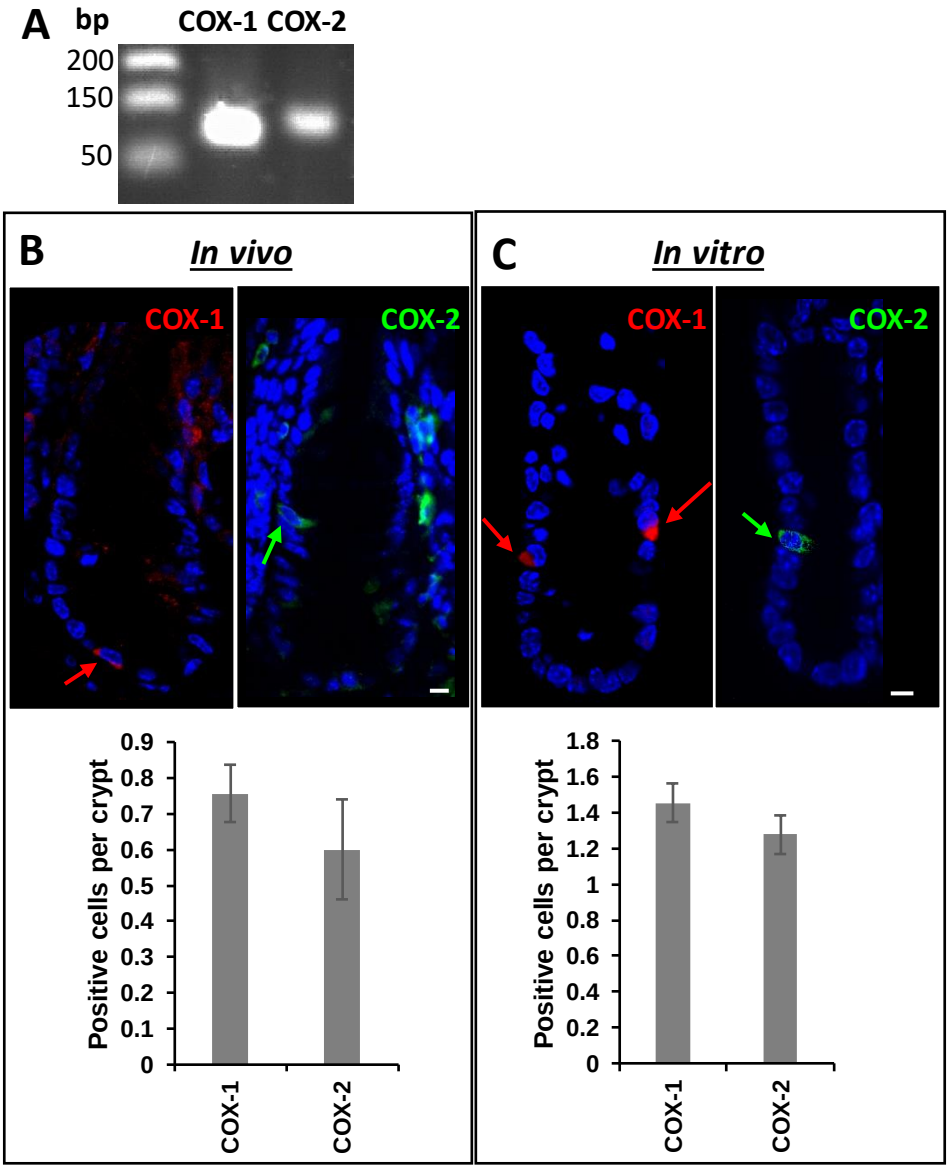


**Figure 4.1. The mouse colonic epithelium expresses COX enzymes for PGE2 synthesis.**

(**A**) PCR gel showing expression of COX-1 and COX-2 transcripts in freshly isolated colonic crypts. Band sizes for PCR products: COX-1=96bp, COX-2=70bp (**B**) Representative confocal images of COX-1 (red, left) and COX-2 (green, mid) expression in colonic crypts *in vivo*, nuclei (DAPI-blue). Histogram showing the number of COX-1 or COX-2 positive cells per crypt *in vivo* (right), (N≥1, n≥6). (**C**) Representative confocal images of COX-1 (red, left) and COX-2 (green, right) expression in cultured colonic crypts, nuclei (DAPI-blue). Histogram showing the number of COX-1 or COX-2 positive cells per crypt in *in vitro* culture (right) (N=3, n≥17) experiments, scale bar 10μm.

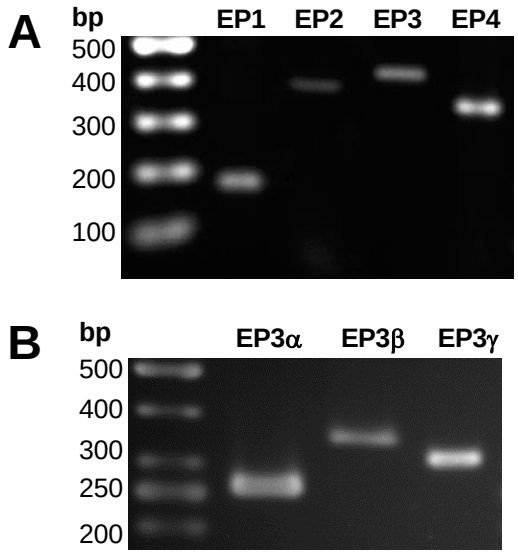


**Figure 4.2. Isolated colonic crypts express mRNA for prostaglandin E2 receptor subtypes.**

**(A)** PCR gel showing expression of EP1-4 receptor mRNA transcripts in freshly isolated colonic crypts. EP2 sample loaded at an approximately 80x greater concentration relative to other samples to enable band visualisation. Band sizes for PCR products EP1= 165bp, EP2= 402bp EP3= 438bp, EP4= 351bp. **(B)** PCR gel showing mRNA expression of EP3 variants ($\alpha$,$\beta$,$\gamma$) in freshly isolated colonic crypts. Band sizes for PCR products EP3$\alpha$= 261bp, EP3$\beta$= 349bp, EP3= 311bp. PCR gels obtained by AG.

We next sought to characterise the expression of EP1, EP2, EP3 and EP4 receptors within the different cell types of the mouse colonic epithelium both *in vivo*, in fresh fixed tissue sections (cut using a cryostat), and in *in vitro* cultured crypts using Immunofluorescent labelling. In order to visualise mucus-secreting goblet cells of the epithelium, and COX-1 expressing cells, an antibody to Muc-2 or COX-1 was utilised; mice were Lgr5EGFP positive for visualisation of stem cells.

The EP1 receptor was shown to be expressed by Lgr5EGFP stem cells (**Figure 4.3A TOP**, yellow arrows), Muc-2 positive cells (**Figure 4.3A MID**, white arrows) and COX-1 expressing cells (**Figure 4.3A BOTTOM**, cyan arrows) in the mouse colonic epithelium *in vivo*. Similar expression of the EP1 receptor was observed *in vitro* in isolated mouse colonic crypts (**Figure 4.3B**), where the EP1 receptor was expressed by Lgr5EGFP (yellow arrows, **TOP**), Muc-2 positive cells (white arrows, **MID**) and COX-1 expressing cells (cyan, **BOTTOM**).

The EP2 receptor (**Figure 4.4A**) was not expressed by Lgr5EGFP cells (yellow arrows, **TOP**), or COX-1 expressing cells (**BOTTOM**). Muc-2 positive cells (**MID**) express the EP2 receptor *in vivo* in the mouse colonic epithelium. When isolated mouse colonic crypts were labelled with

the EP2 receptor antibody (**Figure 4.4B**), a similar labelling pattern was observed to that seen *in vivo*, where only Muc-2 positive cells (**MID**) expressed the EP2 receptor.

Expression of the EP3 receptor (**Figure 4.5A**) was observed in Lgr5EGFP cells (yellow arrows, **TOP**), Muc-2 positive cells (**MID**) and COX-1 expressing cells (**BOTTOM**) *in vivo* in the mouse colonic epithelium. Similarly to that observed *in vivo*, isolated mouse colonic crypts also express EP3 receptor (**Figure 4.5B**) in Lgr5EGFP cells (yellow arrows, **TOP**), Muc-2 positive cells (**MID**) and COX-1 positive cells (**BOTTOM**).

The EP4 receptor (**Figure 4.6A**), was expressed by Lgr5EGFP (**TOP**) and Muc-2 expressing cells (**MID**) *in vivo* in the mouse colonic epithelium. No EP4 receptor expression was observed in COX-1 positive cells (**BOTTOM**) *in vivo*. *In vitro* isolated mouse colonic crypts were shown to express the EP4 receptor in the same cell types as that observed *in vivo*; EP4 was expressed in Lgr5EGFP (**TOP**) and Muc-2 positive cells (**MID**), but not in COX-1 expressing cells (**BOTTOM**) (**Figure 4.6B**).

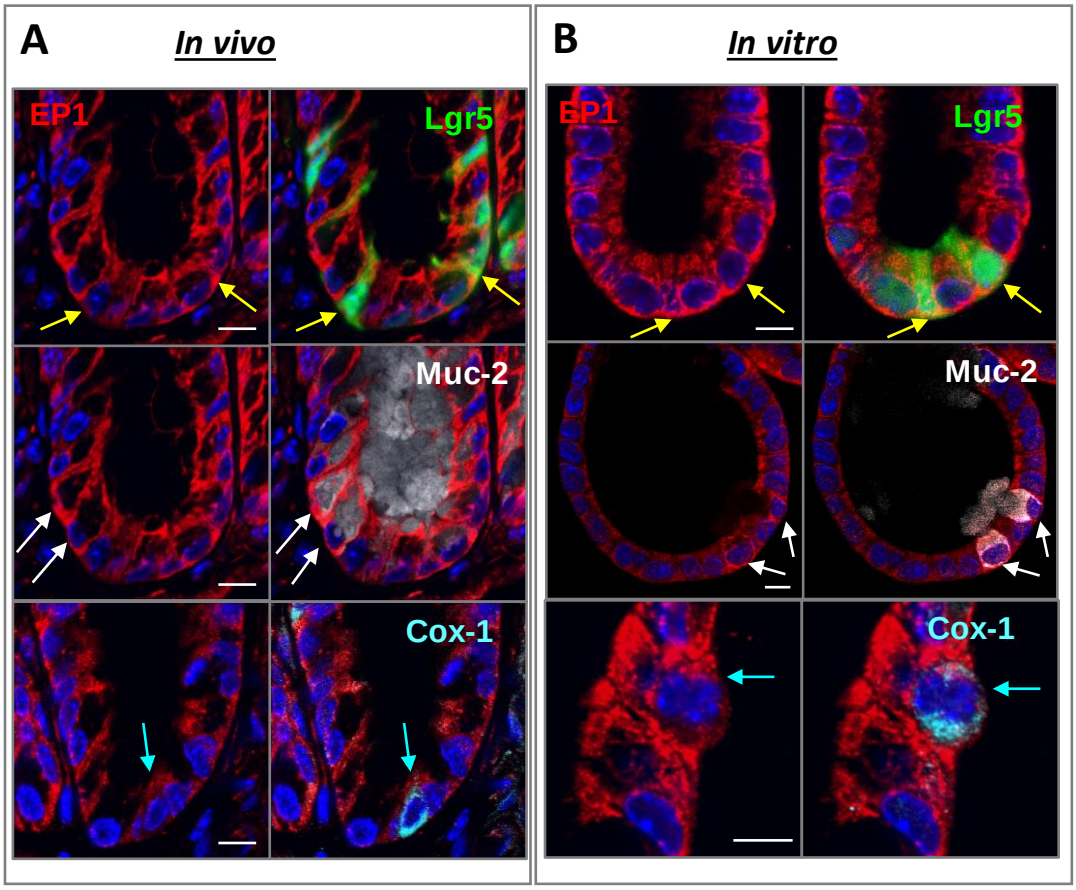


**Figure 4.3. Characterisation of EP1 receptor expression in the colonic epithelium.**

(**A**) Expression of the EP1 receptor *in vivo.* The EP1 receptor (red, left panel) is expressed by Lgr5EGFP positive cells (green, yellow arrows), Muc-2 positive cells (white, white arrows), and COX-1 expressing cells (cyan, cyan arrows), nuclei (DAPI-blue). (**B**) Expression of the EP1 receptor *in vitro*. The EP1 receptor (red, left panel) is expressed by Lgr5EGFP cells (green, yellow arrows), Muc-2 positive cells (white, white arrows) and COX-1 expressing cells (cyan, cyan arrows) *in vitro*, nuclei (DAPI-blue). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

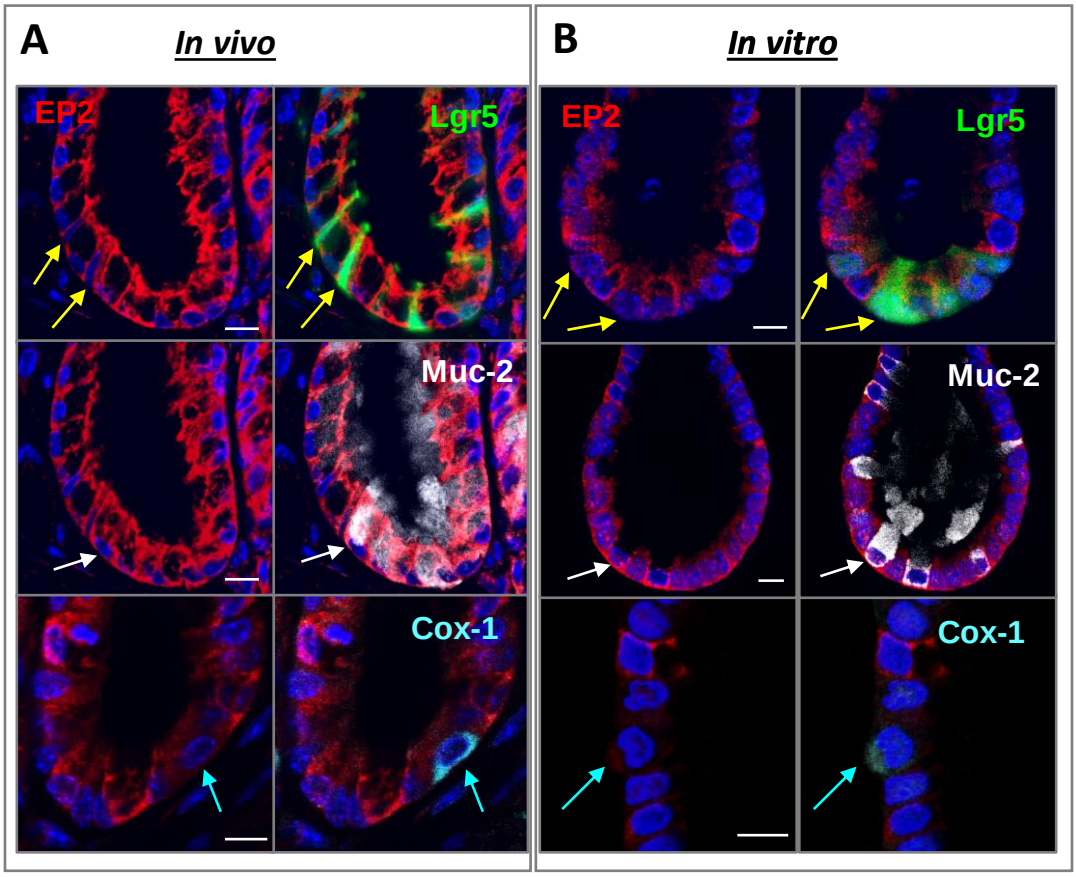


**Figure 4.4. Characterisation of EP2 receptor expression in the colonic epithelium.**

(**A**) Expression of the EP2 receptor *in vivo.* The EP2 receptor (red, left panel) is not expressed by Lgr5EGFP positive cells (green, yellow arrows) or COX-1 positive cells (cyan, cyan arrows). Muc-2 positive cells (white, white arrows) express EP2 receptor (red*) in vivo,* nuclei (DAPI-blue). (**B**) Expression of the EP2 receptor *in vitro*. The EP2 receptor (red, left panel) is not expressed by Lgr5EGFP cells (green, yellow arrows) or COX-1 positive cells (cyan, cyan arrows). Muc-2 positive cells (white, white arrows) express EP2 receptor *in vitro*, nuclei (DAPI-blue). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

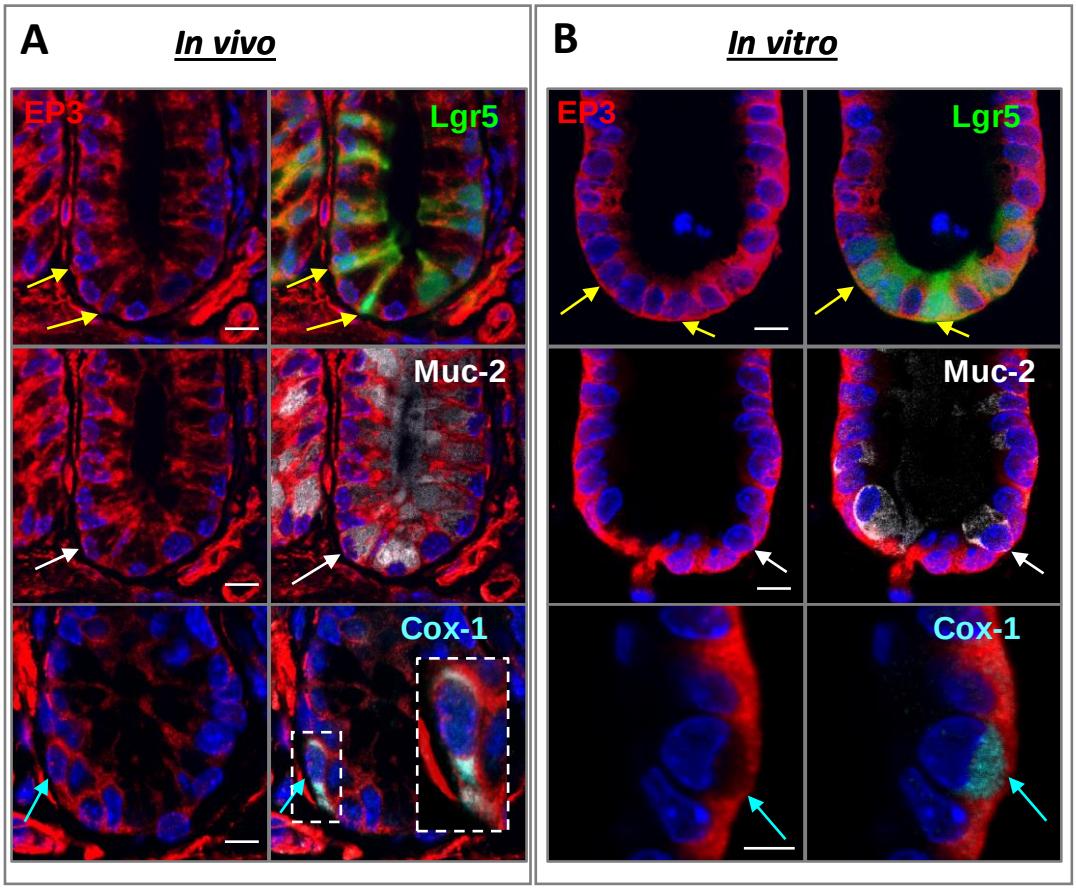


**Figure 4.5. Characterisation of EP3 receptor expression in the colonic epithelium.**

(**A**) Expression of the EP3 receptor *in vivo.* The EP3 receptor (red, left panel) is expressed by Lgr5EGFP positive cells (green, yellow arrows), muc-2 positive cells (white, white arrows), and COX-1 expressing cells (cyan, cyan arrows), nuclei (DAPI-blue). (**B**) Expression of the EP3 receptor *in vitro*. The EP3 receptor (red, left panel) is expressed by Lgr5EGFP cells (green, yellow arrows), Muc-2 positive cells (white, white arrows) and COX-1 expressing cells (cyan, cyan arrows) *in vitro*, nuclei (DAPI-blue). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

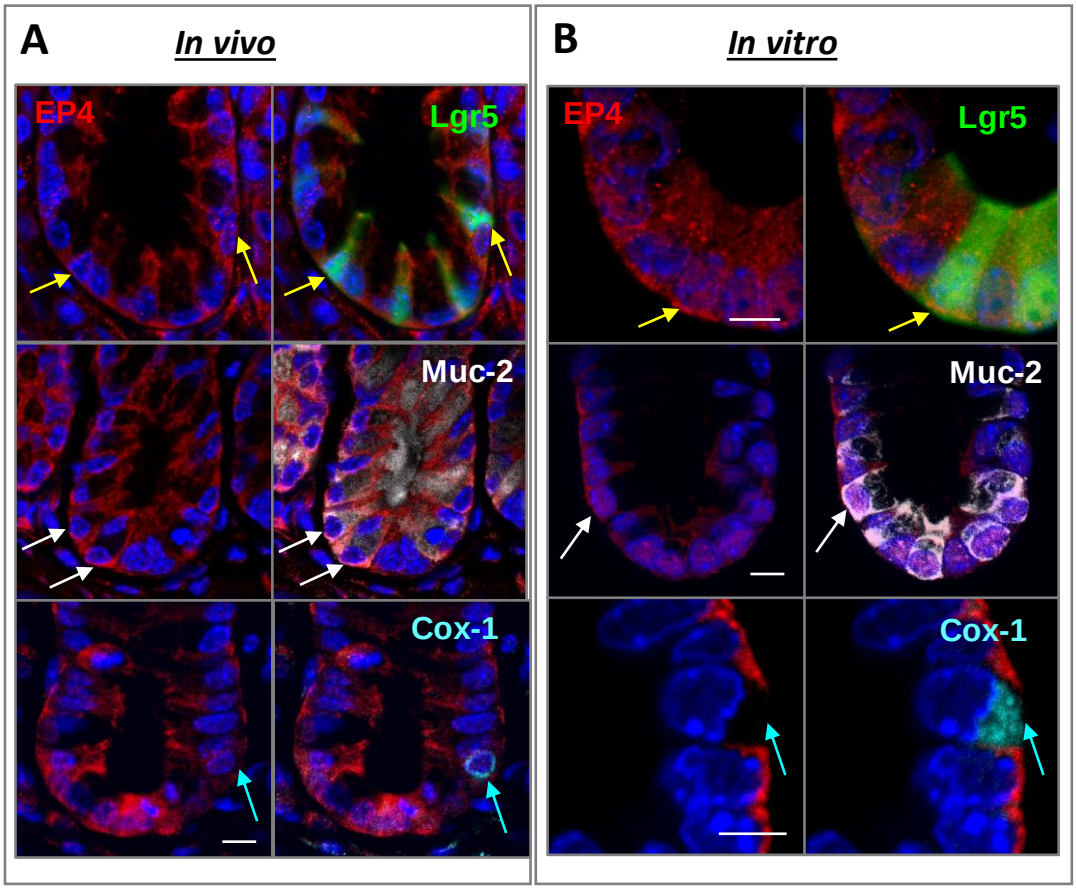


**Figure 4.6. Characterisation of EP4 receptor expression in the colonic epithelium.**

(**A**) Expression of the EP4 receptor *in vivo.* The EP4 receptor (red, left panel) is expressed by Lgr5EGFP positive cells (green, yellow arrows) and Muc-2 positive cells (white, white arrows). No EP4 expression is seen in COX-1 expressing cells (cyan, cyan arrows), nuclei (DAPI-blue). (**B**) Expression of the EP4 receptor *in vitro*. The EP4 receptor (red, left panel) is expressed by Lgr5EGFP cells (green, yellow arrows) and Muc-2 positive cells (white, white arrows). No EP4 expression is observed in COX-1 expressing cells (cyan, cyan arrows) *in vitro*, nuclei (DAPI-blue). Images are representative of N=3 experiments, n≥15, scale bars 10µm.

A summary of EP1, EP2, EP3 and EP4 receptor expression both *in vivo* and *in vitro* is provided in **Table 4.1**.

**Table 4.1. Summary table of EP receptor expression by specific cell types in the mouse colonic epithelium**

| Receptor | *in vivo/ in vitro* | Lgr5EGFP cells | Muc-2 cells | Cox-1 cells |
|---|---|---|---|---|
| **EP1** | *in vivo* | ✓ | ✓ | ✓ |
| | *in vitro* | ✓ | ✓ | ✓ |
| **EP2** | *in vivo* | × | ✓ | × |
| | *in vitro* | × | ✓ | × |
| **EP3** | *in vivo* | ✓ | ✓ | ✓ |
| | *in vitro* | ✓ | ✓ | ✓ |
| **EP4** | *in vivo* | ✓ | ✓ | × |
| | *in vitro* | ✓ | ✓ | × |

### 4.2.2 Autocrine $PGE_2$ signalling induces mouse colonic crypt cell proliferation

The mouse colonic epithelium expresses all the components required for $PGE_2$ signalling during homeostasis; however, the role of autocrine $PGE_2$ signalling in maintaining crypt homeostasis has not yet been determined. Inhibition of COX enzyme activity, and therefore *in vivo* $PGE_2$ synthesis, using NSAIDs has been shown to inhibit tumour growth and increase survival of colorectal cancer patients (Bresalier et al., 2005; Solomon et al., 2005; Arber et al., 2006; Rostom et al., 2007). However, the roles of specific COX enzyme inhibition in colonic crypt renewal during homeostasis remain to be investigated. In order to determine the effects of COX enzyme inhibition on proliferation of mouse colonic crypts, crypts were cultured with specific COX-1 or COX-2 inhibitors (10μM/ 20μM), or a COX-1/COX-2 inhibitor (10μM) for 48h, and EdU (10μM) was added for 17h on Day 1 (as described in **Section 2.3.7**). Concentrations of inhibitors were determined to minimise any non-selective inhibition and were the same or lower than previously utilised in the literature (Liu et al., 2010; Keysselt et al., 2017). A Click-IT reaction was performed on Day 2 for visualisation of EdU incorporating cells (as described in **Section 2.5**).

Inhibition of COX-1 and COX-2 enzymes with aspirin significantly decreased EdU incorporation compared to control (**Figure 4.7A**). To determine the effects of COX-1 enzyme inhibition on EdU incorporation, crypts were incubated with valeryl salicylate, a highly selective COX-1 inhibitor. Treatment of crypts with valeryl salicylate significantly reduced EdU incorporation compared to control treated crypts (**Figure 4.7B**). The effects of inhibition of the COX-2 enzyme on crypt survival, proliferation and budding were determined using the specific COX-2 inhibitor NS-398. NS-398 (20μM) was added to isolated crypts and survival over 6 days was assessed using time-lapse microscopy. A significant reduction in percentage organoid survival following 3 and 6 days in culture with NS-398 was observed compared to control (**Figure 4.7C**). In addition, a significant decrease in the number of nuclei incorporating EdU and the number of new buds formed per organoid was observed following treatment with NS-398 compared to control (**Figure 4.7D** and **E** respectively).

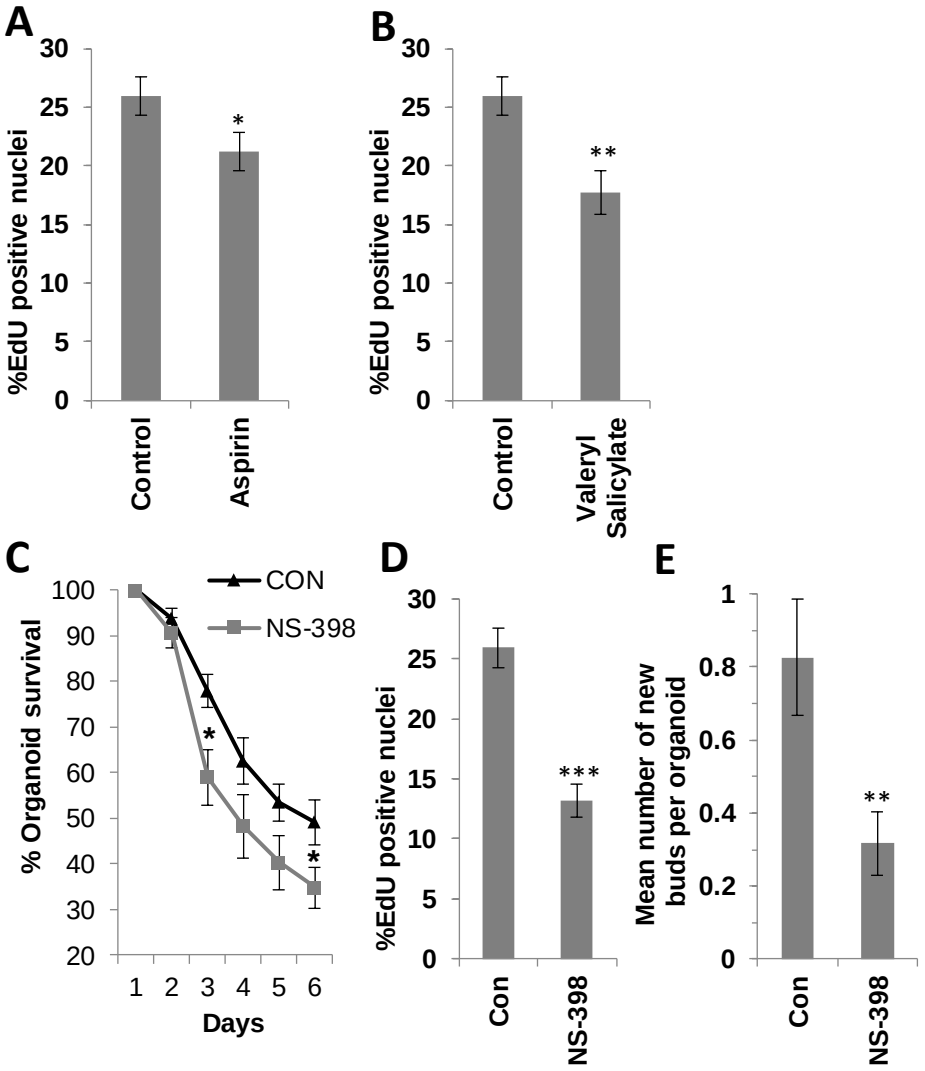


**Figure 4.7. Cox enzyme inhibition inhibits survival, proliferation and budding of mouse colonic crypts.**

Histogram showing a significant decrease in percentage of EdU positive nuclei following treatment with aspirin (10µM) (N=3, n≥210, *P<0.05) (**B**) Histogram showing a significant decrease in EdU incorporation of colonic crypts following treatment with valeryl salicylate (10µM) (N=3, n≥161, **P<0.01). Treatment of colonic crypts in culture with NS-398 (20µM) significantly reduced: **(C)** organoid survival (N=3, n=381, *P<0.05), AL (**D)** EdU incorporation of mouse colonic crypt (N=3, n≥186 ***P<0.001) and **(E)** mean number of new buds formed per organoid (N=3, n=63, **<0.01), compared to control, AL. Data are represented as mean +/- SEM.

The role of autocrine $PGE_2$ signalling in colonic crypt renewal was determined utilising a blocking antibody to $PGE_2$ (Mnich et al., 1995) or IgG control. Following 2 days in culture, EdU was added to crypts for 17h and nuclei incorporating EdU was visualised using a Click-iT reaction and epifluorescent microscopy. A significant reduction in the percentage of nuclei incorporating EdU was observed following treatment with the blocking antibody to $PGE_2$ compared to IgG control (**Figure 4.8**).

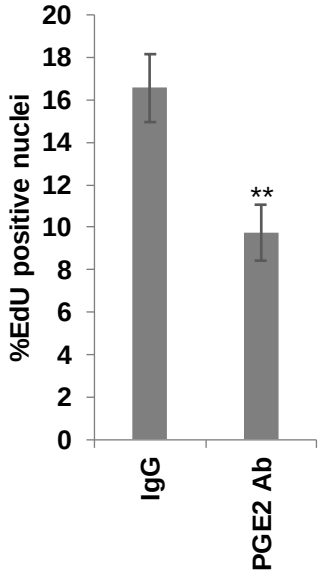


**Figure 4.8. PGE2 is required for mouse colonic crypt cell proliferation in vitro.**

Histogram showing the percentage of EdU positive cells per crypt following treatment with a blocking antibody to $PGE_2$ (50μM) compared to IgG control (N=3, n≥133, **P<0.01).

### 4.2.3 Selective activation of EP1 and EP3 receptor induces mouse colonic crypt renewal

The next step was to determine the role of selective activation of EP receptor subtypes in mouse colonic crypt renewal; therefore, a pharmacological approach utilising selective EP receptor agonists and antagonists was undertaken. The concentrations of agonists and antagonists utilised was selected in order to minimise cross-reactivity between EP receptor subtypes (Funk et al., 1993; Kiriyama et al., 1997; Abramovitz et al., 2000; Juteau et al., 2001; Kabashima et al., 2002; Billot et al., 2003; Gil et al., 2008; Dey et al., 2009; af Forselles et al., 2011; Birrell et al., 2013).

Firstly, in order to determine the role of autocrine EP receptor signalling in proliferation, crypts were incubated for 48h with selective antagonists to inhibit activation of each EP receptor subtype (as described in **Section 2.3.7**). When crypts were treated with a selective antagonist to either the EP2 or EP4 receptor, no difference was observed in the number of new buds formed per organoid (**Figure 4.9A**). Furthermore, EP2 or EP4 receptor inhibition had no effects on the percentage of nuclei incorporating EdU (**Figure 4.9B** and **C** respectively). However, when crypts were treated with antagonists which preferentially inhibit the EP1 or EP3 receptor, a significant reduction in the percentage of nuclei incorporating EdU was observed following treatment with either the EP1 or EP3 receptor antagonist (**Figure 4.10A** and **B** respectively).

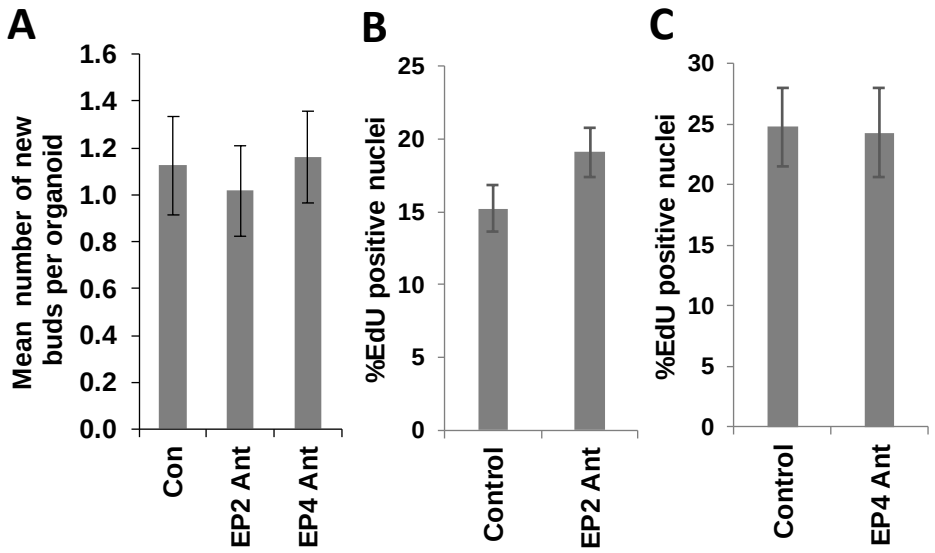


**Figure 4.9. EP2 or EP4 receptor antagonism has no effects on organoid budding or proliferation of mouse colonic crypts.**

(**A**) Histogram showing no difference in the number of new buds formed by colonic crypts following treatment with an EP2 antagonist (PF04418948, 10µM) or and EP4 antagonist (ONO-AE3-208, 10µM), (N=3, n≥57) AL ,(**B**) Histogram showing no change in EdU incorporation of colonic crypts following treatment with an EP2 antagonist (N=5, n≥139), or (**C**) an EP4 antagonist (N=4, n≥45) AL.

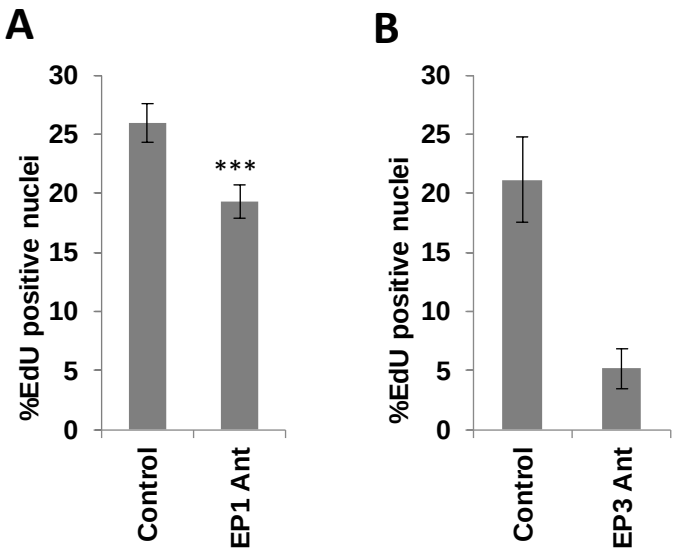


**Figure 4.10. EP1 or EP3 receptor antagonism reduces proliferation of mouse colonic crypts.**

(**A**) Histogram showing a significant reduction in EdU incorporation of colonic crypts following treatment with an EP1 antagonist (SC19220, 20μM) compared to control crypts (N=3, n≥144, ***P<0.001) (**B**) Histogram showing a significant reduction in EdU incorporation of colonic crypts following treatment with an EP3 antagonist (L-798106, 1μM) compared to control crypts (N=1, n≥26), AS.

Next, the effects of selective activation of EP receptor subtypes on mouse colonic crypt cell proliferation were determined (as described in **Section 2.3.7**). When cultured crypts were incubated with a selective EP2 receptor agonist or a selective EP4 receptor agonist for 17h following 5h in growth factor free media, no significant difference in proliferation was observed compared to control treated crypts (**Figure 4.11**). However, treatment of organoids with a selective EP1 receptor agonist or selective EP3 receptor agonist significantly increased proliferation compared to control crypts (**Figure 4.12**).

In order to determine which EP receptor subtype is responsible for the $PGE_2$-induced increase in EdU incorporation, crypts were incubated in growth factor free media for 5h before pre-incubation with the EP1 or EP3 receptor antagonist, or control. Crypts were then stimulated with $PGE_2$ with an EP1 or EP3 receptor antagonist or control for 17h in the presence of EdU. A significant increase in EdU incorporation was observed following treatment with $PGE_2$. Pre-incubation of crypts with either an EP1 or an EP3 receptor antagonist before addition of $PGE_2$ abrogated the $PGE_2$-induced increase in EdU positive nuclei. A significant reduction in EdU incorporation was observed in organoids treated with the EP3 receptor antagonist, however no significant reduction was observed following treatment with the EP1 receptor antagonist utilising this experimental protocol (**Figure 4.13**).

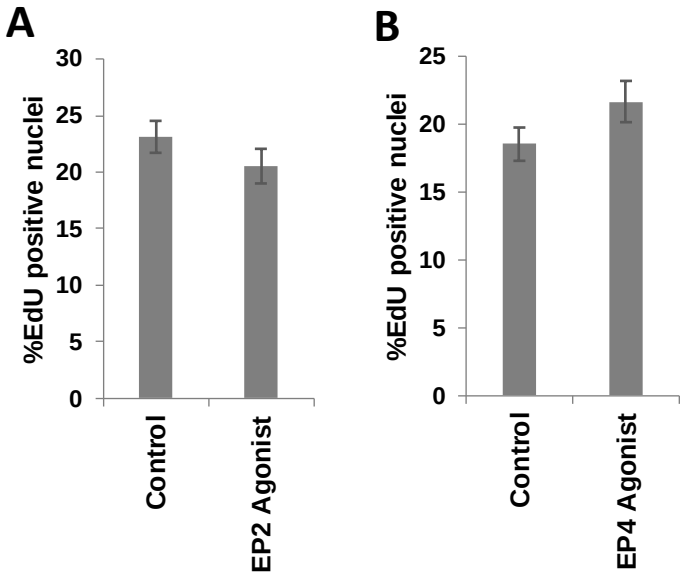


**Figure 4.11. Selective activation of EP2 or EP4 receptor has no effect on mouse crypt cell proliferation.**

Histograms showing no difference in proliferation when cells were treated with (**A**) an EP2 receptor agonist (butaprost, 12µM, N=4, n≥175), or (**B**) an EP4 receptor agonist (TCS-25110, 13µM, N=4, n≥179).

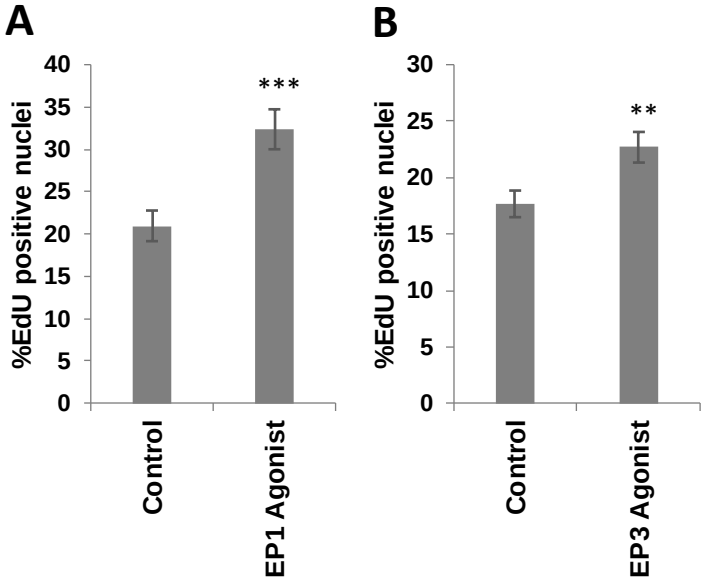


**Figure 4.12. Selective activation of EP1 or EP3 receptor increased mouse crypt cell proliferation.**

Histograms showing a significant increase in proliferation when cells were treated with (**A**) an EP1 receptor agonist (iloprost, 10µM, N=3, n≥93, ***P<0.001), or (**B**) an EP3 receptor agonist (sulprostone, 10µM, N=4, n≥192, **p<0.01).

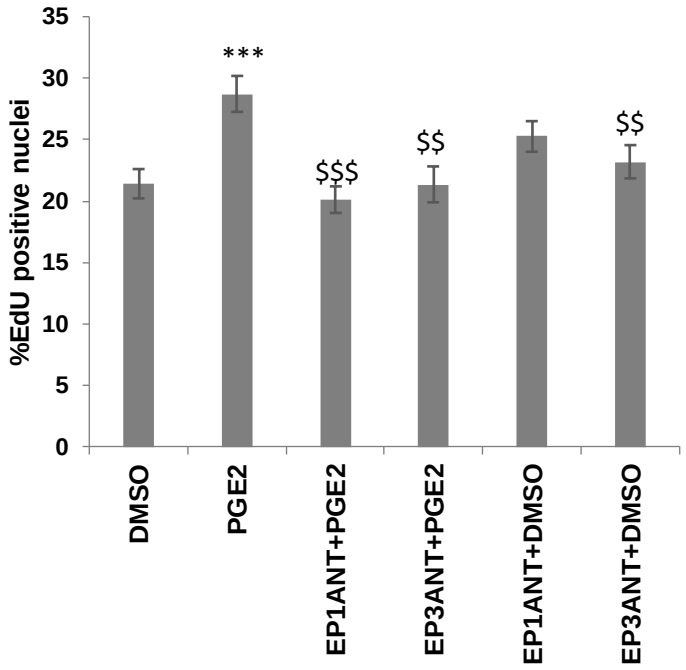


**Figure 4.13. PGE2-induced increase in mouse colonic crypt cell proliferation is abrogated by an EP1 or EP3 antagonist.**

Histograms showing the percentage of EdU positive nuclei in colonic crypts following stimulation with $PGE_2$ (10μM) in the presence or absence of an EP1 antagonist (SC19220, 20μM) or an EP3 antagonist (L-798106, 20μM) and compared to control (N=5, n≥213, ***P<0.001 compared to control, $$$P<0.001, $$P<0.01 compared to $PGE_2$).

### 4.2.4 A role for autocrine $PGE_2$ in human colonic crypt renewal?

We next sought to determine whether a similar paradigm exists in the human colonic epithelium. Human colonic crypts were isolated from tissue samples obtained from patients undergoing colorectal resection at the NNUH and placed in culture in the presence or absence of $PGE_2$ (1μM). On Day 2 of culture, EdU (10μM) was added to the media for 2h before fixation and a Click-iT reaction undertaken to determine EdU incorporation. A significant increase in the percentage of nuclei incorporating EdU per crypt was observed following treatment with $PGE_2$ (**Figure 4.14B**). As described earlier, 15-PGDH is an enzyme expressed by the epithelium which is known to breakdown endogenous $PGE_2$. The expression of 15-PGDH in isolated human colonic crypts or mucosal biopsy was determined using PCR. Expression of mRNA for 15-PDGH was observed in both crypts and biopsy, no expression was observed using a water negative control (**Figure 4.14A**). Recent advances have lead to the development of small molecular inhibitors which are able to antagonist 15-PGDH. In this study, we cultured human crypts with CAY10397, a 15-PGDH inhibitor, in order to determine

the role of autocrine $PGE_2$ signalling in EdU incorporation of crypts. Initial results (N=1), suggest that when crypts were cultured with $PGE_2$ with and without CAY10397, or CAY10397 alone, an increase was observed in the total number of nuclei incorporating EdU per crypt (**Figure 4.14B**). When the percentage of nuclei incorporating EdU was examined in each region of the crypt, a greater increase in EdU incorporation was observed at the base of crypts following treatment with CAY10397, or CAY10397+$PGE_2$. Treatment with $PGE_2$ induced the highest magnitude of increase in the percentage of nuclei incorporating EdU at the supra-base and mid regions of the crypts (**Figure 4.14C**). Further repeats of this experiment are required in order to determine whether the increase in proliferation following treatment with $PGE_2$, with and without CAY10397, is statistically significant.

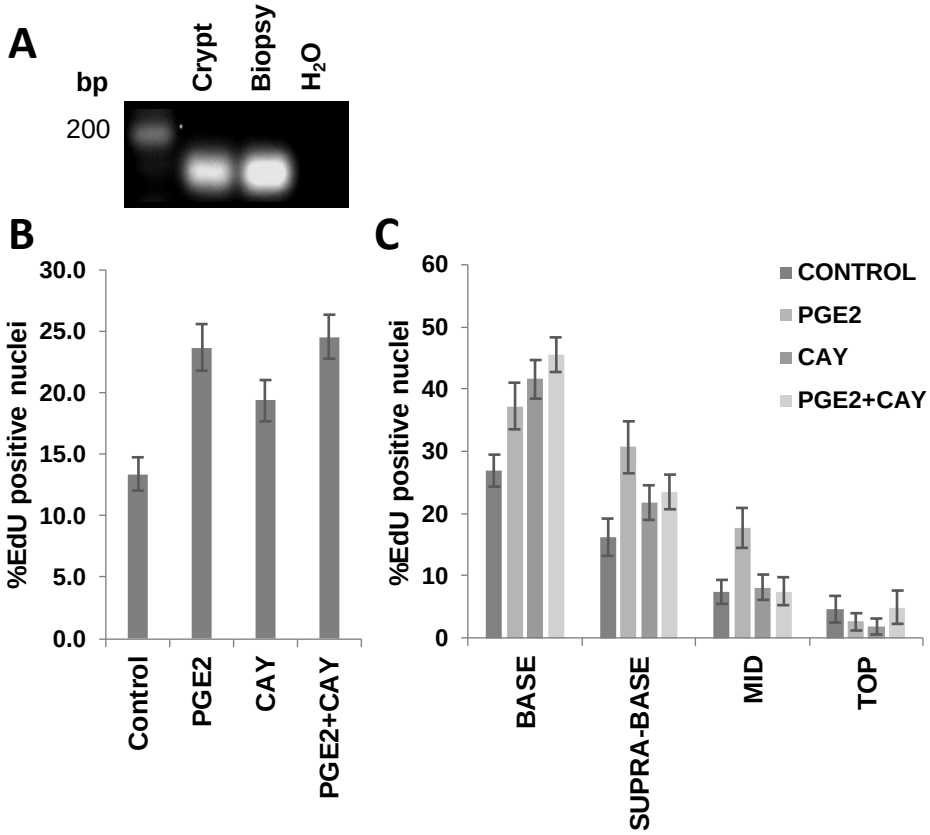


**Figure 4.14. Initial experiments in human colonic crypts suggest that autocrine PGE2 mediates human colonic crypt proliferation.**

(**A**) PCR gel showing 15-PGDH mRNA transcript expression in isolated human colonic crypts or mucosal biopsy, PCR band size 15-PGDH=145bp (**B**) Histogram showing the total percentage of nuclei incorporating EdU per crypt following treatment with $PGE_2$ (1µM), CAY10397 (50µM), or $PGE_2$+CAY10397 compared to control (N=1, n≥18)). (**C**) Histogram showing the percentage of nuclei incorporating EdU at the base, supra-base, mid and top region of crypts following treatment with $PGE_2$, CAY10397 or $PGE_2$+CAY10397 compared to control (N=1, n≥18).

### 4.2.5 EP receptor expression is conserved across the mouse and human colonic epithelium

In order to determine whether results obtained in our mouse study were comparable to human, the expression of EP receptor subtype in human colonic crypts was characterised. Immunofluorescent labelling of isolated human colonic crypts utilising antibodies for EP1-4 receptors, Lgr5 and Muc-2 was undertaken. Similarly to the expression pattern observed in mouse colonic crypts, the EP1 receptor was expressed in Lgr5 positive and Muc-2 positive cells of human colonic crypts (yellow arrows, **Figure 4.15**). The expression of EP2 receptor remained restricted to Muc-2 positive (yellow arrows) cells of human colonic crypts (**Figure 4.16**). In addition, the EP3 receptor was expressed in both Lgr5 positive and Muc-2 positive cells of human colonic crypts (yellow arrows, **Figure 4.17**). The EP4 receptor subtype was expressed by Lgr5 positive cells; less intense labelling of the EP4 receptor was observed in Muc-2 positive cells of human colonic crypts (**Figure 4.18**).

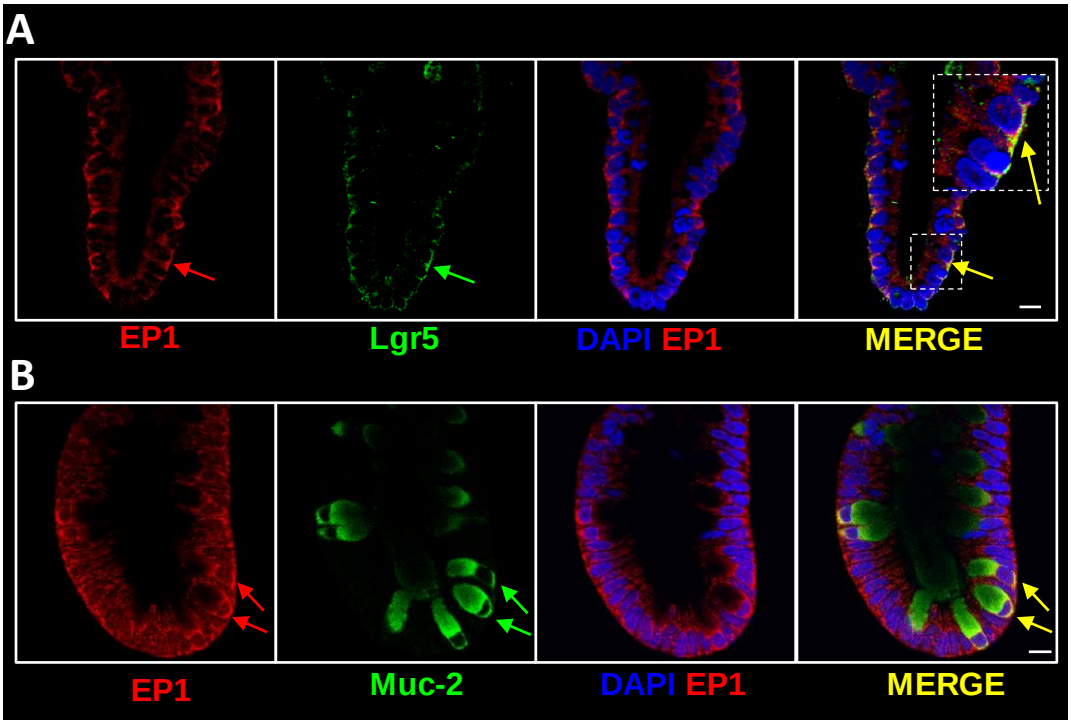


**Figure 4.15. Stem cells and goblet cells of human colonic crypts express EP1 receptor.**

(**A**) Representative confocal image showing EP1 receptor (red, left panel) expression by Lgr5 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). (**B**) Representative confocal image of EP1 receptor (red) in Muc-2 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

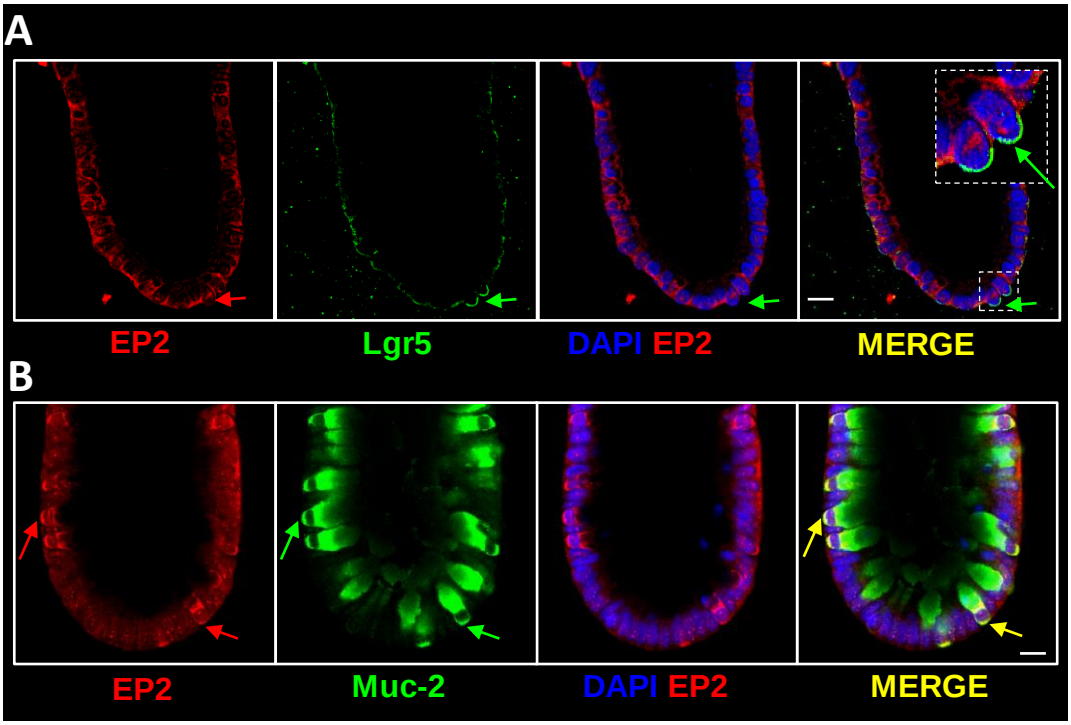


**Figure 4.16. Goblet cells of human colonic crypts express EP2 receptor.**

(**A**) Representative confocal image showing a lack of EP2 receptor (red) expression by Lgr5 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (green arrows). (**B**) Representative confocal image of EP2 receptor (red) in Muc-2 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

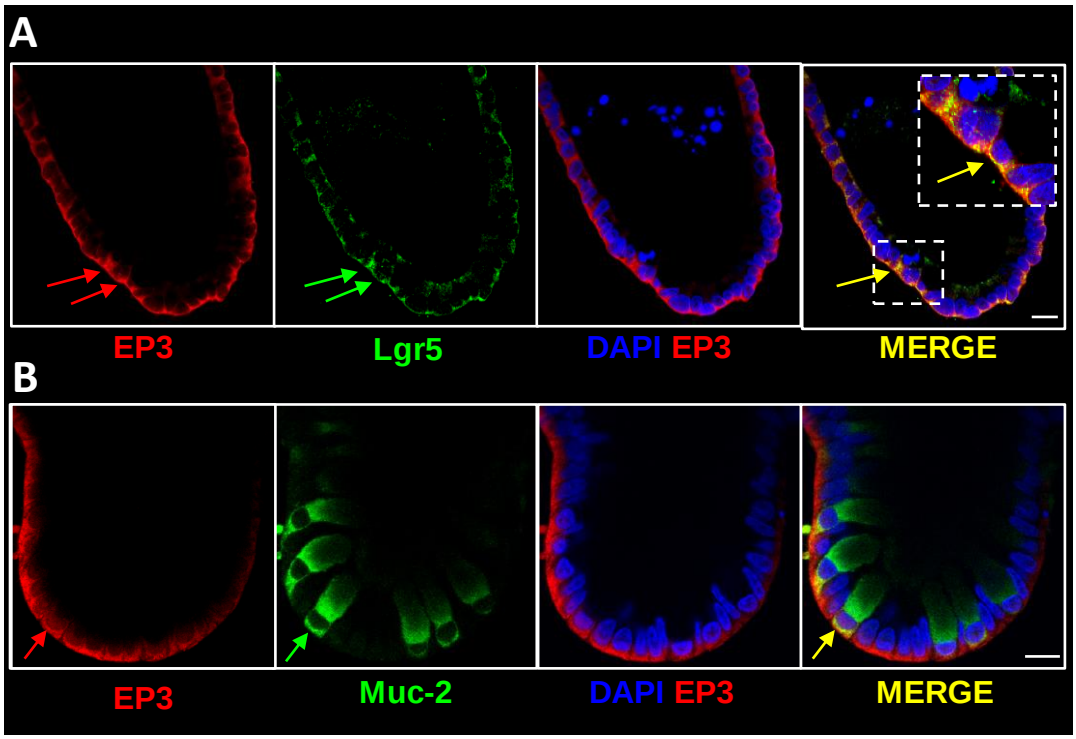


**Figure 4.17. Stem cells and goblet cells of human colonic crypts express EP3 receptor.**

(**A**) Representative confocal image showing EP3 receptor (red, left panel) expression by Lgr5 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). (**B**) Representative confocal image of EP3 receptor (red) in Muc-2 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). Images are representative of N=3 experiments, n≥15, scale bar 10µm.

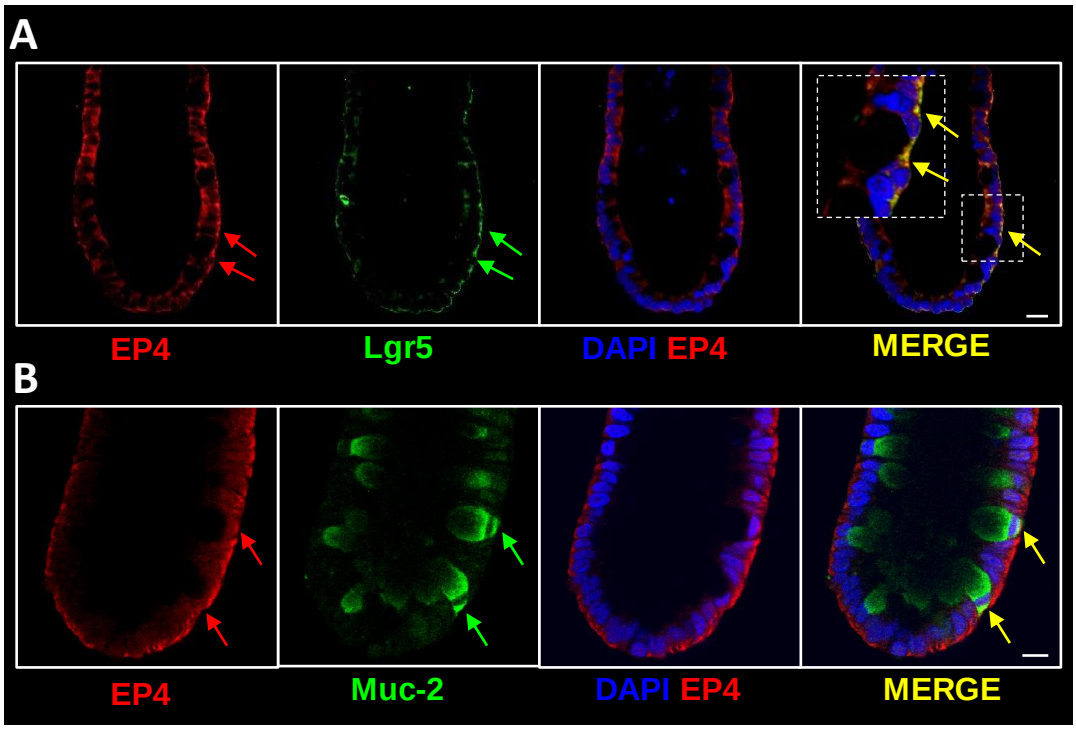


**Figure 4.18. EP4 is expressed by the human colonic epithelium.**

(**A**) Representative confocal image showing EP4 receptor (red, left panel) expression by Lgr5 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). (**B**) Representative confocal image of EP4 receptor (red) in Muc-2 positive cells (green), nuclei (DAPI-blue) in isolated human colonic crypts (yellow arrows). Images are representative of N=2 experiments, n≥15, scale bar 10µm.

A summary table detailing conservation of EP receptor subtype expression in Lgr5 or Muc-2 expressing cells mouse and human colonic epithelium is provided in **Table 4.2**.

**Table 4.2. Summary table demonstrating conservation of EP receptor expression by specific cell types in human and mouse colonic crypts.**

| Receptor | Human/ Mouse | Lgr5+ cells | Muc-2 cells |
|---|---|---|---|
| **EP1** | Human | ✓ | ✓ |
| | Mouse | ✓ | ✓ |
| **EP2** | Human | low | ✓ |
| | Mouse | × | ✓ |
| **EP3** | Human | ✓ | ✓ |
| | Mouse | ✓ | ✓ |
| **EP4** | Human | ✓ | ✓ |
| | Mouse | ✓ | ✓ |

## 4.3 Discussion

In this chapter, we demonstrate for the first time a role for autocrine $PGE_2$ signalling in the maintenance of colonic homeostasis (**Figure 4.19**). The mouse colonic epithelium expresses COX enzymes for the synthesis of $PGE_2$ both *in vivo* and in our *in vitro* crypt culture system. The receptors for $PGE_2$, EP1, EP2, EP3 and EP4 were all shown to be expressed by the colonic epithelium both *in vivo* and *in vitro*. EP1 and EP3 receptors expression was observed on stem cells, goblet cells, and COX-1 expressing cells. Inhibition of endogenous $PGE_2$ using a blocking antibody approach *in vitro* reduced colonic crypt cell proliferation. In addition, inhibition of COX-1 and/or COX-2 enzymes reduced crypt cell proliferation, budding and organoid survival. $PGE_2$ induced colonic crypt cell proliferation, which was abrogated by inhibition of EP1 or EP3 receptor signalling using selective antagonists. These data suggest that $PGE_2$ signalling through the EP1/3 signalling axis is important for mouse colonic crypt homeostasis.

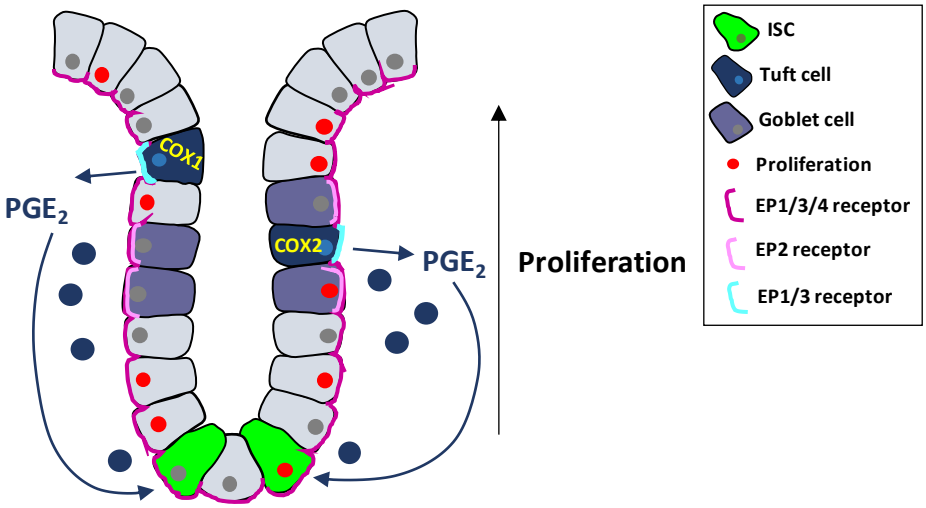


**Figure 4.19. PGE2 induces colonic crypt cell proliferation during homeostasis.**

The colonic epithelium expresses COX enzymes for $PGE_2$ synthesis, and EP1-4 receptor subtypes for $PGE_2$ signalling. EP1 and EP3 are expressed on all cell types in the colonic epithelium; restricted expression of EP2 is observed on goblet cells, EP4 receptor is expressed on all cell types with the exception of COX-1-expressing cells. Autocrine $PGE_2$ induced colonic crypt cell proliferation through activation of the EP1/EP3 receptor subtypes.

The differential expression of Cyclooxygenase enzymes by Doublecortin-like kinase 1 protein+ tuft cells of the intestinal epithelium has recently been described (Gerbe et al., 2011); COX-1 is constitutively expressed, and COX-2 is inducible upon tissue stress or damage (Wang and Dubois, 2010). Interestingly, when the expression of COX-1 and COX-2 were

characterised in the mouse colonic epithelium *in vivo* and in *in vitro* isolated colonic crypts, no significant difference between the numbers of cells positive for COX-1 or COX-2 was observed. Previous studies have suggested that COX-1 and COX-2 are co-expressed by the same cells; unfortunately, we were unable to confirm COX-1 and COX-2 co-expression due to differences in antigen retrieval methods required. Gerbe *et al* (2011) reported that a rare population of cells exists within the epithelium which are DCLK1 positive and COX-1 negative. These cells were located mainly in the base of crypts, and were defined as differentiating tuft cells. Whilst COX-2 positive cells were confirmed to be tuft cells as determined by co-expression of the tuft cell marker SOX-9, no data was provided as to the number of COX-2 positive cells per crypt compared to COX-1 positive cells, or indeed if these cells co-expressed COX-1 (Gerbe et al., 2011). Further experiments are required in order to determine whether COX-1 cells *in vivo* co-express COX-2 during homeostasis, and to explore the possibility that the distinct subset of differentiating tuft cells which do not express COX-1 may be the COX-2 positive cells.

Previously, the use of NSAIDs, which inhibit both COX-1 and COX-2 enzyme activity, have been shown to significantly reduce the risk of developing colorectal cancer (Flossmann et al., 2007; Rostom et al., 2007; Chan et al., 2008). However, the long-term use of NSAIDs was associated with an increased risk of cardiovascular thromboembolic events. We therefore sought to determine the effects of selective COX enzyme inhibition on mouse colonic crypt cell proliferation. In line with the literature, inhibition of COX-1 and COX-2 enzymes using aspirin induced a significant decrease in proliferation. Interestingly, we also observed a significant reduction in proliferation of colonic crypts following treatment with a selective inhibitor of COX-1 (valeryl salycilate) or COX-2 (NS-398). This suggests that the basal activity of either COX-1 or COX-2 enzymes alone is insufficient to maintain homeostatic levels of proliferation. In contrast to what has been previously described in the literature, this suggests a role for both COX-1 and COX-2 enzyme activity for the maintenance of crypt renewal during health. Although further experiments would be required to confirm this, we would speculate that selective inhibition of COX-1 or COX-2 as an anti-cancer therapy would not necessarily prevent side effects observed following treatment with NSAIDs.

Studies have suggested that all four EP receptor subtypes are expressed at the mRNA level in colonic mucosal tissue (Shoji et al., 2004; Sugimoto and Narumiya, 2007; Olsen Hult et al., 2011). The EP3, EP2 and EP4 receptor subtypes were also previously shown to be expressed by epithelial cells within the colonic epithelium using an antibody labelling approach (Shoji et al., 2004; Olsen Hult et al., 2011), however no previous studies have characterised the

differential expression of EP receptor subtypes in cell types of the mouse intestinal epithelium. In this thesis, the presence of all four EP receptor subtypes were confirmed to be expressed in isolated mouse colonic crypts, at the mRNA level and in specific cell types at the protein level. The EP3 receptor is known to have three different isoforms, EP3α, β and γ (Namba et al., 1993), all of which were shown in our studies to be expressed at the mRNA level by the colonic epithelium. The EP3 receptor isoforms are known to signal through slightly different pathways; EP3α and β inhibit adenylate cyclase whereas the EP3γ variant inhibits adenylate cyclase at low $PGE_2$ concentrations, and stimulates adenylate cyclase at high $PGE_2$ concentrations. Overexpression of each of EP3 receptor splice variant in tumour cells has been described to decrease tumorigenic potential *in vivo*. In this study, no differences were observed between the effects of modulating EPα, β and γ in tumour cells (Macias-Perez et al., 2008). It would be of great interest to determine the relative expression levels of each of these isoforms in the healthy colonic epithelium using quantitative RT-PCR in order to further elucidate the role of EP3 receptor activation in homeostasis.

The drivers of intestinal tissue renewal are stem cells, which are located at the base of colonic crypts. Interestingly, only EP1, EP3 and EP4 receptor subtypes were expressed by Lgr5EGFP stem cells in the mouse colonic epithelium, which suggests that these receptor subtypes may be important for stem cell driven tissue renewal. In agreement, when autocrine signalling through the EP1 or EP3 receptor was inhibited using addition of selective antagonists into culture immediately following crypt isolation, a significant decrease in mouse crypt cell proliferation was observed. Selective activation of the EP1 or EP3 receptor induced a significant increase in proliferation, suggesting that signalling through the EP1/3 receptors are important for colonic crypt renewal during homeostasis. However, some cross-reactivity between the EP1 and EP3 receptor subtypes has previously been described with use of the EP1 agonist (iloprost) and EP3 agonist (sulprostone), therefore further experiments are required to determine whether selective EP1 or EP3 receptor activation induces crypt cell proliferation (Abramovitz et al., 2000). The $PGE_2$-induced increase in crypt cell proliferation observed in our studies was abrogated by the addition of a selective EP1 or EP3 antagonist, suggesting that $PGE_2$ signals through EP1 and EP3 receptor subtypes to induce proliferation. However, in this experiment, treatment of colonic crypts with the EP1 or EP3 receptor antagonist in the absence of $PGE_2$ did not decrease crypt cell proliferation (**Figure 4.13**). This is likely to be due to differences in experimental protocol, whereby a shorter incubation with EP1 or EP3 receptor antagonists was utilised following 24h in culture. Addition of the EP1 receptor antagonist for a short temporal stimulation did not significantly decrease crypt cell

proliferation compared to $PGE_2$ stimulated crypts. This suggests that a longer incubation time is required for EP1 receptor inhibition to induce a decrease in crypt cell proliferation. The concentrations of EP1 and EP3 receptor antagonists in this experiment were higher than previously utilised in the literature (Bassil et al., 2008; Rodriguez-Lagunas et al., 2010); however, no significant cross-reactivity with other EP receptor subtypes is thought to occur following the use of SC19220 or L-798106 at higher concentrations (Funk et al., 1993; Juteau et al., 2001).

Signalling through the EP1 receptor has previously been described to promote cell invasion and migration associated with tumour metastasis (Yang et al., 2010; Kim et al., 2011; Zhang et al., 2014). However, the findings described in this chapter indicate that EP1 receptor signalling is also important for maintaining crypt homeostasis. This suggests that inhibition of EP1 receptor signalling for the prevention of tumour metastasis may be associated with a downregulation of normal intestinal tissue renewal and therefore may not be a suitable target for anti-cancer therapy. Interestingly, previous studies have demonstrated a decrease in tumour cell proliferation following selective activation of EP3 receptor signalling (Macias-Perez et al., 2008). This finding, along with our studies which suggest a role for EP3 receptor signalling in mouse crypt cell proliferation during homeostasis, suggest that EP3 receptor activation may be important only for proliferation of cells within the healthy colonic epithelium. Therefore, future treatment of cancer patients with an EP3 receptor agonist may represent an attractive target to reduce tumour growth whilst protecting normal crypt cell proliferation. However, further work would be required to determine whether EP3 receptor activation also induces crypt cell proliferation during homeostasis in human colonic crypts.

Expression of the EP1 and EP3 receptor on COX-1 expressing cells in the mouse colonic epithelium suggests that $PGE_2$ signalling through the EP1 and EP3 signalling axis may be involved in a positive feedback loop, whereby activation of EP1 and EP3 receptors may be able to induce further $PGE_2$ secretion. This remains the subject of future work. As described earlier, COX-2 is thought to be induced in tissue stress, and is elevated in colorectal cancer. It would therefore be of interest to determine the expression of EP receptors on COX-2 positive cells, as this may be indicative of which EP receptor subtypes are important for $PGE_2$ signal transduction in cancer.

The presence of EP4 receptor expression on Lgr5EGFP stem cells indicated a possible role for EP4 in stem cell driven tissue renewal, although semi-quantitative analysis revealed that the EP4 receptor expression is significantly lower in the base, supra-base and mid regions of

mouse colonic crypts compared to top *in vivo* and *in vitro* (**Appendix A**). This suggests that although the EP4 receptor is expressed by stem cells, this expression is lower than in other cells of the crypt suggesting that the primary role of EP4 receptor activation may not be in stem cell renewal. Indeed, when the EP4 receptor was inhibited no change in proliferation, organoid survival or budding was observed. Previous studies by Hawcroft et al. (2007) showed that treatment with an EP4 agonist (TCS-2510) had no effects on cell proliferation in a colon cancer cell line (Hawcroft et al., 2007). In agreement with this, no change in crypt cell proliferation was observed following treatment with the EP4 receptor agonist in these studies. However, previous studies have identified a role for EP4 receptor signalling in the crypt regenerative response to injury through the differentiation of WAE cells (Manieri et al., 2012; Manieri et al., 2015). EP4 receptor signalling has also previously been shown to ameliorate colitis in DSS-induced colitis (Kabashima et al., 2002), but is implicated in pathogenesis of colitis induced by T cell transfer into recombinase-activation gene 2 deficient mice (Yao et al., 2013). It is possible that in tissue injury or inflammation, $PGE_2$ activates the EP4 receptor on stem cells to induce proliferation and crypt regeneration; it would therefore be of interest to observe the effects of EP4 receptor modulation in crypts isolated from colitic tissue.

The localisation of EP2 receptor subtype was restricted to Muc-2 positive goblet cells of colonic crypts. Interestingly, no changes in proliferation were observed following treatment of colonic crypts with either a selective EP2 agonist or EP2 antagonist, suggesting that this receptor subtype is not important for this aspect of intestinal tissue renewal. We would speculate that the restricted expression of EP2 receptor to goblet cells may suggest a role for activation of the EP2 receptor subtype in mucus secretion and therefore maintenance of the epithelial barrier during homeostasis. In agreement with the EP2 receptor playing a role in epithelial barrier function, previous studies by Lejeune et al. (2014) demonstrated, using a pharmacological interrogation of Caco-2 cells, that downregulation of the EP2 receptor was associated with a decrease in TER by inducing the degradation of the tight junction protein Claudin-4 (Lejeune et al., 2014). However, these studies were limited to the use of cell lines, and as such the effects on tight junction protein expression following EP2 receptor inhibition of colonic crypts would be required to further elucidate the mechanism of EP2 receptor signalling. A role for EP2 receptor signalling has been described in intestinal polyposis (Sonoshita et al., 2001) and the lack of changes in homeostatic proliferation suggests that specific abrogation of EP2 receptor signalling may be a promising target for the development of anti-cancer therapies. However, the effects of inhibition of EP2 receptor signalling on

epithelial barrier function would need to be fully evaluated before the development of such treatments.

As described previously, the WNT signalling pathway is thought to be the master regulator of intestinal renewal. Activation of $PGE_2$ is known to augment WNT signalling in order to induce hematopoietic stem cell proliferation and differentiation during development and in organ regeneration (Goessling et al., 2009). It is therefore possible that the effects of autocrine $PGE_2$ signalling on colonic crypt cell proliferation described in this thesis are also mediated through the WNT signalling pathway. However, previous studies suggest that $PGE_2$ effects on WNT signalling are mediated through cAMP/PKC signalling, indicating involvement of EP2 and EP4 receptor transduction pathways (Goessling et al., 2009). This is in contrast to results shown in this thesis, where activation of EP2 and EP4 receptor signalling did not induce crypt cell proliferation. During steady-state renewal in the colonic crypt, autocrine $PGE_2$ was shown to signals through the EP1 and EP3 receptor subtypes to induce proliferation. The downstream effects of activation of EP1 and EP3 receptor subtypes are known to induce an increase in intracellular calcium through binding of $G_\alpha$ proteins (Sugimoto and Narumiya, 2007), which has previously been associated with the crypt regenerative response to injury (Chen et al., 2015). Calcium signalling is required for a number of cellular processes, including proliferation and apoptosis in all mammalian cell types (Roderick and Cook, 2008). However, previous studies have identified that the EP3 receptor also binds $G_i$ proteins, which have been linked to inhibitory functions of EP3 receptor activation through inhibition of adenylate cyclase. Future work aims to identify the downstream signalling transduction pathways through EP1/EP3 receptor activation induces crypt cell proliferation. Initial experiments will determine whether intracellular calcium signalling is increased following EP1/EP3 receptor activation during calcium sensitive dyes such as Fura-2-AM in combination with live imaging. Furthermore, enzymatic fluorometric assays may be utilised to determine adenylate cyclase activity in response to treatment with EP1/EP3 receptor activation.

### 4.3.1 Autocrine $PGE_2$ signalling is important for human colonic crypt proliferation

The validation of our mouse studies with human experiments are of critical importance when considering the interrogation of this pathway for the development of treatments for human disease. In agreement with findings observed in mouse studies, prostaglandin-$E_2$ was also shown to play a role in human colonic crypt cell proliferation. Addition of $PGE_2$ induced an increase in human colonic crypt cell proliferation. Inhibition of autologous $PGE_2$ breakdown

using an antagonist to 15-PGDH induced crypt cell proliferation. The expression of EP receptor subtypes by the colonic epithelium was highly conserved across the mouse and human epithelium.

These findings demonstrate a role for $PGE_2$ signalling in colonic crypt homeostasis. Exogenous addition of $PGE_2$ was shown to induce a significant increase in proliferation, which was most evident in the supra-base and mid regions of the crypts, suggesting that it may play a role in the proliferation of progenitor cells and differentiated cells which are present in these regions. Utilising a newly developed small molecular inhibitor, CAY10397, to inhibit the breakdown of endogenous $PGE_2$ by the 15-PGDH enzyme, autocrine $PGE_2$ signalling was shown to be important for colonic crypt renewal. This effect was most evident at the base of crypts, where intestinal stem cells reside. The regenerative potential of small molecule inhibitors of 15-PGDH has previously been applied to mouse models of liver injury and colitis, where SW033291-treated mice had an increased rate of liver regeneration and were protected from colitis by maintaining cell proliferation within DSS-treated mucosa (Zhang et al., 2015). However, the 15-PGDH enzyme is lost in colorectal cancers and FAP, therefore further studies are required to determine the effects of small molecule inhibition of 15-PGDH on the risk of developing cancer.

The differential expression of EP receptor subtypes was highly conserved across mouse and human samples. In agreement with our mouse data, stem cells of human colonic crypts expressed EP1, EP3 and EP4 whereas the EP2 receptor was restricted to Muc-2 positive goblet cells. The next steps for the human studies are to determine the roles of selective EP receptor activation on colonic crypt renewal in the human colonic epithelium.

### 4.3.2 Limitations of the study and next steps

A pharmacological approach was undertaken in this study to determine the contribution of selective EP receptor activation and inhibition on colonic crypt renewal during homeostasis. Whilst we were able to conclude that $PGE_2$ promotes colonic crypt cell proliferation, which is likely to be through the EP1 and EP3 receptor signalling pathways, the study is limited by the specificity of pharmacological agents commercially available. The concentrations of drugs utilised were optimised to minimise activation of other receptor subtypes, however the exact efficacies of each agonist and antagonist to specific receptor subtype in our system were not determined. Agonist and antagonist efficacies could be determined in the future using a ligand binding affinity assay (Strange, 2008). Future work in our lab will add

antagonists for EP receptor subtypes along with each agonist in order to further minimise any cross-reactivity. Furthermore, the development of novel agents which are specific to each EP receptor subtype would further support our findings.

A future aim of this area of work would be to utilise a combination of selective activation and inhibition of EP receptor subtypes in order to determine the relative contributions of each EP receptor subtype in cell proliferation of healthy and tumour tissue. The development of 3D tumoroid culture in the Williams' laboratory amongst others, enables these studies to be completed on paired samples from tumour and 'healthy' tissue obtained from the same patient. The development of elegant techniques which allow gene knock out in organoids, such as the use of Cre recombinase-inducible retrovirus vectors or CRISPR/Cas9 system, provides useful tools to investigate the roles of specific EP receptor deletion in organoid and tumoroid growth (reviewed in (Driehuis and Clevers, 2017). The use of these techniques in order to determine the functional role of EP receptor subtypes in health and cancer remains the focus of future work.

## 4.4 Conclusions

This chapter has identified a novel role for autocrine $PGE_2$ in the maintenance of colonic crypt homeostasis through the EP1/3 signalling axis. Human studies in this chapter support findings from mouse studies, and validate the use of mouse models for the interrogation of the $PGE_2$ pathway.

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The next chapter looks at a potential source of IL-6 and $PGE_2$ in the intestinal mucosa. An abundance of immune cells are resident in the lamina propria underlying the intestinal epithelium. One immune cell subtype, which is resident along the gastrointestinal tract from birth and is known to secrete both IL-6 and $PGE_2$, is the eosinophil. Whilst eosinophils have been best characterised for their role in allergy, parasitic infection and inflammatory diseases, a role for eosinophils in the maintenance of homeostasis is beginning to be recognised. The next chapter examines the spatial characteristics of eosinophils in the intestinal mucosa, and begins to look at the effects of tissue resident eosinophils on the epithelium.

# 5 Chapter 5. The role of eosinophils in human colonic crypt homeostasis

## 5.1 Introduction

The intestinal epithelium forms a vital barrier between luminal microbial content and underlying immune cells in the lamina propria. A plethora of immune cells reside in the lamina propria during health. Immune cells are known to express factors important for crypt cell renewal (Rosenberg et al., 2013), and evidence is beginning to emerge for a role of intestinal immune cells in the modulation of crypt homeostasis renewal (Pull et al., 2005; Seno et al., 2009; Kuhn et al., 2014; Skoczek et al., 2014). Approximately 20-40% of the immune cells residing within the intestinal lamina propria are eosinophils (Carlens et al., 2009). Eosinophils are potent effector cells, most commonly associated with helminth infection, allergic asthma and Inflammatory Bowel Diseases (IBDs); however, they are also present in abundance during health and in quiescent IBD (Al-Haddad and Riddell, 2005). As such, roles are emerging for eosinophils in tissue remodelling (Rosenberg et al., 2013), the regulation of inflammation namely through secretion of IL-6 (Chu et al., 2011), and maintenance of the epithelial barrier (Furuta et al., 2005; Wallon et al., 2011). However, the roles of eosinophils in maintenance of colonic crypt homeostasis remain largely unknown.

The accumulation of eosinophils in sites of renewal and regeneration led Lee et al. (2010) to suggest that the role of eosinophils is to modulate Local Immunity And/or Remodelling/Repair; the LIAR hypothesis. Lee et al. (2010) speculated that signals derived from cells undergoing apoptosis recruit eosinophils to the site and induce accumulation; proliferating cells secrete factors which are sufficient to prolong survival and maintain eosinophils in the niche. This suggests that eosinophil accumulation is therefore not simply a defence mechanism against pathogenic challenge and in allergic exacerbations as has been described in the past, but a strategy to maintain tissue homeostasis in health and disease (Lee et al., 2010).

When eosinophils are activated in tissues, the cytotoxic granule content is released into the surrounding environment, where components including MBP, ECP and EDN are known to have cytotoxic effects on both pathogens and host tissues (Gleich et al., 1979; Levy et al., 2001; Kato et al., 2012). As well as cytotoxic effects, activated eosinophils have also been shown to increase epithelial cell permeability though the secretion of MBP and CRF (Furuta

et al., 2005; Wallon et al., 2011). However, the granule content also contains a number of other factors, including EGF, which is known to be important for epithelial crypt cell regeneration (Sato et al., 2011; Rosenberg et al., 2013). Eosinophils also express a wide variety of receptors and ligands which have importance in health and disease (reviewed in Rosenberg et al. (2013)). Of note, eosinophils are a major source of secreted IL-6, and lipid bodies present in eosinophil cytoplasm contain $PGE_2$, which have been shown in this thesis to modulate intestinal renewal during homeostasis (**Chapter 3** and **Chapter 4** respectively).

Eosinophils have a major function in the regulation of immune cells during homeostasis. Recent studies have shown that eosinophils maintain plasma cells in the bone marrow and lamina propria through secretion of IL-6 and APRIL (Chu et al., 2011). The recruitment of $T_{H2}$ cells in response to chemoattractants CCL17 and CCL22 is promoted by eosinophils (Jacobsen et al., 2008). Furthermore, eosinophils also promote dendritic cell maturation through the granule protein EDN (Yang et al., 2003; Lotfi and Lotze, 2008; Yang et al., 2008). Release of IL-5 and IL-13 by eosinophils recruits alternatively activated macrophages to adipose tissues, where they have an increased survival (Wu et al., 2011).

Previous studies have utilised isolated eosinophils from the blood of healthy donors and co-cultured them with an epithelial cell line in order to determine the effects of eosinophil secreted products on epithelial cells (Jawien et al., 2002; Furuta et al., 2005). However, phenotypic differences are observed between blood and tissue resident eosinophils, including an increased survival (Wu et al., 2011) and expression of different receptors (Carlens et al., 2009; Wallon et al., 2011). Therefore, the gold standard for the investigation of the effects of tissue resident eosinophils on crypts would be to isolate eosinophils from the underlying lamina propria and add them into co-culture with isolated intestinal crypts. Previous studies have isolated eosinophils from the lamina propria of the mouse intestine for the study of effects of eosinophils on plasma cell survival (Chu et al., 2011); however, there is no documented literature on the isolation of human mucosal eosinophils and co-culture with isolated intestinal crypts.

Interactions between immune cells in the underlying lamina propria and ISCs at the base of colonic crypts have previously been shown to induce crypt regeneration during homeostasis (Skoczek et al., 2014). Skoczek et al. (2014) demonstrated that following luminal microbial input, monocytes and eosinophils move closer to the stem cell niche, where monocytes were shown to induce stem cell expansion and an increase in crypt cell proliferation. In an *in vitro* co-culture system of isolated mouse colonic crypts and bone marrow derived monocytes or

THP-1 cells, monocytes were shown to induce crypt cell proliferation (Skoczek et al., 2014). Whether similar interactions between eosinophils and human colonic crypts occur remains to be elucidated.

The aim of this chapter was to determine the roles of eosinophils in intestinal homeostasis. Therefore, a protocol for the isolation and purification of tissue resident eosinophils was attempted. The data presented in this chapter demonstrate a spatial relationship between eosinophils and functional regions along the colonic crypt-axis *in vivo*.

## 5.2 Results

An Immunofluorescent labelling approach was undertaken in order to determine the spatial characteristics of eosinophils within the human colonic lamina propria during health and IBD. A human isolated crypt co-culture system with HL-60-eosinophils or isolated tissue resident eosinophils was developed and initial experiments to investigate the effects of eosinophils on human colonic crypt cell proliferation undertaken.

### 5.2.1 Eosinophils reside in sites of renewal and regeneration *in vivo*

Eosinophils have previously been described to reside in sites of tissue renewal and regeneration, including the intestinal lamina propria (Lee et al., 2010). However, the spatial distribution of eosinophils along the crypt axis in the lamina propria surrounding human colonic crypts remained to be characterised. Tissue was obtained from patients undergoing colorectal resection at the NNUH, immediately fixed in paraformaldehyde and embedded in paraffin and 5µm thick sections obtained with a microtome (described in **Section 2.4.2**). Immunofluorescent labelling of lamina propria eosinophils was undertaken utilising the markers C-C chemokine receptor type 3 (CCR3), major basic protein (MBP) and Sialic acid-binding Ig-like lectin 8 (Siglec-8) (as described in **Section 2.3.5**). The number of CCR3, MBP or Siglec-8 positive cells in the lamina propria surrounding the base, mid and top regions of crypts was quantified. Quantification of the number of CCR3+ cells in the base, mid and top regions along the crypt-axis (white dashed line) revealed a significant increase in the number of CCR3 positive cells at the base of crypts compared to mid and top regions of crypts (**Figure 5.1**). Whilst CCR3 is a marker specific to eosinophils, it is also expressed on other immune cell subtypes which are known to reside in the colonic lamina propria (Agace et al., 2000), therefore further characterisation of eosinophils was determined using an antibody for the eosinophil granule protein MBP. The number of MBP positive cells in the lamina propria surrounding crypts was significantly increased in the base and top regions compared to mid region (**Figure 5.2**). Another marker which is specific to eosinophils is Siglec-8. Quantification of the number of Siglec-8+ cells in the base, mid or top region (see white dashed lines, **Figure 5.3A**) of the crypt revealed that significantly less Siglec-8+ cells were observed at the mid and top regions of the crypt compared to base region. A significant increase in the number of Siglec-8+ cells was observed at the top region of crypts compared to mid region (**Figure 5.3B**). Co-expression of eosinophil markers was not possible in this system due to the inherent autofluorescent properties of eosinophils using

excitation wavelengths 525nm and 595nm, described below, therefore Immunofluorescent labelling of eosinophils was only possible using filters to detect emission at 647nm.

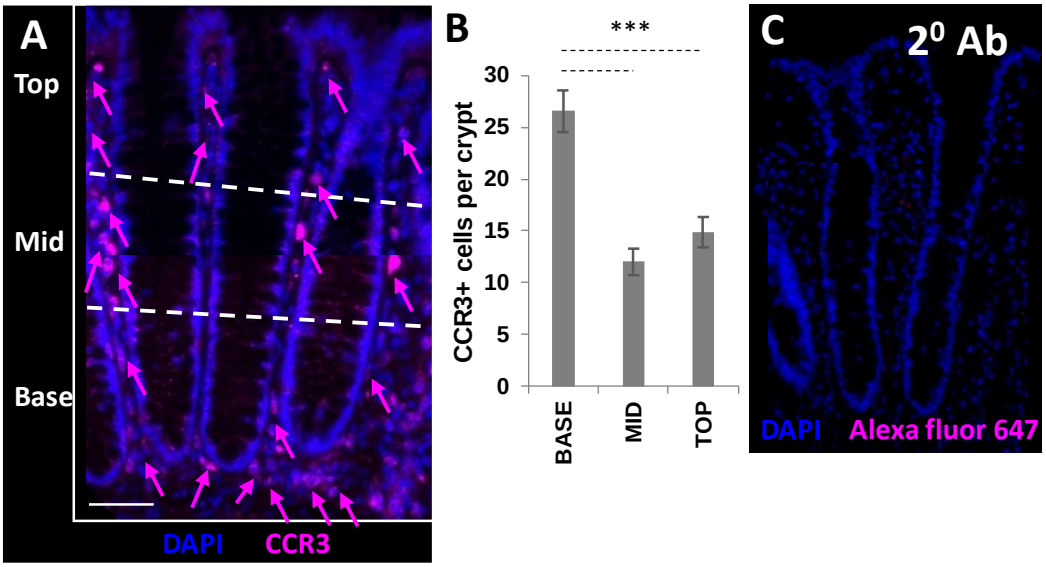


**Figure 5.1. Spatial distribution of CCR3+ cells in the healthy colonic mucosa.**

(**A**) Immunofluorescent labelling of CCR3+ cells (far red) and nuclei/DAPI (blue) in the human colonic mucosa. Scale bar 50µm. (**B**) Histogram showing the number of CCR3+ cells in the base, mid and top regions of colonic crypts (N=4, n=29, ***P<0.001 compared to base region). Mean values ± SEM were plotted for each crypt region. (**C**) No labelling was observed following utilising the Alexa Fluor 647 secondary antibody ($2^0$ Ab) alone.

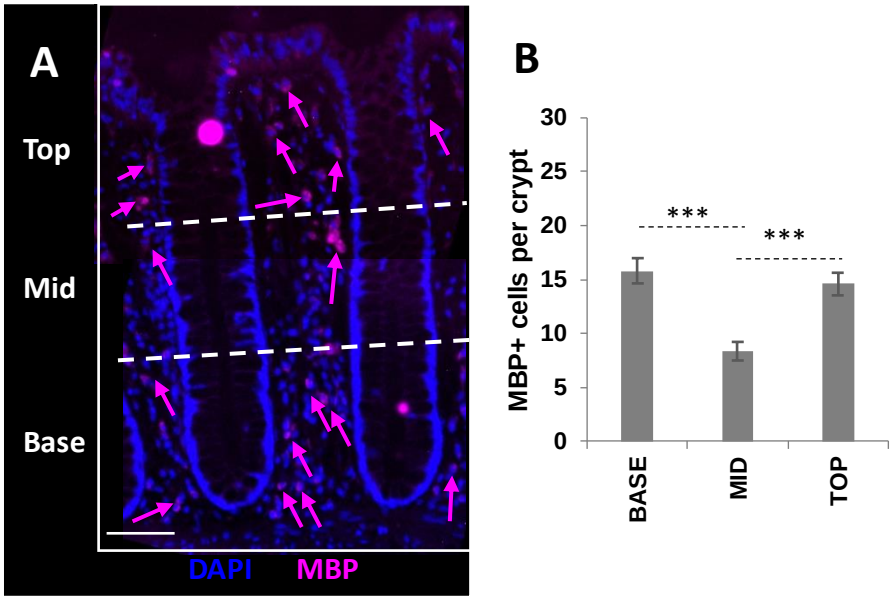


**Figure 5.2. Spatial distribution of MBP+ cells in the healthy colonic mucosa.**

(**A**) Immunofluorescent labelling of MBP+ cells (far red) and nuclei/DAPI (blue) in the human colonic mucosa. (**B**) Histogram showing the number of MBP+ cells in the base, mid and top regions of colonic crypts (N=4, n=34, ***P<0.001 compared to mid region). Mean values ± SEM were plotted for each crypt region. Scale bar 50µm.

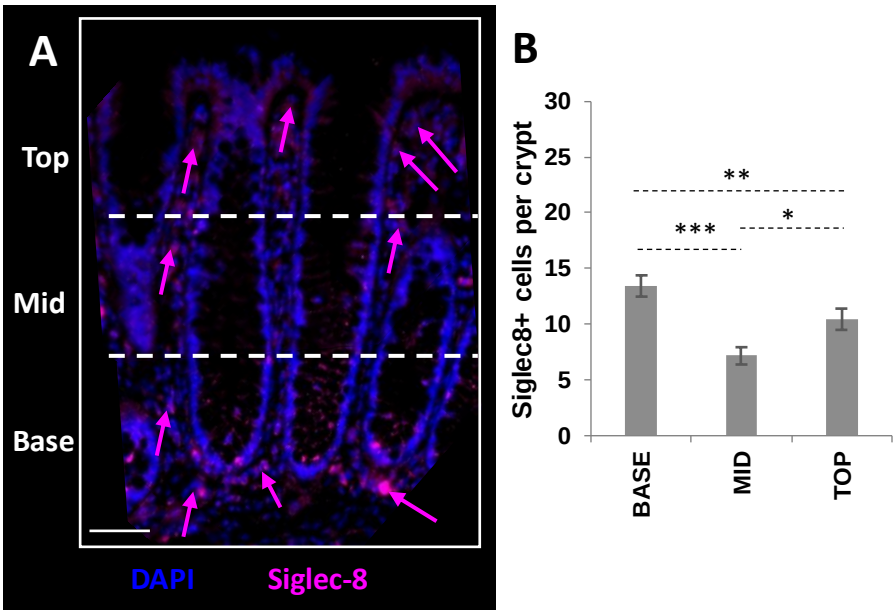


**Figure 5.3. Spatial distribution of Siglec-8+ cells in the healthy colonic mucosa.**

(**A**) Immunofluorescent labelling of Siglec-8+ cells (far red) and nuclei/DAPI (blue) in the human colonic mucosa. Images representative of N=2 experiments. (**B**) Histogram showing the number of Siglec-8+ cells in the base, mid and top regions of colonic crypts in control compared to mid region (N=2, n=21,***P<0.001, **P<0.01, *P<0.05 compared to indicated region). Mean values ± SEM were plotted for each crypt region. Scale bar 50µm.

During the Immunofluorescent antibody experiments, a population of autofluorescent cells was observed in the colonic lamina propria using epifluorescent filters to detect light in the green and red channels, and excitation wavelengths of 525nm and 595nm using laser scanning microscopy. A representative image of autofluorescent cells in the lamina propria between colonic crypts (white dashed line) using excitation wavelengths 525nm and 595nm for multiphoton microscopy is provided in **Figure 5.4A**. Merging the 525nm and 595nm images revealed co-localisation of autofluorescence between the two wavelengths (**Figure 5.4A**). Similarly to the spatial relationship observed following quantification of Siglec-8 positive cells in the colonic lamina propria (**Figure 5.3**), when the number of autofluorescent cells were quantified in each region of crypts, a significant increase in the number of autofluorescent cells residing in the regions surrounding the base region was observed compared to mid and top (**Figure 5.4B**). The identity of these cells was then investigated using Immunofluorescent labelling of immune cells. Approximately 90% of autofluorescent cells were shown to co-localise with Siglec-8+ cells in the lamina propria surrounding crypts in the base, mid and top regions (**Figure 5.4C**), suggesting that eosinophils are autofluorescent in the colonic lamina propria. This paves the way for some exciting studies whereby eosinophil dynamics can be studied in real-time (without the need for immunofluorescent labelling of eosinophils) and the effects of luminal addition of PAMPS or commensal/pathogenic microbes on spatial and temporal characteristics of eosinophils may be determined.

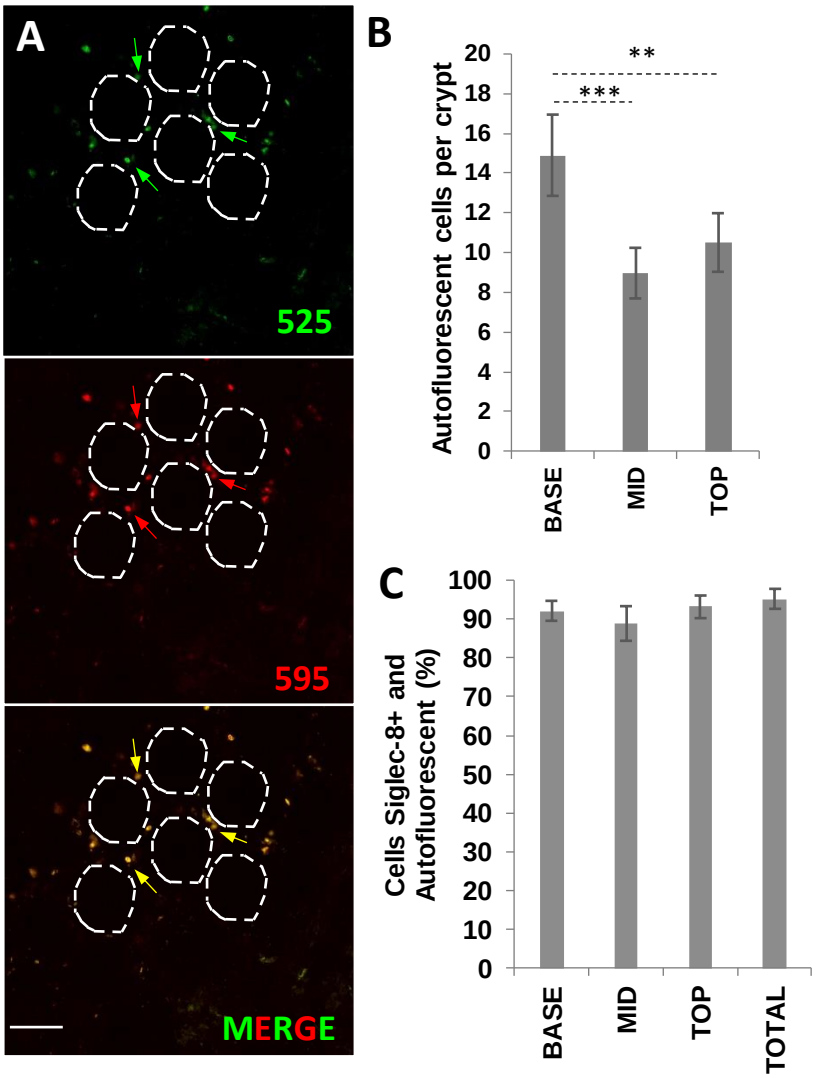


**Figure 5.4. Autofluorescent cells in the lamina propria of healthy colonic tissue are Siglec-8+ eosinophils.**

(**A**) Representative fluorescent image of autofluorescent cells in the lamina propria of healthy colonic explants using excitation wavelengths 525nm and 595nm, white denotes the colonic epithelium. (**B**) Histogram showing the number of autofluorescent cells in the base, mid and top regions of colonic crypts in control compared to mid region (N=2, n=21, ***P<0.001 compared to mid region, $$P<0.01 compared to base region). Mean values ± SEM were plotted for each crypt region. (**C**) Histogram showing the percentage of autofluorescent cells that were also Siglec-8 positive (N=2, n=21). Multi-photon imaging data is representative of N=2 biopsy samples. Scale bar 50µm

Whilst eosinophils are known to be an abundant population in the intestinal lamina propria during health (Carlens et al., 2009), eosinophils have been shown to accumulate in IBD (Desreumaux et al., 1999). The next step was to determine whether a similar spatial relationship was observed between eosinophils and the colonic epithelium during active IBD. Therefore, tissue from patients with active IBD was obtained, and Immunofluorescent labelling for Siglec-8 undertaken (**Figure 5.5A**). Quantitative analysis of the number of Siglec-8 positive cells in the lamina propria surrounding the base, mid and top region of colonic crypts revealed that a significant increase in the number of Siglec-8 positive cells was present at the base region of crypts compared to mid and top regions in sections obtained from IBD tissue. Furthermore, a significant increase in the number of Siglec-8 positive cells were observed at the base, mid and top regions of crypts compared to the respective regions in sections from healthy tissue (**Figure 5.5B**). A significant increase in the total number of Siglec-8+ cells surrounding each crypt was observed in IBD tissue compared to control (**Figure 5.5C**).

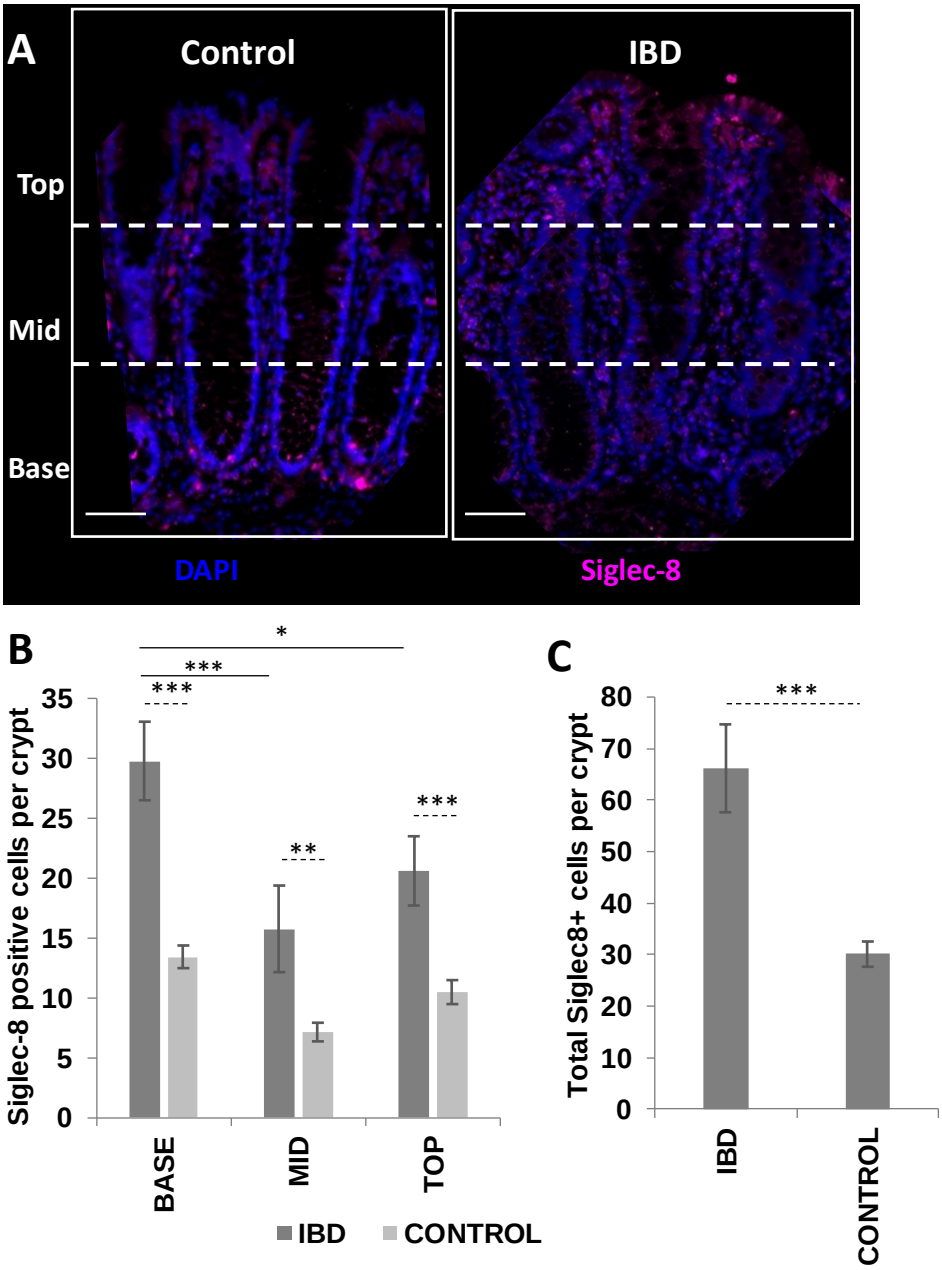


**Figure 5.5. Characterisation of tissue resident Siglec-8+ cells in the colonic mucosa of healthy and IBD patients.**

**(A**) Immunofluorescent labelling of Siglec-8+ cells (pink) and nuclei (DAPI-blue) in the colonic mucosa of tissue obtained from healthy (control, left) and IBD (right) patients, white dashes represent base, mid and top regions. (**B**) Histogram showing the number of Siglec-8+ cells observed in the base, mid and top regions colonic crypts *in vivo* of IBD and control sections (N≥2, n≥21, ***P<0.001, **P<0.01, *P<0.05 compared to indicated regions) mean values ± SEM were plotted for each crypt region. (**C**) The total number of Siglec-8+ cells per crypt in IBD samples compared to control (N≥2, n≥21***P<0.001). Scale bars 50µm.

### 5.2.2 Distribution of eosinophils *in vitro* is similar to that observed *in vivo*

As described previously, HL-60 cells differentiate into an eosinophil-like phenotype following 5 days in culture with 0.5mM butyric acid (Fischkoff, 1988). In order to determine the effects of eosinophils on colonic crypts *in vitro*, HL-60-eosinophils were added into co-culture with isolated human colonic crypts. Following 24h in co-culture, images were taken using timelapse microscopy (**Figure 5.6A**). Crypts were split into three equal regions to represent the base, mid and top hierarchy of the crypts and HL-60-eosinophils within a 2-cell thick radius (see white dotted line, **Figure 5.6A**) were counted in order to characterise the spatial distribution of HL-60-eosinophils along the crypt axis *in vitro*. Interestingly, initial results suggested that following 24h in co-culture with human colonic crypts, HL-60-eosinophil distribution mirrored that observed *in vivo*; HL-60-eosinophils were present in high numbers in the base and top regions compared to mid region surrounding human colonic crypts (**Figure 5.6B**). This suggests that the HL-60-eosinophil-crypt co-culture model is a good representation of *in vivo* eosinophil-crypt behaviour and therefore a good system to investigate the effects of eosinophils on human colonic crypts *in vitro*.

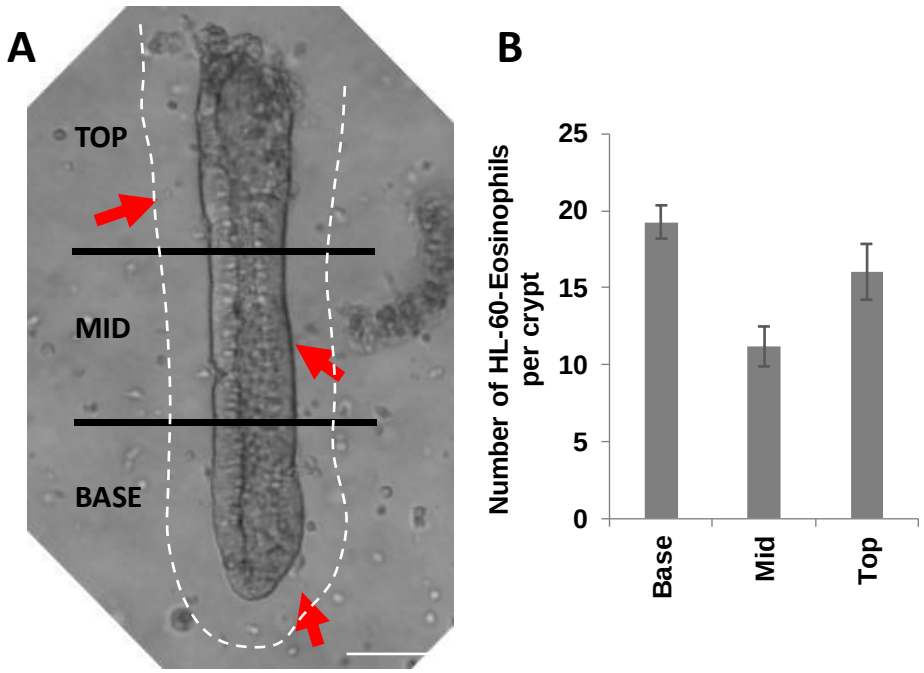


**Figure 5.6. Distribution of HL-60-eosinophils observed in in vitro co-culture is similar to that observed in vivo.**
(**A**) DIC image of immune cells (red arrows) in co-culture with human colonic crypts following 24h in co-culture. (**B**) Histogram showing the number of HL-60-eosinophils surrounding human colonic crypts at the base and top region of crypts compared to mid region following 24h in co-culture. Scale bar 50µm (N=1, n=15).

Previous studies have shown that physical interactions occur between immune cells and stem cells in the base of mouse colonic crypts (Skoczek et al., 2014). Immunofluorescent labelling of MBP and OLFM4 revealed that physical associations were observed between MBP+ cells and the base of crypts close to the stem cell niche *in vivo* (**Figure 5.7A,** red arrow). Similarly physical interactions were observed between HL-60-eosinophils and the colonic crypt base in *in vitro* co-culture studies (**Figure 5.7B**). It has previously been shown that Lgr5EGFP positive stem cells extend epithelial processes into the surrounding lamina propria to physically interact with monocytes *in vitro* (Skoczek et al., 2014). Similarly, in this study E-cadherin+ processes were shown to extend into the surrounding matrix and physically interact with HL-60-eosinophils (**Figure 5.7C**).

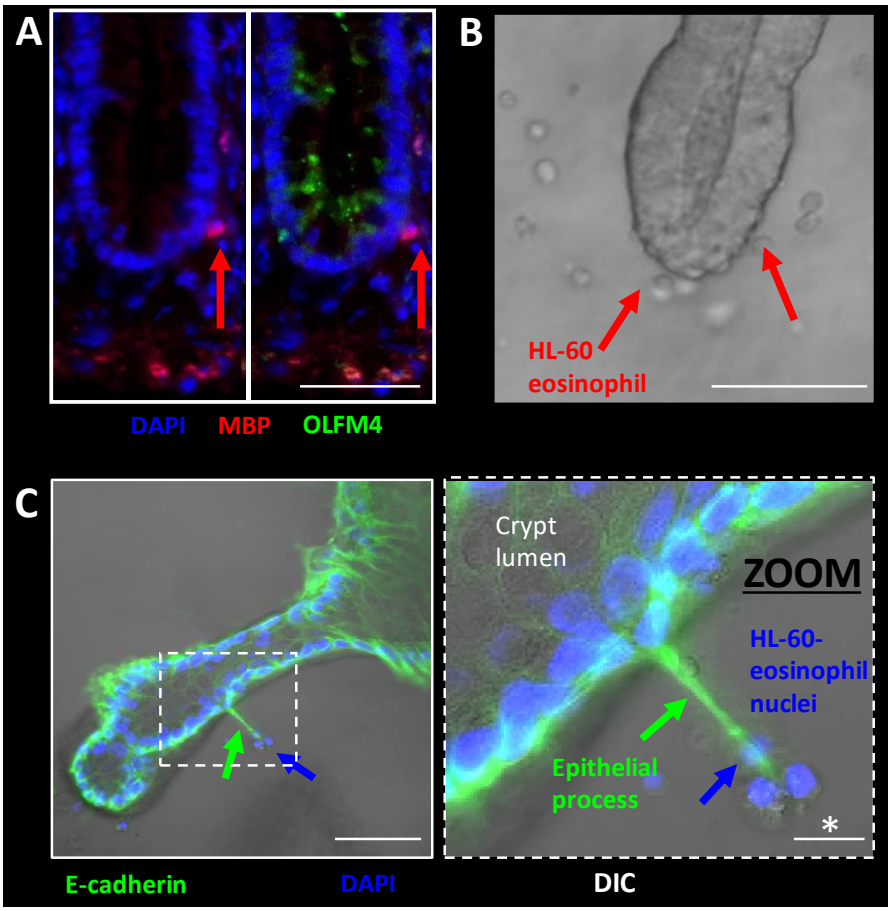


**Figure 5.7. Physical interactions between eosinophils and crypt stem cells are observed in vivo and in in vitro co-culture.**
(**A**) Representative confocal image showing MBP (red) positive cell in the lamina propria residing in close proximity to OLFM4 (green) positive cells at the base of colonic crypts *in vivo*. (**B**) White light DIC image of HL-60-eosinophil-crypt co-culture. HL-60-eosinophils form interactions (red arrows) with the stem cell niche of isolated colonic crypts in co-culture. (**C**) Representative immunofluorescent image of HL-60-eosinophil crypt co-culture. Crypts extend E-cadherin (green) positive processes towards HL-60-eosinophils in surrounding matrigel, nuclei (DAPI-blue). Confocal imaging data are representative of N=3, n≥10, experiments. Scale bars 50µm, *scale bar 10µm.

### 5.2.3 Characterisation of isolated human tissue resident eosinophils

The observation of physical interactions between eosinophils and human colonic crypts begs the question of the effects of eosinophils on crypt cell renewal. The use of reductionist co-culture studies provides the ideal methodology to determine the roles of immune cells in crypt renewal. However, no previous co-culture studies have been attempted using immune cells isolated from the intestinal lamina propria in co-culture with isolated human crypts. Therefore, the aim of this study was to develop a protocol for the isolation of immune cells from the mucosal lamina propria and subsequent purification of eosinophils (see **Section 2.6.2**), with the intention of adding purified eosinophils into reductionist co-culture with isolated human colonic crypts. Previously, the immune cell content of the intestinal lamina propria has been described to be comprised 20-40% eosinophils (Carlens et al., 2009), and data has suggested that the number of eosinophils present falls in the descending colon from the ascending colon (Saad, 2011; Matsushita et al., 2015). However, no data was available regarding the percentage composition of immune cells in the lamina propria from different regions of the large intestine i.e. ascending or descending colon. Therefore, immune cells were isolated from the underlying lamina propria of tissue taken from the ascending and descending colon. The proportion of each immune cell subtype was quantified using morphological characterisation of H&E stained cytospins. Immune cell content included eosinophils, neutrophils, basophils, macrophages, monocytes, dendritic cells and lymphocytes. The percentage of eosinophils in the mixed immune cell population was increased in the ascending colon lamina propria compared to the descending colon, although this was not statistically significant (**Figure 5.8A**). Eosinophils have been shown to be present with a greater abundance in tissue taken from patients with IBD (**Figure 5.5**); therefore, immune cells were also isolated from the colon of patients with IBD or no IBD. As suggested earlier, an increased proportion of eosinophils was observed in immune cells isolated from patients with IBD compared to healthy, although these results are not yet significant (**Figure 5.8B**).

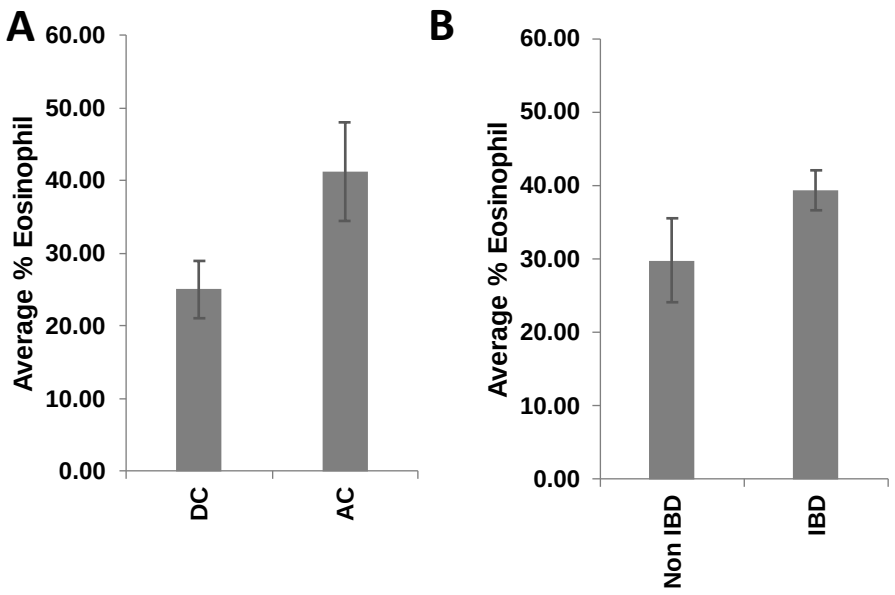


**Figure 5.8. More eosinophils are present in the ascending colon than descending colon, and in IBD compared to non-IBD.**

(**A**) Histogram showing the average percentage of eosinophils present in a mixed immune cell population isolated from the mucosal lamina propria of descending colon (DC) or ascending colon (AC) before purification. (**B**) Histogram showing the average percentage of eosinophils present in a mixed immune cell population isolated from the ascending colon lamina propria of IBD or non-IBD patients. Data was obtained from a minimum of N=3 experiments, n>1200 immune cells counted.

### 5.2.4 The effects of eosinophils on human colonic crypt renewal

In order to determine whether eosinophils induced crypt cell proliferation, and the number of eosinophils required in order to illicit a response, HL-60-eosinophils were added into co-culture with isolated human colonic crypts. Addition of 50,000 HL-60-eosinophils induced a marginal increase in crypt cell proliferation at the base of crypts, that was not statistically significant (**Figure 5.9A**). Therefore, isolated human colonic crypts were co-cultured with either 50,000 or 100,000 HL-60-eosinophils. No significant increase in crypt cell proliferation was observed at the base of crypts following co-culture with 50,000 HL-60-eosinophils, or 100,000 HL-60-eosinophils (**Figure 5.9B**).

In order to determine whether HL-60-eosinophils express factors that can potentially induce renewal of colonic crypts, RT-PCR using primers for crypt regenerative factors was undertaken. HL-60-eosinophils expressed mRNA for WNT3A (**Figure 5.10A**). The expression of COX-1 mRNA by HL-60-eosinophils was also confirmed (**Figure 5.10B**).

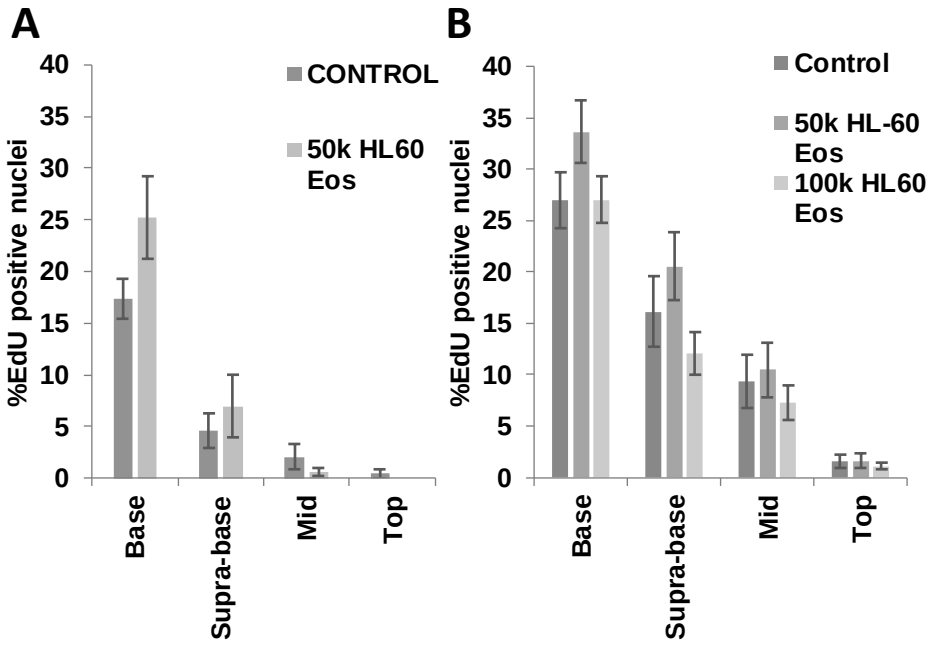


**Figure 5.9. HL-60-eosinophils in co-culture with human colonic crypts induces a slight increase in proliferation.**

(**A**) Histogram showing the percentage of nuclei incorporating EdU following co-culture with 50,000 HL-60-eosinophils, (N=1, n>14) (**B**) Histogram showing the percentage of nuclei incorporating EdU following co-culture with 50,000 or 100,000 HL-60-eosinophils, (N=4, n≥20).

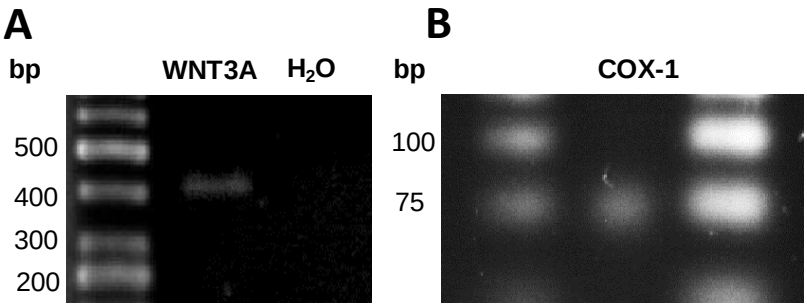


**Figure 5.10. HL-60-eosinophils express crypt regenerative factors.**

(**A**) Expression of WNT3A mRNA by RT-PCR using cDNA from butyric acid treated HL-60 cells; expected PCR band size WNT3A=404 bp (**B**) Expression of COX-1 mRNA by RT-PCR using cDNA from HL-60/Eos; expected PCR band size COX-1=76bp.

In this chapter, a novel protocol was developed to isolate immune cells from the underlying lamina propria of the colonic mucosa, and purify the tissue resident eosinophils for use in co-culture studies with isolated colonic crypts (summarised in **Section 2.6.2**). The protocol remains in the developmental phase, and future work aims to further purify and increase the yield from the mucosa. For initial experiments, a population of immune cells with an eosinophil purity of 85% were added into co-culture with isolated human colonic crypts. Siglec-8 positive eosinophils isolated from the human colonic mucosa were added into co-culture with isolated human colonic crypts and the effects on crypt proliferation assessed using EdU incorporation (**Figure 5.11A**). The percentage of nuclei incorporating EdU in the base, supra-base, mid and top regions of the crypts was quantified. Addition of 50,000 immune cells per well induced no change in human colonic crypt cell proliferation (**Figure 5.11B**).

In order to determine whether the population of purified eosinophils express markers characteristic of eosinophils, RT-PCR using primers for markers specific to eosinophils was utilised. Isolated tissue resident immune cells, with an eosinophil purity of 55-65%, expressed a number of receptors for and ligands for chemotaxis, including CCR1, CCR3, CCR7, CCR9, CCL2, CCL5 (**Figure 5.12**); which have all been previously described to be expressed by eosinophils (Akuthota et al., 2013; Rosenberg et al., 2013; Baumann et al., 2015). The important roles of eosinophils in the maintenance of plasma cells through secretion of IL-6 begs the question of whether eosinophils isolated from the mucosa of healthy individuals express IL-6. Using PCR, tissue resident immune cells, of which 55-65% were eosinophils, were shown to express mRNA for IL-6 (**Figure 5.12**). The mixed immune cell population was also shown to express CCR10 and CCRL2, which have not previously been described to be expressed by eosinophils (**Appendix B**).

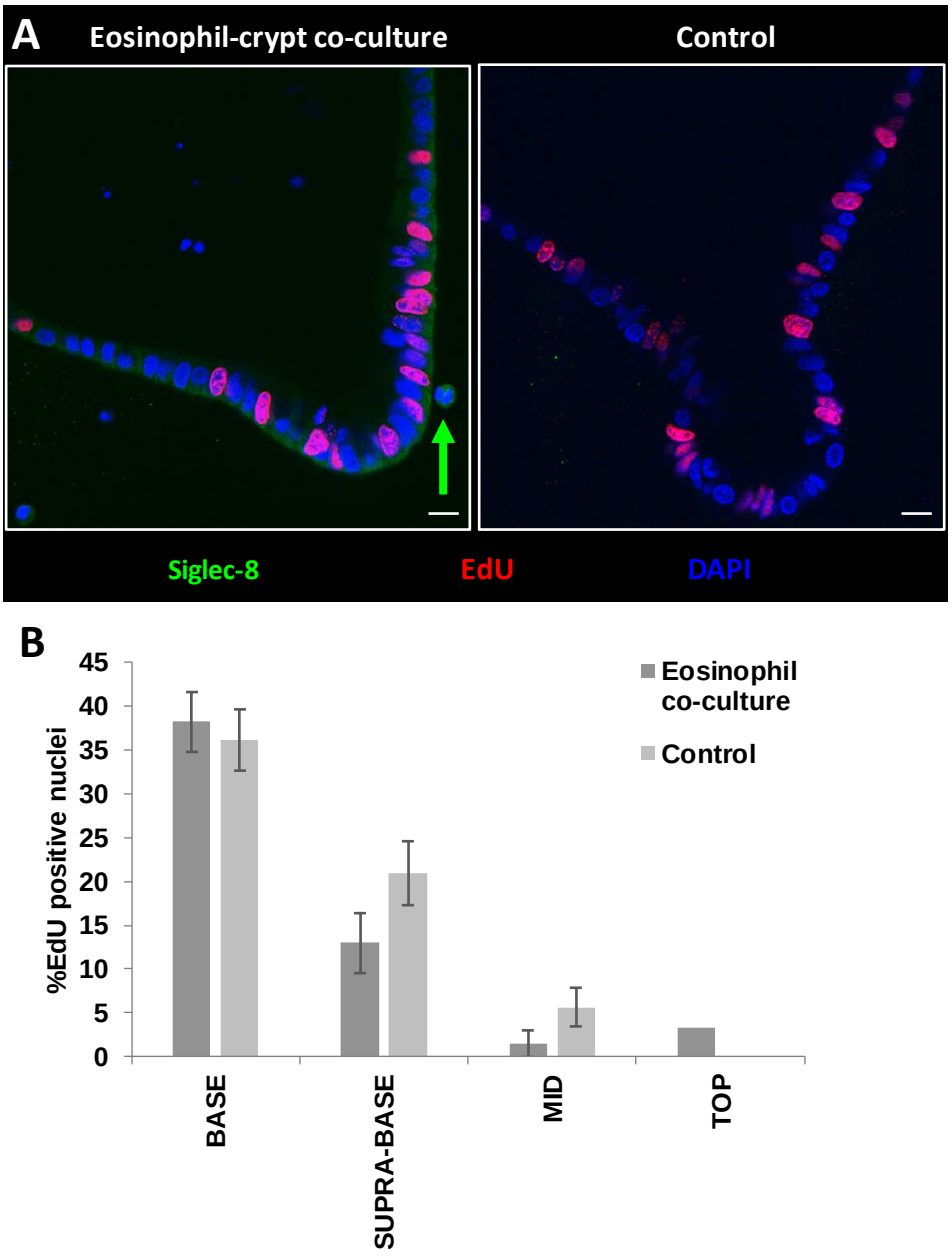


**Figure 5.11. Co-culture of tissue resident eosinophils induced no change in proliferation of human colonic crypts.**

(**A**) Representative confocal image of EdU (red) incorporating nuclei (DAPI-blue) of human colonic crypts following co-culture with Siglec-8 (green) positive cells. (**B**) Histogram showing the percentage of nuclei incorporating EdU following co-culture with 85% pure eosinophil population, compared to control with no immune cells, N=1, n≥9. Scale bar 10µm.

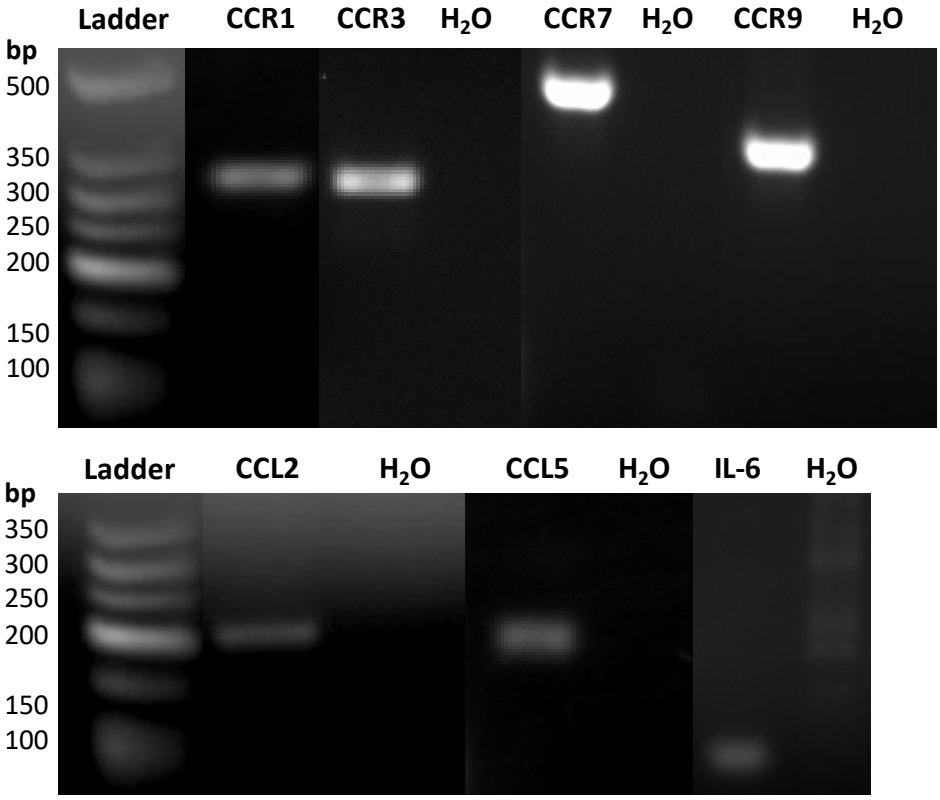


**Figure 5.12. Isolated tissue resident immune cells express characteristic markers of eosinophils, including IL-6.**

Expression of eosinophil characteristic marker mRNA by RT-PCR using cDNA from isolated tissue resident immune cells, eosinophil purity was between 55 and 65% for all samples used. Expected PCR band sizes: CCR1=358bp; CCR3=323bp; CCR7=497bp; CCR9=413bp; CCL2=219bp; CCL5=201bp; IL-6=86bp

## 5.3 Discussion

In this chapter, a novel spatial relationship between eosinophils and the colonic epithelium has been described; with more eosinophils present at the colonic stem cell niche and at the surface epithelium (**Figure 5.13**). An accumulation of eosinophils was observed in tissue obtained from IBD patients, and for the first time, this chapter quantifies a 1.2 fold increase in the number of eosinophils present at the base of crypts from IBD patients compared to healthy patients. The use of a reductionist system whereby HL-60-eosinophils were co-cultured with isolated human colonic crypts revealed that the spatial distribution following 24h in culture was the same *in vitro* as that observed *in vivo*, with physical interactions observed between the epithelium and HL-60-eosinophils (**Figure 5.13**). A human tissue resident eosinophil isolation technique was also developed, whereby immune cells expressing a number of markers characteristic of eosinophils were isolated from the colonic mucosa. Furthermore, this chapter describes for the first time, the development of the reductionist co-culture system to include tissue-resident eosinophils in co-culture with isolated human colonic crypts.

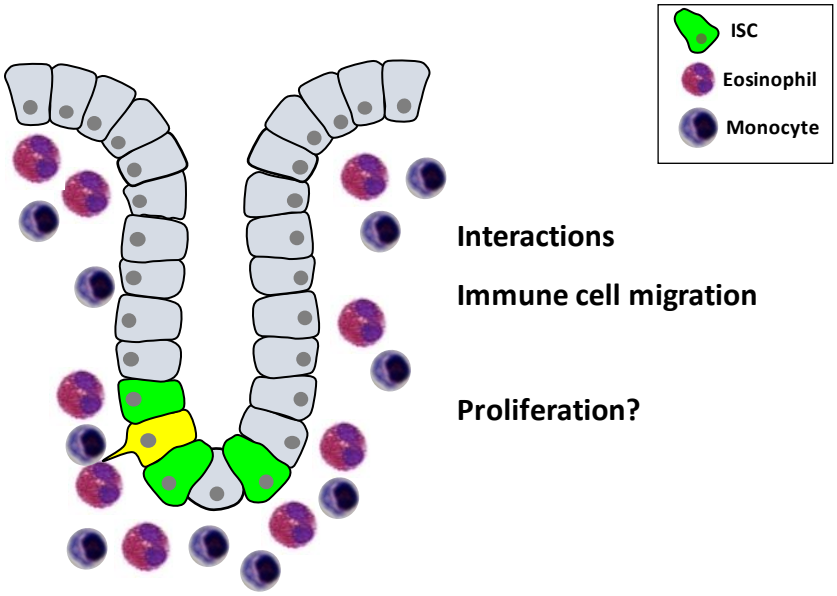


**Figure 5.13. Eosinophils reside in sites of renewal and regeneration during homeostasis.**

Eosinophils and monocytes reside at the base (site of renewal) and top (site of regeneration) along the crypt-axis in the human colonic mucosa. In *in vitro* co-culture, eosinophils and monocytes migrate to the crypt base and top following 24h in co-culture, where the epithelium is seen to extend epithelial processes towards immune cells in the underlying lamina propria.

These findings demonstrate that eosinophils specifically home to sites of renewal and regeneration during health. We observed a significant increase in the number of eosinophils present in the lamina propria at the base, the major site of stem cell driven tissue renewal, and top, where cells undergo apoptosis and are shed into the gut lumen, regions of crypts *in vivo*. This complements the LIAR hypothesis, previously described by Lee et al. (2010), whereby eosinophils are thought to be recruited to sites of renewal and regeneration through factors secreted when cells undergo apoptosis, and retained at the site through factors derived from proliferating cells (Lee et al., 2010). In our *in vitro* co-culture system, HL-60-eosinophils were shown to reside at the base and top regions of the crypt following 24h in culture. Human colonic crypts have previously been shown to express Eotaxin-1, the major chemoattractant for eosinophils (El Hadi, personal communication). The expression of CCR3 by isolated eosinophils indicates that they are able to respond to Eotaxin-1 expression by the epithelium, which may induce migration towards crypts and possibly potentiate physical interactions between eosinophils and epithelial cells.

Previously, physical interactions between monocytes and stem cells were shown to induce stem cell expansion and crypt cell proliferation in the mouse colonic mucosa (Skoczek et al., 2014). The presence of physical interactions between HL-60-eosinophils and colonic epithelial cells in our *in vitro* co-culture suggests that a similar paradigm may exist between eosinophils and human colonic crypts. Furthermore, HL-60-eosinophils were shown to express mRNA for factors known to induce crypt cell proliferation; COX-1 for $PGE_2$ synthesis, shown in **Chapter 4** of this thesis to induce proliferation, and WNT3a, which is the master regulator of intestinal renewal (Schepers and Clevers, 2012). However, initial experiments revealed no differences in proliferation of isolated human colonic crypts following addition of 100,000 HL-60-eosinophils into co-culture. When 50,000 HL-60-eosinophils were added into co-culture a small but not significant increase was observed in crypt cell proliferation compared to crypts alone. Skozcek et al. (2014) undertook a dose response experiment whereby 50,000-600,000 monocytes were added into co-culture with isolated mouse colonic crypts. In this system, addition of any number of monocytes between 50,000 and 600,000 per well induced a significant increase in crypt cell proliferation (Skoczek et al., 2014). It is possible that a higher number of HL-60-eosinophils is required to induce crypt cell proliferation through increased secretion of crypt regenerative factors. Future work aims to determine whether eosinophils induce crypt regeneration, and if so, use a conditioned media experimental approach in order to determine whether functional effects are dependent on physical interactions or secreted crypt regenerative factors.

Another crypt regenerative factor, which was shown in this thesis to induce crypt cell proliferation through autocrine and paracrine pathways, is IL-6 (**Chapter 3**). In mouse studies, eosinophils have been shown to be a major paracrine source of IL-6, which modulates gut immunity through increasing plasma cell survival; however, the role of eosinophil secreted IL-6 on epithelial renewal was not determined (Chu et al., 2011). In this chapter, isolated tissue resident eosinophils were shown to express mRNA for IL-6 thus confirming eosinophils as a paracrine source of IL-6 in the human intestine. A role for eosinophils in modulating gut immunity was supported by data in this Chapter showing that isolated tissue resident eosinophils express factors and receptors for chemotaxis including CCR1, CCR3, CCR7, CCR9, CCL2 and CCL5. This suggests that eosinophils play a major role in the recruitment of other immune cells to the intestinal stem cell niche. The purified immune cell population also expressed CCR10 and CCRL2 (**Appendix B**); CCR10 expression by eosinophils was previously shown to be induced in IBD (Rehman et al., 2013), and CCRL2 expression is characteristic of dendritic cells and macrophages, and has not previously been shown to be expressed by eosinophils (Otero et al., 2010). Due to the significant contaminating immune cell population in these samples, it is possible that CCR10 and CCRL2 are expressed on contaminating immune cells. Further experiments are required to determine the functional expression of chemoattractant factors and receptors on eosinophils in the mixed immune cell population.

Future work aims to utilise the reductionist co-culture system in order to investigate the role of paracrine eosinophil secretion of IL-6 and $PGE_2$ in epithelial renewal. The addition of antibodies to block the IL-6 receptor or neutralise secreted IL-6, or block $PGE_2$, as used in *in vitro* studies in Chapters 3 and 4 respectively, will be added into eosinophil-crypt co-culture and the effects on intestinal crypt cell proliferation and intestinal stem cell expansion will be determined. Furthermore, the reductionist co-culture system provides an ideal platform in order to investigate the relative contribution of eosinophils and other immune cells in epithelial renewal. Indeed, other immune cell populations i.e. monocytes, B cells could also be isolated and added into co-culture and the effects on crypt renewal, chemotactic factor secretion and immune cell migration investigated.

### 5.3.1 Roles for eosinophils in IBD

Accumulation of eosinophils in the intestinal lamina propria is characteristic of acute IBD and has been associated with disease severity and a poorer prognosis (Desreumaux et al., 1999). Enteric neural dysfunction and associated mucus secretion is characteristic of IBD, and

eosinophils were previously shown to co-localise with neurons in the intestinal mucosa during active IBD (Smyth et al., 2013). In our system, we observed a 1.2 fold, significant increase in the number of eosinophils present at the base of crypts in the colonic lamina propria of patients with acute IBD. The accumulation of eosinophils could be associated with an increase in the frequency of physical interactions occurring between eosinophils and epithelial cells, an increase in secreted crypt regenerative factors or increased mucus secretion through neural interactions. Previous studies have suggested that eosinophils are present in higher numbers in IBD remission than in acute IBD, suggesting that eosinophils play a role in epithelial repair in response to tissue injury (Lampinen et al., 2005). It is also possible that differences in the secreted crypt regenerative factors or chemotactic factors expressed by eosinophils are observed in acute IBD compared to IBD remission and health, as has been previously suggested (Carlens et al., 2009). For example, during health eosinophils are known to express the IL-6R on their surface membrane, suggesting that they are able to induce and respond to IL-6 classical signalling (Melo et al., 2013). IL-6R cleavage is associated with activation of IL-6 trans signalling and inflammation. It would be of interest to characterise whether expression of the IL-6R is lost on eosinophils obtained from IBD tissue. This could indicate that eosinophils contribute to IL-6 classic signalling in homeostasis, and IL-6 trans signalling during inflammation. In addition, eosinophils are thought to induce tissue damage through activation and release of toxic granule contents in active IBD. Previously, the accumulation of eosinophils was shown to increase epithelial permeability through MBP secretion (Furuta et al., 2005). Therefore, 'healthy eosinophils' may be protective and have roles in crypt regeneration, but 'IBD eosinophils' may induce tissue damage and altered barrier function.

Future work aims to explore the possibility that eosinophils resident in the inflamed mucosa confer a different function on cells within the colonic epithelium to eosinophils resident in the healthy colonic mucosa. In order to investigate this, a future program of work aims to isolate tissue resident eosinophils from the inflamed mucosa of IBD patients, and co-culture these cells with isolated colonic crypts from either inflamed or healthy mucosa. Similarly, eosinophils isolated from the healthy colonic mucosa can be added into culture with IBD crypts. In these co-culture studies the effects of eosinophils on crypt proliferation, barrier function through mucus secretion and cell permeability, and the reciprocal effects of the epithelium on eosinophil survival, migration and differential receptor expression will be determined in health and inflammation (depicted in **Figure 5.14**).

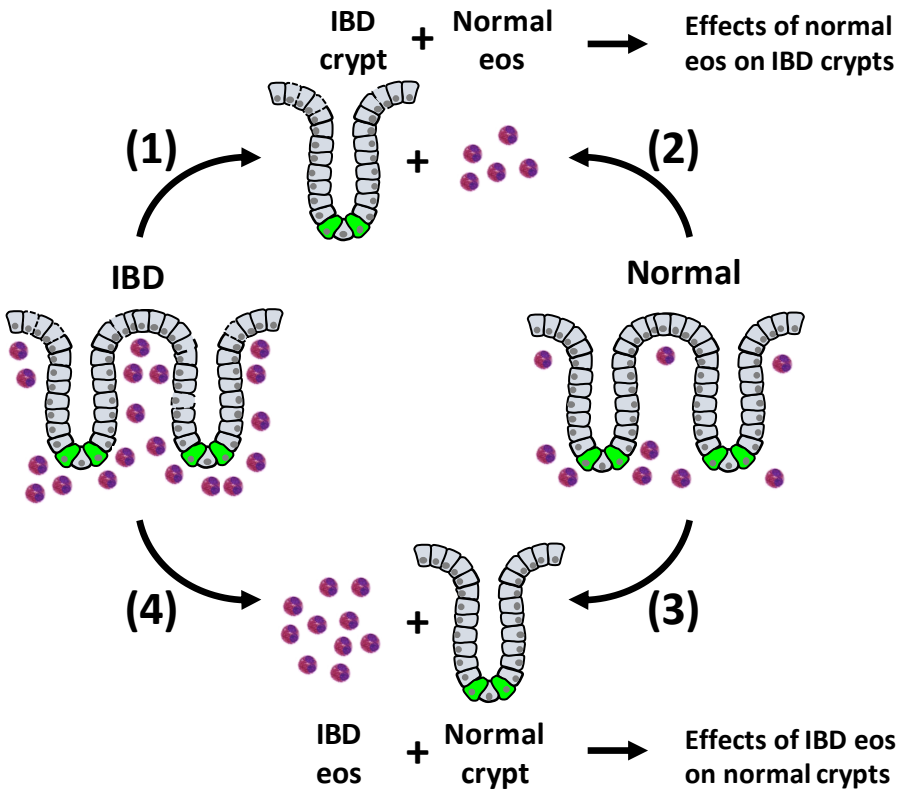


**Figure 5.14. Proposed experimental plan for tissue resident eosinophil-crypt co-culture.**

Crypts isolated from IBD patients (**1**), can be added into co-culture with eosinophils isolated from patients with normal histopathology (**2**) to determine the effects of normal eosinophils on IBD crypts. Crypts isolated from individuals with normal histopathology (**3**) can be added into co-culture with eosinophils isolated from patients with IBD (**4**) to determine the effects of IBD eosinophils on normal crypts.

### 5.3.2 Limitations of the current study & next steps

In this study, human tissue resident eosinophils were isolated and purified from the colonic mucosa. The development of this protocol paves the way for a number of elegant co-culture studies with healthy and inflammatory eosinophils and isolated crypts. However, the maximum purity achieved using the protocol developed in this programme of work (described in detail in **Chapter 2, Section 6.2**), was 85% eosinophils. Therefore, whilst the addition of this population into co-culture with crypts is able to give an insight into the roles of eosinophils in the maintenance of renewal, we are unable to define the relative contribution of contaminating cell types within the 'pure' population. This is of particular importance given the recent findings from Skoczek et al. (2014), which describe an important role for monocytes in the maintenance of mouse colonic crypt homeostasis.

The generation of a pure tissue resident eosinophil population would provide the gold standard for the investigation of the functional roles of tissue resident eosinophils in a number of systems. Therefore, the employment of additional techniques in order to further purify the eosinophil population isolated from the intestinal lamina propria is a crucial next step for the eosinophil studies. In this study, flow cytometric cell sorting using the autofluorescent property of eosinophils was attempted. However, this technique was unsuccessful due to an induction of apoptosis in the mixed immune cell population when attempted, likely due to the mechanical stress imposed on the cells during the sorting procedure (data not shown). Therefore, further work is required in order to attempt to optimise the procedure for autofluorescence-based eosinophil cell sorting. Flow cytometric cell sorting has previously been utilised for the purification of isolated mouse intestinal eosinophil, using specific antibodies for markers of immune cells, which were used for co-culture studies with other immune cells (Carlens et al., 2009; Chu et al., 2011). This suggests that eosinophils are able to withstand the mechanical process of cell sorting. Detailed functional analysis would be required following flow cytometric cell sorting of eosinophils in order to confirm that crucial receptors and factors are not lost from eosinophils during the sorting process. Of particular interest to our future studies are the roles of eosinophil paracrine secretion of $PGE_2$ and IL-6 in epithelial renewal, therefore the expression of these factors would be confirmed at the mRNA and protein levels in the pure eosinophil population. Upon the generation of a pure eosinophil population, eosinophils would be added into co-culture with isolated human colonic crypts in order to assess the role of eosinophils in colonic crypt renewal.

Another exciting next step for the eosinophil studies is the dynamic real-time imaging of eosinophil migration using multiphoton microscopy and the inherent autofluorescent properties of eosinophils (Ethier et al., 2014). Multiphoton microscopy uses two photons of light to excite fluorophores within tissue, which induces emission of a photon with a longer wavelength than confocal microscopy. The longer wavelength ensures less scattered light and less photobleaching of fluorophores. This allows for deep tissue imaging up to 500µm which is extremely valuable when considering *in vivo* imaging (Oheim et al., 2006). Eosinophils were shown in our system to home to sites of renewal (crypt base) and cell shedding (crypt top) both *in vivo* and *in vitro* co-culture. In *in vitro* co-culture, epithelial processes were shown to extend into the surrounding matrix and physically interact with eosinophils. This suggests that a dynamic spatial relationship is present between eosinophils and the epithelium, which begs the question of whether this process could be imaged live.

We therefore conducted proof of principle experiments whereby a colonic biopsy was fixed and imaged using a multiphoton microscope without any addition of fluorescent probes. We were able to visualize autofluorescent cells (which were shown in this study to be eosinophils) deep within the tissue into the submucosal layers. This paves the way for exciting live tissue imaging of tissue resident eosinophils within the colonic mucosa. Lamina propria monocytes and eosinophils migrate towards the stem cell niche following addition of luminal microbes to the air apical interface of a mouse colonic explant culture (Skoczek et al., 2014). Future work aims to identify the spatial and temporal effects of eosinophils following addition of commensal or pathogenic microbes to the luminal surface of live human colonic mucosa biopsies using multi-photon microscopy. The roles of eosinophils in wound healing could also be determined using this imaging technique through mechanical wounding of the air apical surface of the biopsy and eosinophil migration visualised in real time.

## 5.4 Conclusions

Eosinophils are known to have a number of functions in health and disease. In this chapter, a previously undefined spatial relationship between eosinophils and the base and top of crypts was described. A novel tissue resident eosinophil-crypt co-culture methodology was developed and initial experiments undertaken to begin to determine the relationship between eosinophils and human colonic crypts. The role of tissue resident eosinophils on isolated human colonic crypt renewal in inflammation and health remains the subject of future work. Previous work in this thesis has identified a novel role for autocrine IL-6 and $PGE_2$ signalling in the maintenance of small intestinal and colonic crypt renewal (**Chapter 3** and **Chapter 4** respectively). The roles of paracrine IL-6 and $PGE_2$ signalling were also investigated, however the source of paracrine IL-6 and $PGE_2$ *in vivo* remains to be determined. Future work aims to elucidate the role of eosinophil-secreted IL-6 and $PGE_2$ on small intestinal and colonic crypt renewal.

# 6 Chapter 6. General Discussion and Future Work

The major findings described in the preceding Chapters demonstrate that IL-6 (**Chapter 3**) and $PGE_2$ (**Chapter 4**) contribute to homeostasis in the small and large intestine in an autocrine and paracrine manner, and suggest a dynamic role for eosinophils (**Chapter 5**). Overall, these findings provide a more comprehensive view of how autocrine and paracrine signalling pathways act in concert to maintain intestinal renewal. In keeping with this notion, the findings from each chapter are now discussed in a more integrated fashion. Moreover, a model for the concerted regulation of intestinal barrier function by IL-6 and $PGE_2$ is formulated and is based on findings from this thesis in conjunction with other published work. In doing so, significant gaps in our current understanding are highlighted and suggestions are made on how these may be addressed. The implications for restoration of homeostasis in diseased states is also commented upon.

## 6.1 Key Findings and Concepts

The intestinal epithelium forms a vital barrier between host tissues and the microbial content of the gut lumen *in vivo*. Continuous epithelial renewal is modulated by innate sensing of gut luminal microbes, which induces cytokine secretion by epithelial cells or recruited immune cells residing in the underlying lamina propria. This initiates crypt cell proliferation, cell migration, differentiation and cell shedding in order to maintain the epithelial barrier and therefore intestinal homeostasis. Results described in detail in **Chapters 3** and **4** suggest that intestinal renewal is modulated by IL-6 and $PGE_2$ during homeostasis, and are summarised in **Figure 6.1**. The next step is to explore how autocrine and paracrine actions of IL-6 and $PGE_2$ converge on epithelial cell types to maintain intestinal barrier function. In order to explore how these pathways interact, the cellular sources, cellular targets, signal transduction pathways and physiological outcomes of IL-6 and $PGE_2$ must be considered. To a first approximation, conservation of findings emanating from investigation of the small and large intestine (e.g. a goblet cell in the small intestine is similar to a goblet cell in the large intestine) will be assumed in order to facilitate a discussion of the possible interactions between IL-6 and $PGE_2$.

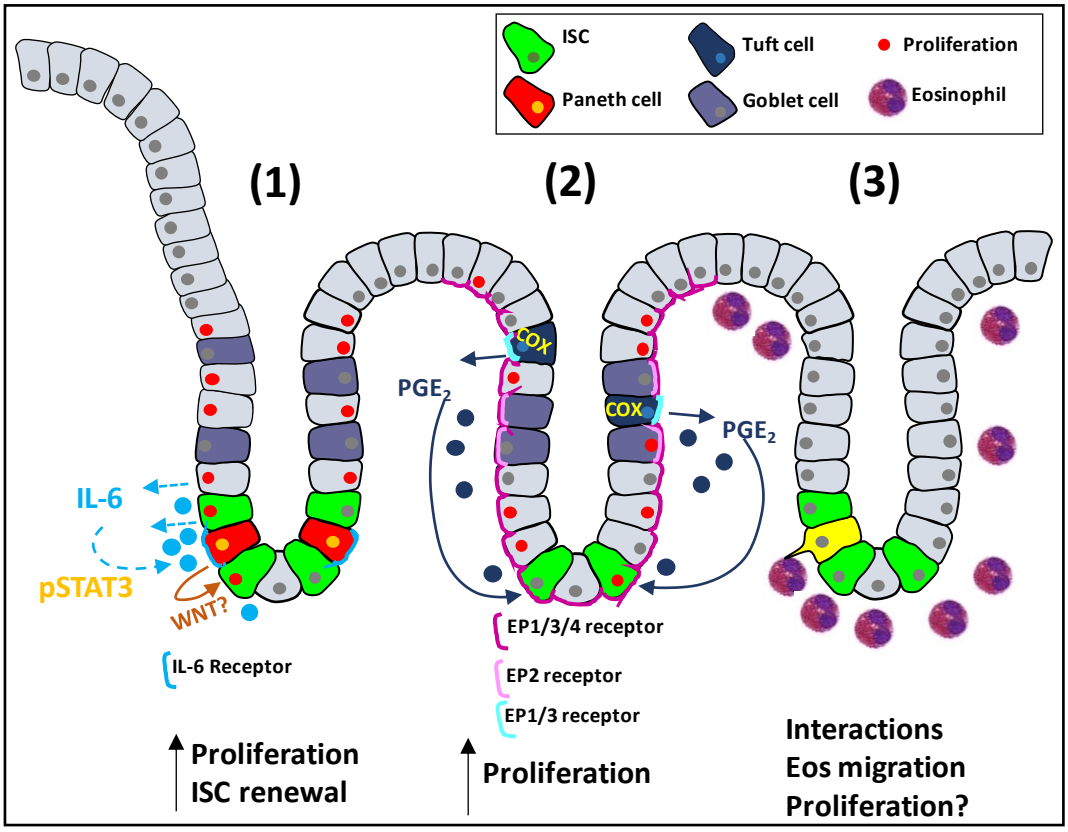


**Figure 6.1. Summary of key findings**

**(1)** Autocrine IL-6 signalling induces small intestinal crypt cell proliferation through activation of the IL-6 receptor on Paneth cells and pSTAT3 activation in a WNT dependent manner (**Chapter 3**). (**2**) The colonic epithelium expresses all the components required for $PGE_2$ signalling, COX enzymes and EP1-4 receptors. Autocrine $PGE_2$ signalling induced colonic crypt proliferation through EP1 and EP3 receptor subtypes (**Chapter 4**). (**3**) Eosinophils reside along the crypt-axis and home to the colonic crypt base and top during homeostasis where they form physical interactions with epithelial cells (**Chapter 5**).

### 6.1.1 Sources of IL-6 and $PGE_2$ in intestinal mucosa

Identifying the cellular sources of IL-6 and $PGE_2$ can give clues regarding the stimuli that trigger their release and their proximal cellular targets. The epithelium was shown to be an important autocrine source of both IL-6 and $PGE_2$ in *in vitro* organoid culture; the tuft cell source of $PGE_2$ synthesis was visualised by COX-1/2 enzyme expression, and discrete pools of IL-6 surrounding the basal epithelial membrane at the stem cell niche were observed by immunolabelling. Given that tuft cells are located above the stem cell niche, it is unlikely that $PGE_2$ and IL-6 originate from the same cell types. Nevertheless, immunolabelling to determine co-expression of IL-6 protein and COX-1/2 cells would confirm whether COX-1/2 cells are a source of IL-6. Furthermore, RT-PCR detection of IL-6 transcripts within mRNA isolated from small intestinal organoids suggest that the epithelium is a source (at least in part) of IL-6. Single cell RT-PCR could be used as an approach to identify the cellular sources of IL-6, and could also be used to confirm tuft cells as a source of $PGE_2$ (i.e. enriched for COX-1/2 expression).

The proximal location of IL-6 and $PGE_2$ cellular sources suggest that the secretion of one cytokine could influence the secretion of the other, and vice versa. In a model of breast cancer, expression of COX-2 by tumour-associated macrophages (TAM) induced the reciprocal expression of COX-2 and secretion of $PGE_2$ by cancer cells (Li et al., 2015). Significantly, epithelial COX-2 activity also stimulated IL-6 secretion by TAM (Gan et al., 2016). Moreover, IL-6 has been shown to induce COX-2 expression and $PGE_2$ secretion in macrophages (Falcone et al., 2007). Hence, the potential exists for a positive feedback loop between IL-6 expression/secretion and COX-2 expression/$PGE_2$ secretion in the same (i.e. autocrine) and/or neighbouring cells (paracrine). This paradigm could form an important amplification step in the mucosal immune surveillance system. **Figure 6.2A** illustrates a scenario where sensing of low levels of a given Microbe-Associated Molecular Pattern (MAMP) by tuft cells stimulates low levels of $PGE_2$ secretion and, similarly, sensing of a different MAMP by a nearby cellular source of IL-6, stimulates low levels of IL-6 secretion. It is feasible that in isolation, low levels of each MAMP would not induce sufficient secretion of either IL-6 or $PGE_2$ in order to modulate barrier function. However, in the presence of a positive feedback loop i.e. amplification, the levels of IL-6 and $PGE_2$ secreted would be more likely to reach threshold levels to enact an appropriate response to maintain epithelial barrier homeostasis. Therefore, subthreshold levels of different MAMPs could potentially modulate barrier function by recruitment and cooperation of cytokines by different cellular sources through the formation of a positive feedback loop.

A similar paradigm could apply to paracrine input from subepithelial sources of IL-6 and $PGE_2$. Immune cells residing in the underlying lamina propria during health, including eosinophils, monocytes and macrophages, represent a paracrine source of potential crypt regenerative factors. Published evidence, in combination with data obtained in these studies, suggests that eosinophils may be an important paracrine source of IL-6 and $PGE_2$ in the intestinal mucosa (Rosenberg et al., 2013; Chu et al., 2014). Eosinophils were shown in **Chapter 5** to home to the colonic stem cell niche *in vivo* and in a reductionist *in vitro* co-culture model. It is possible that innate sensing of MAMPs stimulates the epithelium to secrete an intermediate factor or chemoattractant, which induces the recruitment of eosinophils (and other immune cells) to the stem cell niche (**Figure 6.2B**). Recruited or resident eosinophils (or other immune cells) at the stem cell niche are well positioned to secrete paracrine factors for crypt regeneration, including IL-6 and $PGE_2$, where the close proximity to the stem cell niche allows easy access of IL-6 and $PGE_2$ ligand to potential targets, i.e. receptors expressed by specific cell types within the epithelium. In support of this, the epithelium expresses Eotaxin-1 (El Hadi, personal communication) for the recruitment of eosinophils and CCR3 (the receptor for eotaxin-1) was expressed by eosinophils isolated from the intestinal lamina propria. The expression of IL-6 and COX enzymes were confirmed in primary eosinophils and HL-60-eosinophils, which indicates that following recruitment to the crypt base, eosinophils potentially secrete IL-6 and synthesise $PGE_2$ in close proximity to the stem cell niche (**Figure 6.2B**). This indicates a possible dynamic spatial relationship between eosinophils and the epithelium at the stem cell niche, which may contribute to tissue homeostasis through the secretion of IL-6 and $PGE_2$.

Alternative paracrine sources of IL-6 and $PGE_2$ are monocytes, macrophages or myofibroblasts (**Figure 6.2B**) (Ishikawa et al., 2011; Roulis et al., 2014; Hunter and Jones, 2015; Li et al., 2015; Villarino et al., 2015). A role for monocytes in crypt renewal during homeostasis in response to innate microbial sensing has previously been described (Skoczek et al., 2014). Monocytes are known to be a major source of IL-6 *in vivo*, as such IL-6 represents a potential candidate as a regenerative factor secreted by monocytes in this system, although this has not yet been investigated. Macrophages and myofibroblasts have also been shown previously to secrete crypt regenerative factors, including IL-6 and $PGE_2$, and have been associated with induction of epithelial repair processes in response to intestinal injury (Ishikawa et al., 2011; Roulis et al., 2014; Li et al., 2015). However, Ishikawa *et al.* (2011) induced a COX-2 deletion in all cells of myeloid origin, therefore the specific immune cell sources of COX-2 in epithelial regeneration was not fully investigated. Specific

genetic ablation of COX enzymes in each myeloid cell subtype would be required to determine the individual roles of eosinophils, neutrophils, monocytes and macrophages in $PGE_2$-induced crypt regeneration.

Regardless of the cellular sources of $PGE_2$ and IL-6, their impact on epithelial barrier function can be predicted from the relationship between epithelial cell function and receptor subtype expression.

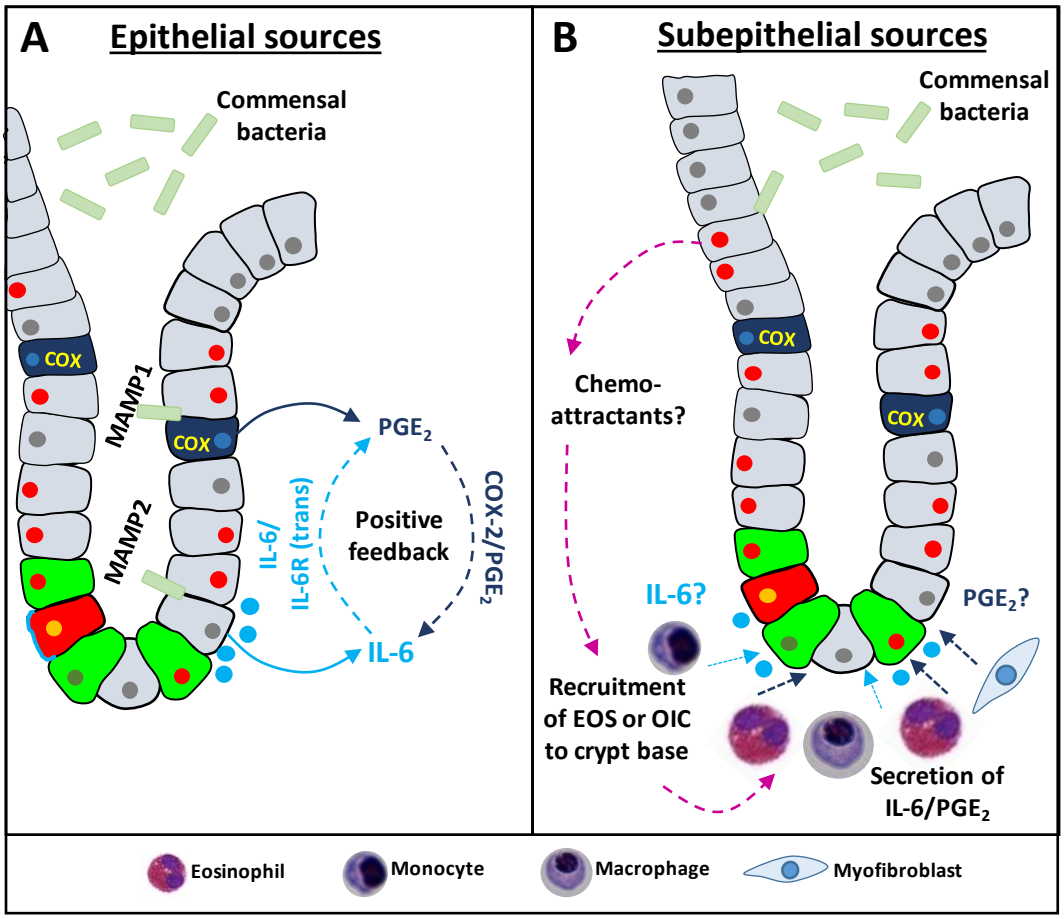


**Figure 6.2. Potential sources of IL-6 and PGE2 in the intestinal mucosa.**

(**A**) Following innate sensing of MAMPs, the epithelium secretes IL-6 and synthesises $PGE_2$ in order to induce a physiological response. IL-6 is secreted in discrete pools surrounding the ISC niche, and $PGE_2$ is synthesised by COX enzymes expressed on tuft cells. Whether IL-6 is able to induce $PGE_2$ secretion and vice versa, in a positive feedback loop, remains to be determined (**B**) Alternatively, microbial sensing induces expression of chemoattractants (or other factors) which recruit immune cells including eosinophils to the stem cell niche. Eosinophils (EOS) or other immune cells (OIC) may then secrete IL-6 and $PGE_2$ in a paracrine manner.

### 6.1.2 Differential expression of IL-6R and EPR subtypes in the intestinal epithelium

The intestinal epithelium is comprised of a number of epithelial cell types, which have distinct functions that are highly conserved across the small and large intestine. It is logical to suggest that the co-expression of receptors for IL-6 and $PGE_2$ on the same cell is predictive for regulation of physiological function by these cytokines. Also, the cell type and identity of the co-expressed cytokine receptors will be indicative of the cellular signals that are permissive for a given physiological outcome following receptor activation.

For example, restricted expression of the IL-6R was observed in Paneth cells of the small intestine. Whilst there are no Paneth cells in the large intestine, CD24+ Goblet-Like Cells (GLCs) which reside in the stem cell niche at the base of crypts are thought to be the Paneth cell equivalent in the colon (Sato et al., 2011), suggesting that GLCs may be the site of IL-6R expression in the colon. GLCs express markers of goblet cells including Muc-2 and cKit (King et al., 2012; Rothenberg et al., 2012). All four EP receptor subtypes were expressed on Muc-2 positive cells at the base of colonic crypts (interestingly, EP2 was exclusively expressed on goblet cells). This suggests the EP1-4 receptor subtypes and IL-6R may be co-expressed by GLCs in the colonic epithelium (**Figure 6.3**). Future experiments could utilise an immunolabelling approach to determine whether co-expression of the IL-6R and EP1-4 receptors is observed on CD24+ GLCs in the colon, and Paneth cells in the small intestine.

Whilst expression of the IL-6R was not observed on any other cell types of the SI epithelium, endogenous expression of the gp130 receptor is observed throughout the epithelium, suggesting that IL-6 trans signalling may be activated in other epithelial cell types, which express differential EP receptor subtypes. Lgr5+ colonic stem cells were shown to express EP1, EP3 and EP4 receptor subtypes. Lgr5 expression in stem cells is conserved across the colon and small intestine, therefore it is likely that EP1, EP3 and EP4 receptor subtype expression on stem cells is also conserved in the small intestine. The EP1 and EP3 receptor subtypes were also expressed on COX-1 positive cells of the colonic epithelium, suggesting that they may induce an autocrine positive feedback loop in order to induce further $PGE_2$ synthesis. It remains to be seen whether EP1 and EP3 receptor expression of COX-1 positive cells is conserved in the small intestinal epithelium.

Co-expression of EP receptors and the IL-6R/gp130 on the same cell type(s) in the small intestine and colon suggests the possibility of integrated $PGE_2$ and IL-6 signalling transduction

in the modulation of various physiological functions required to maintain epithelial homeostasis in the intestine.

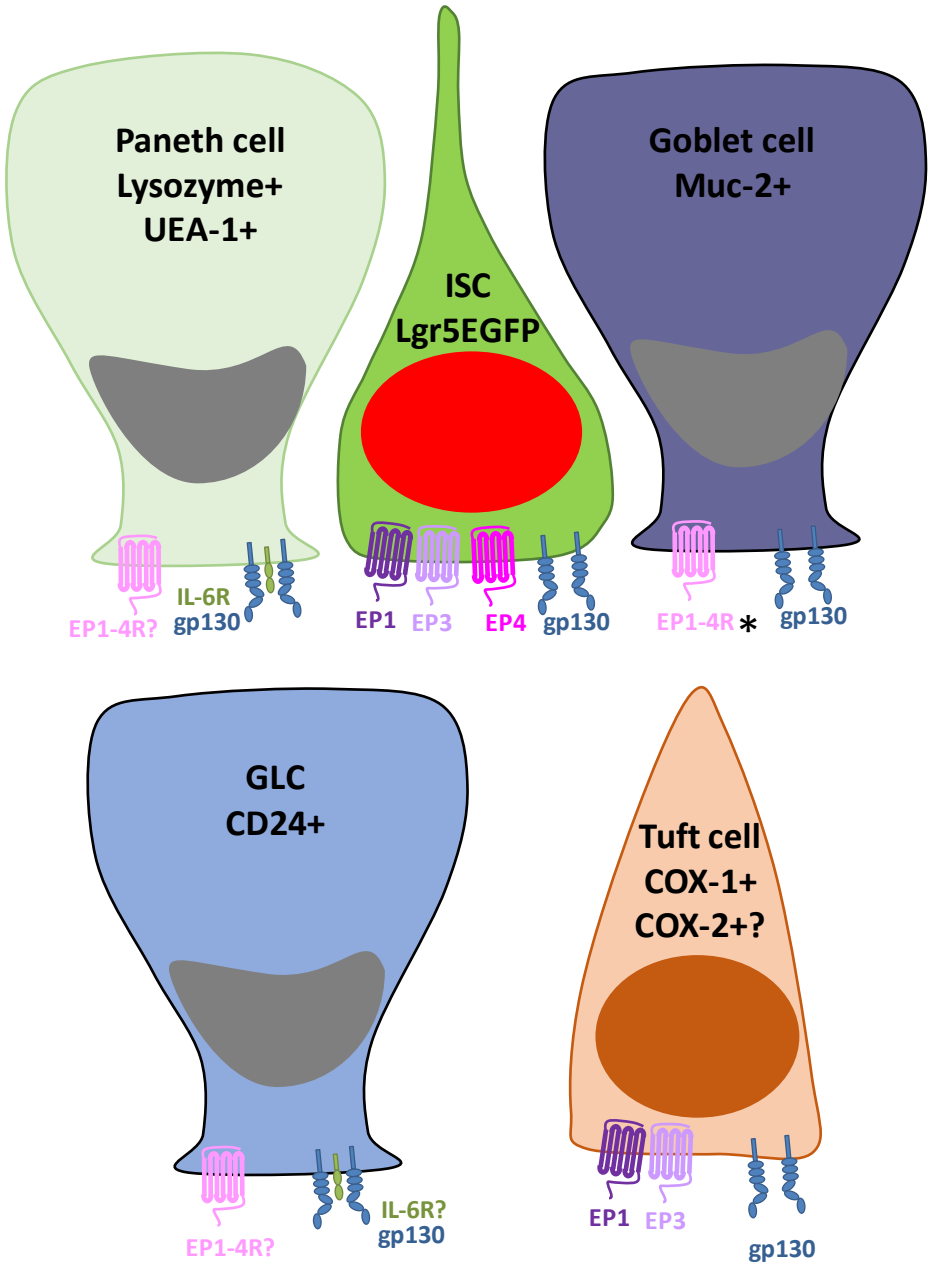


**Figure 6.3. Differential IL-6 and EP receptor expression by specific cell types in the intestinal epithelium.**

Paneth cells are the site of restricted IL-6R expression in the SI epithelium. The expression of EPR on Paneth cells remains to be determined. ISC express EP1, EP3 and EP4 receptor subtypes. Muc-2 positive goblet cells expressed all EP receptor subtypes EP1-4. NB.* EP2 expression was restricted to Muc-2 positive goblet cells. CD24+ goblet-like cells (GLCs) likely express EP1, EP2, EP3 and EP4 receptors, and the IL-6R as GLCs are to be the Paneth cell equivalent in the colon. COX-1 positive cells expressed the EP1 and EP3 receptor subtypes. The gp130 receptor is endogenously expressed by all cell types in the intestinal epithelium.

### 6.1.3 Integrated signalling transduction pathways of IL-6 and $PGE_2$

Another question, which remains to be investigated, is whether downstream integration of the IL-6 and $PGE_2$ signalling pathways occurs during homeostasis. Following receptor activation, the downstream signalling cascades of IL-6 and $PGE_2$ are activated. Up to this point, the pathways through which IL-6 and $PGE_2$ transduce signals have been described as largely independent; IL-6 through the JAK/STAT pathway, and $PGE_2$ through individual EP receptor subtypes to activate downstream intracellular calcium mobilisation or adenylate cyclase stimulation. In reality, convergence is likely to occur between the signal transduction cascades. This may be directly through co-activation of similar downstream signal transduction pathways, or through induction of a common intermediate signalling molecule (summarised in **Figure 6.4**).

Activation of the JAK/STAT pathway by IL-6 induces phosphorylation of STAT3 and translocation into the nucleus (Scheller et al., 2011; Garbers et al., 2015). Previous studies have suggested that $PGE_2$ also induces pSTAT3 activation in colorectal cancer cells through transactivation of the EGF signalling pathway (Oshima et al., 2011). In order to investigate whether $PGE_2$/EP activates pSTAT3 during homeostasis, a similar approach to that undertaken in **Chapter 3** could be utilised e.g. intestinal organoids/crypts could be stimulated with $PGE_2$ or EP receptor selective agonists, and pSTAT3 activation in specific cell types determined using immunofluorescent labelling.

Following activation of JAK/STAT signalling, and translocation into the nucleus, pSTAT3 is known to directly induce transcription factors including *c-Myc, CyclinD1, CyclinB1* and *Cdc2* (Bromberg et al., 1999; Bowman et al., 2001; Masuda et al., 2002). Activation of these target genes directly induces cell proliferation (**Figure 6.4**, scenario **1**), which again is testable in the crypt/organoid *in vitro* culture systems. IL-6 stimulation of small intestinal organoids was shown to induce crypt cell proliferation. However, in the absence of intestinal tissue injury, Paneth cells (which were identified as the site of IL-6R expression, and pSTAT3 activation) are not thought undergo proliferation (Roth et al., 2012). This indicates that IL-6-induced increase in proliferation is likely to be through pSTAT3-mediated induction of an intermediate signalling molecule that induces proliferation of neighbouring stem cells.

The WNT signalling pathway is the master regulator of intestinal tissue renewal (Schepers and Clevers, 2012). Therefore, it is not surprising that downstream signalling of both IL-6 and $PGE_2$ is now thought to be mediated by involvement of the WNT signalling pathway (**Chapter 3** and (Goessling et al., 2009)). It is therefore possible that IL-6 and $PGE_2$ signalling are

integrated at the level of secretion of WNT as a common intermediate signalling molecule. It remains to be seen whether canonical or non-canonical WNT signalling transduction is initiated following activation by IL-6 or $PGE_2$, and whether the secretion of common or different WNT ligands are induced following activation of IL-6 and $PGE_2$ signalling.

The major activator of canonical WNT signalling is WNT3 (Sato et al., 2011). Although results in this thesis did not show a detectable change in WNT3 expression in SI organoids following IL-6 stimulation, this may have been due to limitations in the sensitivity of the Immunofluorescent labelling technique used. Future studies aim to utilise a more sensitive detection method, i.e. ELISA or q-RT-PCR in order to determine whether WNT3 secretion is induced following IL-6 or $PGE_2$ stimulation. WNT3 secretion by Paneth cells (or potentially CD24+ cells in colon) is known to bind to Frizzled receptors on neighbouring ISC (Farin et al., 2016). This leads to inhibition of GSK3, allowing nuclear accumulation of β-catenin. β-catenin then induces downstream transcription of TCF/LEF transcription factors, which subsequently induces proliferation (reviewed in Nusse (2012)). This potential signalling transduction pathway is depicted in **Figure 6.4,** scenario **2**.

Previous studies have alluded to a role for non-canonical WNT5A in epithelial regeneration (Miyoshi et al., 2012). IL-6 trans-signalling has also previously been shown to upregulate WNT5A in models of chronic inflammation and melanoma cell migration (Linnskog et al., 2014). It is therefore possible that in this system, pSTAT3 activation through IL-6 classical signalling also activates WNT5A secretion of Paneth/CD24+ cells and the non-canonical WNT pathway in order to induce downstream effects on proliferation (**Figure 6.4** scenario **3**). WNT signalling augmented by $PGE_2$ (with or without STAT3 involvement) may also be through the WNT5A non-canonical pathway. Future studies aim to use a WNT array to determine whether expression levels of canonical and non-canonical WNT associated genes are altered in response to IL-6 or $PGE_2$ stimulation of organoids.

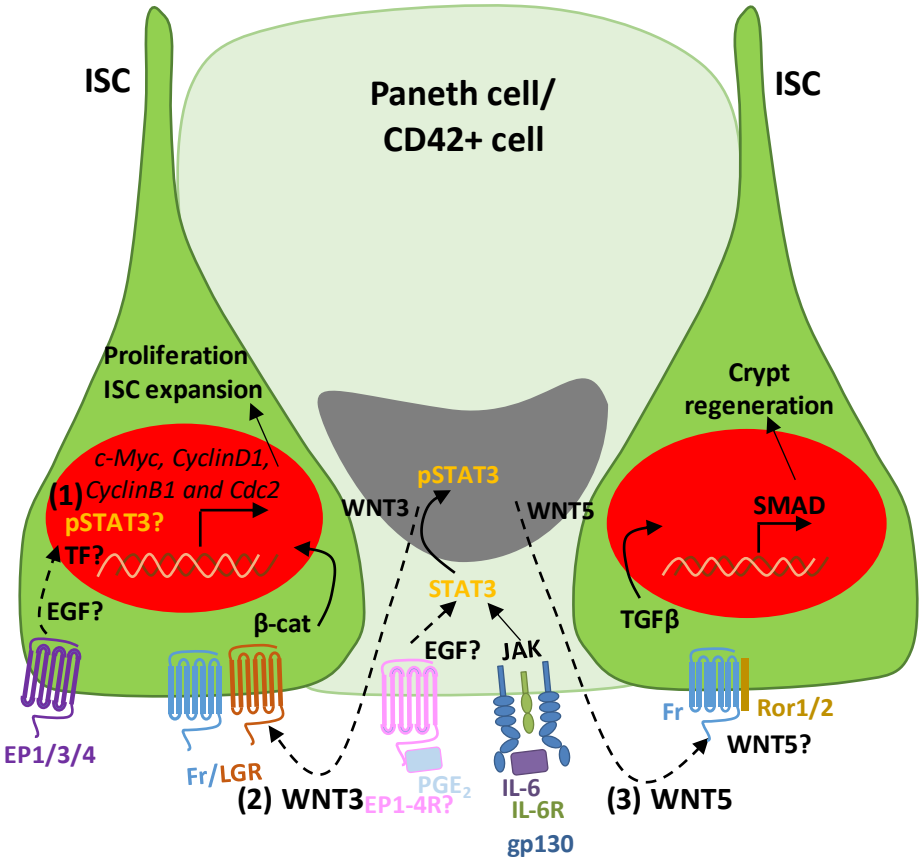


**Figure 6.4. Integrated signalling of IL-6 and PGE2 induces crypt cell proliferation?**

Potential activation of STAT3 by $PGE_2$ activation of EPR on ISC induces phosphorylation of STAT3 and translocation into the nucleus where (**1**) pSTAT3 induces direct activation of proliferation-associated target genes. IL-6 and $PGE_2$ have both been shown to require involvement of the WNT signalling pathway. (**2**) A potential scenario is that IL-6/$PGE_2$ activates pSTAT3, which induces WNT3 secretion from CD24+ cells (or Paneth cells in SI) where WNT binds to Frizzled receptors on ISC. This activates canonical WNT signalling, which subsequently induces proliferation. (**3**) In contrast, non-canonical WNT signalling could become activated by secretion of WNT5A, which has also been shown to induce crypt regeneration.

### 6.1.4 Physiological Functions of IL-6 and $PGE_2$ signalling activation

The physiological functions investigated within this thesis were largely focussed on cellular proliferation and ISC expansion required for epithelial renewal, in response to IL-6 and $PGE_2$ stimulation of small and large intestinal crypts/organoids, respectively. Further to the data presented in **Chapter 3** detailing a role for IL-6 in small intestinal homeostasis, initial experiments have shown that exogenous addition of IL-6 to isolated mouse colonic crypts in culture also induces colonic crypt proliferation during homeostasis (**Figure 6.5**). The role of autocrine IL-6 in crypt cell proliferation and stem cell expansion in the colon remain to be determined. The experiments undertaken in **Chapter 4** were focussed on the role of $PGE_2$ activation in crypt cell renewal in the colonic epithelium, as this is the site of colon cancer initiation, which $PGE_2$ has been shown to be a driving factor of (Wang and Dubois, 2010). However, the role of $PGE_2$ signalling in small intestinal renewal during homeostasis and disease has not yet been investigated.

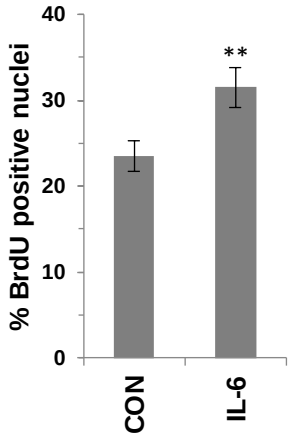


**Figure 6.5. IL-6 induces proliferation of colonic organoids**

Histogram showing the percentage of BrdU positive nuclei in colonic organoids following 24 hours stimulation with IL-6 (100 ng/ml) compared to control (n=3, $^{**}P<0.01$; $^{***}P<0.001$) figure obtained by AS.

Other physiological functions required for maintenance of the epithelial barrier, i.e. apoptosis and cell shedding or mucus secretion, have not yet been fully investigated. Activation of the STAT3 pathway is known to induce the production of factors to prevent apoptosis and prolong survival (reviewed in Jarnicki et al. (2010)). Data in this thesis alluded to a role for STAT3 in prevention of apoptosis in small intestinal organoids (quantified using

caspase-3 as a marker of apoptosis); however, the percentage change in caspase-3 positive cells was minimal in comparison to the percentage change in BrdU incorporation, indicating that the major physiological function initiated following STAT3 activation was cell proliferation. Furthermore, the restricted expression of the EP2 receptor subtype on goblet cells of the colonic epithelium suggests that EP2 receptor signalling may play a major role in mucus secretion, which is required for maintenance of epithelial barrier homeostasis (Johansson et al., 2013). The effects of IL-6 or $PGE_2$ signalling in apoptosis, cell differentiation and mucus secretion remain the focus of future studies.

It is possible that physiological processes required to maintain epithelial barrier homeostasis require combined activation by these two cytokines. Alternatively, compensatory mechanisms may exist between IL-6 and $PGE_2$ during homeostasis in order to ensure sufficient proliferation, i.e. where IL-6 is lost in the SI, $PGE_2$ induces SI renewal to maintain crypt homeostasis. An obvious initial experiment to conduct in order to determine the role of IL-6 cross-talk with $PGE_2$ signalling in intestinal homeostasis, would be to simultaneously add exogenous IL-6 and $PGE_2$ to intestinal crypt culture and determine the effects on the signalling pathways discussed above and physiological processes underpinning barrier function such as cell proliferation and stem cell expansion. In addition, treatment of colonic/SI organoids with the IL-6R-blocking antibody or IL-6-neutralising antibody in combination with exogenous addition of $PGE_2$ would be required in order to determine whether $PGE_2$-induced increase in proliferation is dependent on IL-6. Similarly, addition of the $PGE_2$ blocking antibody to organoid culture in combination with exogenous IL-6 addition would identify whether the IL-6-induced increase in proliferation is dependent on $PGE_2$.

These experiments could be conducted in combination with experiments detailed in **section 6.1.3** for investigation into the downstream signalling integration between IL-6 and $PGE_2$. For example, the potential activation of pSTAT3 in response to $PGE_2$ may require input from endogenous IL-6 signalling. Therefore, immunofluorescent pSTAT3 labelling experiments in response to $PGE_2$ stimulation would be conducted in the presence and absence of an IL-6 blocking antibody.

### 6.1.5 An Integrated Model for IL-6/$PGE_2$-mediated regulation of epithelial barrier homeostasis

The discussion in the preceding sections gives rise to a model whereby IL-6 and $PGE_2$ signalling act in concert to maintain epithelial barrier homeostasis (depicted in **Figure 6.6**). Innate sensing of microbial patterns from commensal bacteria residing in the gut lumen initiates physiological processes which are required for the maintenance of the epithelial barrier (**1**). The epithelium is an important autocrine source of IL-6 and $PGE_2$ (**2**). Whether a positive feedback loop exists between IL-6 and $PGE_2$, through induction of endogenous IL-6 signalling in response to $PGE_2$ secretion, or vice versa remains to be seen. Potential subepithelial paracrine sources of $PGE_2$ and IL-6 are eosinophils, which home to the crypt stem cell niche and crypt top along the crypt-axis in the underlying lamina propria of the intestinal mucosa (**3**). Provision of IL-6 or $PGE_2$ ligand in close proximity to the stem cell niche, through epithelial or subepithelial sources, induces receptor subtype(s) activation in differential cell types of the epithelium. Potential receptor co-expression of IL-6R and EP receptor subtypes on CD24+ cells in the colon represents possible integration between $PGE_2$ and IL-6 signalling transduction for physiological function (**4**). IL-6 and $PGE_2$ (via EGFR transactivation) could act in synergy to activate STAT3 (**5**). STAT3 activation mediates crypt cell proliferation, possibly through the secretion of WNT ligands derived from the stem cell niche, which could serve as common intestinal (stem) cell mitogens downstream of IL-6 and $PGE_2$ receptor activation. The roles of IL-6 and $PGE_2$ signalling in the regulation of other processes that underpin epithelial barrier function such as cell differentiation, apoptosis or mucus secretion remain to be elucidated.

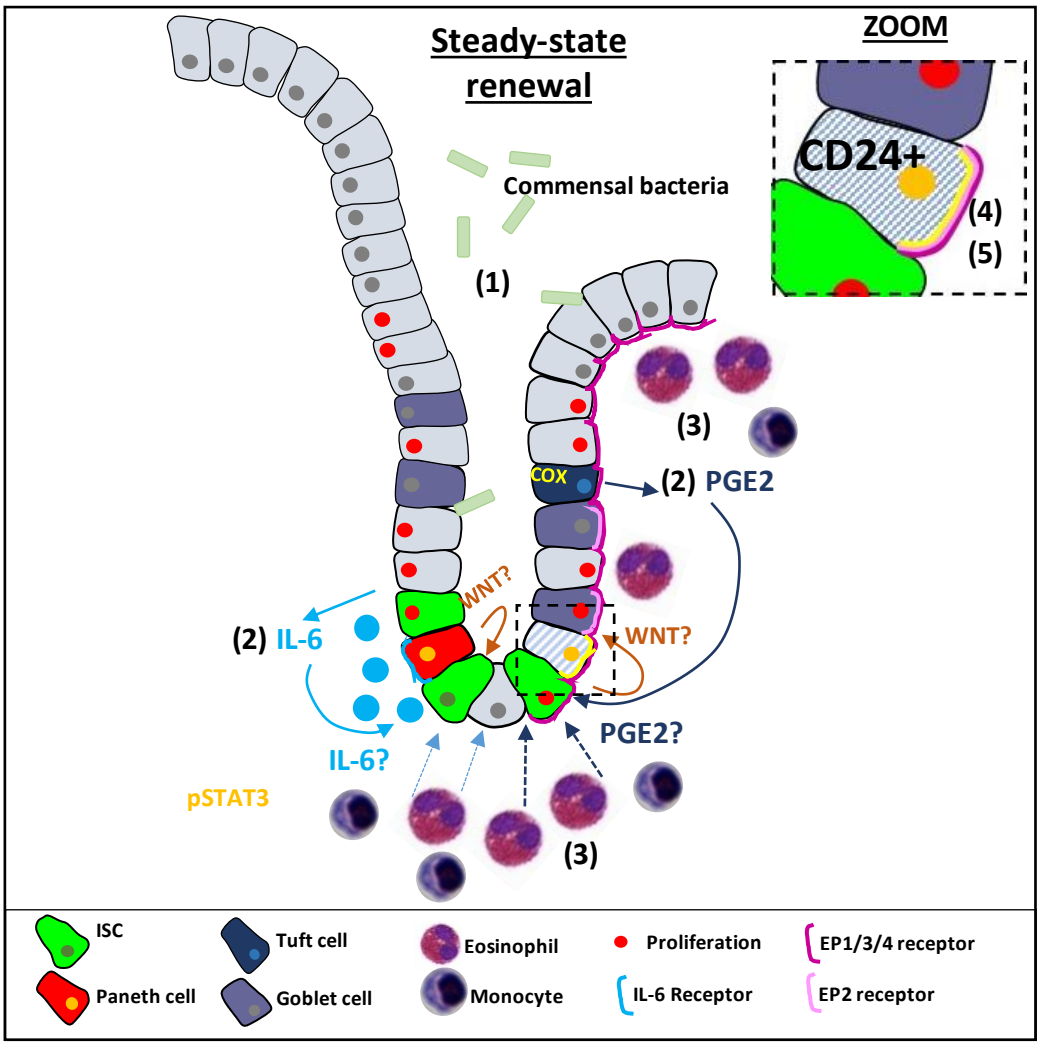


**Figure 6.6. Integrated model for the dynamic regulation of intestinal homeostasis by IL-6 and PGE2.**

**(1)** Innate sensing of MAMPS initiates physiological processes for epithelial barrier maintenance. (**2**) The epithelium is an autocrine source of IL-6 and $PGE_2$. (**3**) Potential subepithelial sources of IL-6 and $PGE_2$ are eosinophils, which reside at the intestinal stem cell niche and crypt top during health. (**4**) Potential co-expression exists for the IL-6R and EP1-4 receptor subtypes on CD24+ cells of the colon. (**5**) IL-6 (and $PGE_2$) activates STAT3 which mediates cell proliferation, possibly through the secretion of WNTs as a potential common intermediate signalling molecule.

## 6.2 Homeostasis versus Disease

Previous studies have suggested that a number of signalling pathways required for epithelial renewal during health are conserved during inflammation and cancer. Indeed, both IL-6 and $PGE_2$, which modulated crypt renewal during health, have been shown to induce inflammation and tumorigenesis. It is possible that increased concentrations of IL-6 and $PGE_2$

secreted (through an increase in immune cells) results in increased signalling through the same pathway, and therefore induce a higher magnitude of the same physiological responses observed in health. Alternatively, different signal transduction pathways may be activated in health and disease to induce different (patho) physiological functions.

### 6.2.1 Increase in concentration of factors secreted in IBD and cancer

Eosinophils are a paracrine source of both IL-6 and $PGE_2$ in the intestinal lamina propria that are known to reside in high numbers in the lamina propria in health and in IBD (Rosenberg et al., 2013; Chu et al., 2014). The striking spatial relationship observed between eosinophils and the ISC niche described in this thesis suggests that eosinophils may play a role in maintaining the stem cell niche during health. This spatial relationship was also observed between monocytes and the crypt base of the human colonic epithelium *in vivo* and in *in vitro* co-culture with the THP-1 monocytic cell line (**Appendix C** and **D**), in agreement with published work (Skoczek et al., 2014). In tissue sections obtained from patients with active IBD, a 1.2 fold increase in the number of eosinophils was observed at the stem cell niche (29.8+/-3.3 IBD compared to 13.4+/- 0.9 control, $P<0.001$). In IBD tissue, a significant increase in the number of CD14+ monocytes was also observed in all functional regions compared to healthy tissue *in vivo* (**Appendix C**). The increase in numbers of eosinophils and monocytes present at the ISC in IBD suggests may indicate an increase in secreted crypt regenerative factors (IL-6 and $PGE_2$).

Cell shedding is another process of vital importance to maintain the balance of cell growth and apoptosis in the intestine, which prevents the accumulation of cells and cancer-driving genetic mutations. Interestingly, a significant increase in the number of eosinophils and monocytes present at the top region of crypts (the site of cell shedding) was observed in IBD samples compared to control. This indicates that in IBD, eosinophils and monocytes may play a role in re-establishing homeostasis following inflammation through modulating stem-cell driven proliferation and cell shedding.

Future studies aim to identify whether a potential increase in secreted concentrations of IL-6 and $PGE_2$ by eosinophils, monocytes (and macrophages) at functional regions of crypts play a role in the modulation of crypt renewal to re-establish homeostasis following inflammation. Alternatively, increased paracrine secretion of IL-6 and $PGE_2$ at functional regions of crypts by immune cells may induces a pathogenic response, i.e. inflammation or uncontrolled crypt cell proliferation, in inflammation and cancer.

### 6.2.2 Differential activation of IL-6 trans-signalling or the EP2/4 receptor signalling axis in inflammation and cancer

The data presented in this thesis, in combination with previous work in the literature, suggests that in health and disease, IL-6 and $PGE_2$ may act through different signal transduction pathways in order to induce physiological functions in the prevention or pathogenesis of inflammation or cancer. Novel data has been described in this thesis, to support the paradigm that IL-6 classic signalling is important for epithelial renewal during homeostasis. The pro-inflammatory responses of IL-6 signalling are thought to be mediated through IL-6 trans-signalling (Jones et al., 2005; Scheller et al., 2011), which could be investigated in future experiments utilising the hIL-6 fusion protein (IL-6 linked to IL-6R by a flexible peptide chain) (Fischer et al., 1997). We speculate that addition of hIL-6 into organoid culture would induce global pSTAT3 activation in all cells of organoids through binding to endogenously expressed gp130, potentially mimicking the result obtained by Aden et al (2016), where hIL-6 induced an increase in pSTAT3, which was detectable by western blot (Aden et al., 2016). It would be of interest to compare the magnitude of epithelial response to IL-6 to hIL-6 with respect to proliferation, apoptosis and inflammatory markers or tissue damage. IL-6 trans-signalling has also previously been shown to enhance the growth of tumour initiating cells. It is possible that the paradigm we observe during homeostasis, whereby IL-6 binds the IL-6R on Paneth cells, which activates pSTAT3 and induces crypt cell proliferation, induces a tightly regulated renewal required to maintain the epithelial barrier. We speculate that in cancer, the IL-6R is cleaved from the membrane of Paneth cells and lamina propria immune cells, which enables activation of IL-6 trans signalling in all cells of the small intestinal epithelium. This may then induce uncontrolled proliferation through increased secretion of WNTs or direct activation of proliferative genes. Immunofluorescent labelling of the IL-6R in tissue derived from tumours would enable us to determine whether IL-6 trans-signalling contributes to tumorigenesis.

Cleavage of the IL-6R in IL-6 trans signalling is dependent on ADAM10/17 proteases (Matthews et al., 2003; Chalaris et al., 2010). Interestingly, EP4-mediated $PGE_2$ signalling was previously shown to upregulate expression of ADAM proteases (including ADAM10/17), which was shown to induce transactivation of the EGFR pathway (Oshima et al., 2011). Transactivation of the EGFR by $PGE_2$ also induces STAT3 activation (Normanno et al., 2006). The upregulation of ADAM proteases provides a potential mechanism for the $PGE_2$-induced transactivation of STAT3 through activation of IL-6 trans signalling, and suggests the presence

of positive feedback loop between $PGE_2$ and IL-6 signalling which may be implicated in cancer (**Figure 6.7**).

The roles of IL-6 classic and trans signalling in cell shedding have not yet been determined. STAT3 activation also plays a role in the regulation of anti-apoptotic and pro-survival factors (Yu et al., 2009). In agreement with this, the number of cells undergoing apoptosis (determined by caspase-3 activation) was increased following STAT3 inhibition with STATTIC-treatment of SI organoids. In the case of tumorigenesis, it is possible that a cumulative effect of uncontrolled proliferation, combined with prevention of apoptosis, is initiated by IL-6 trans-signalling which induces tumour growth in the intestine. The novel roles described in this thesis for IL-6 signalling in the maintenance of small intestinal renewal during homeostasis are of importance when considering the perturbation of the IL-6 signalling pathway as a therapeutic target. For these reasons, the abrogation of inflammatory IL-6 trans signalling, whilst maintaining IL-6 classical signalling required for intestinal renewal during homeostasis appears to be an attractive strategy for developing treatments for inflammatory diseases and cancer.

A novel role for differential EP1/EP3 receptor activation by $PGE_2$ during health has been described. Autocrine and paracrine $PGE_2$-induced crypt cell proliferation was mediated through activation of EP1 and EP3 receptor subtypes, and EP2 and EP4 receptor subtype activation/inhibition induced no change in basal crypt cell proliferation compared to control crypts during homeostasis. Previous studies have suggested that signalling through the EP2 and EP4 receptor subtypes induce intestinal polyposis, tumour cell proliferation and tumour invasion (Sonoshita et al., 2001; Kamiyama et al., 2006; Hawcroft et al., 2007; Chang et al., 2015; Lee et al., 2016). Therefore, $PGE_2$ may activate EP1/3 receptor subtypes during health, and EP2/4 receptor subtypes during cancer in order to induce crypt cell proliferation. This suggests that targeting $PGE_2$ signalling through the EP2 or EP4 receptor subtypes may inhibit tumorigenesis and tumour cell proliferation, whilst maintaining EP1/3 receptor signalling required for basal cell proliferation in steady state renewal. However, EP2 receptor signalling has previously been associated with maintenance of the mucosal barrier (Lejeune et al., 2014), a paradigm supported by our finding that expression of the EP2 receptor was restricted to goblet cells of the colonic epithelium, which are vital for the replenishment of mucus layers to protect the epithelium (Johansson et al., 2013). In addition, the EP4 receptor subtype has also been described to ameliorate severe colitis and reduce mucosal damage following DSS-induced colitis (Kabashima et al., 2002). Therefore, whilst EP2/EP4 receptor signalling abrogation may reduce proliferation in tumour cells, the effects on epithelial

barrier function and inflammation would need to be fully investigated before targeting of this pathway for the development of anti-cancer therapies.

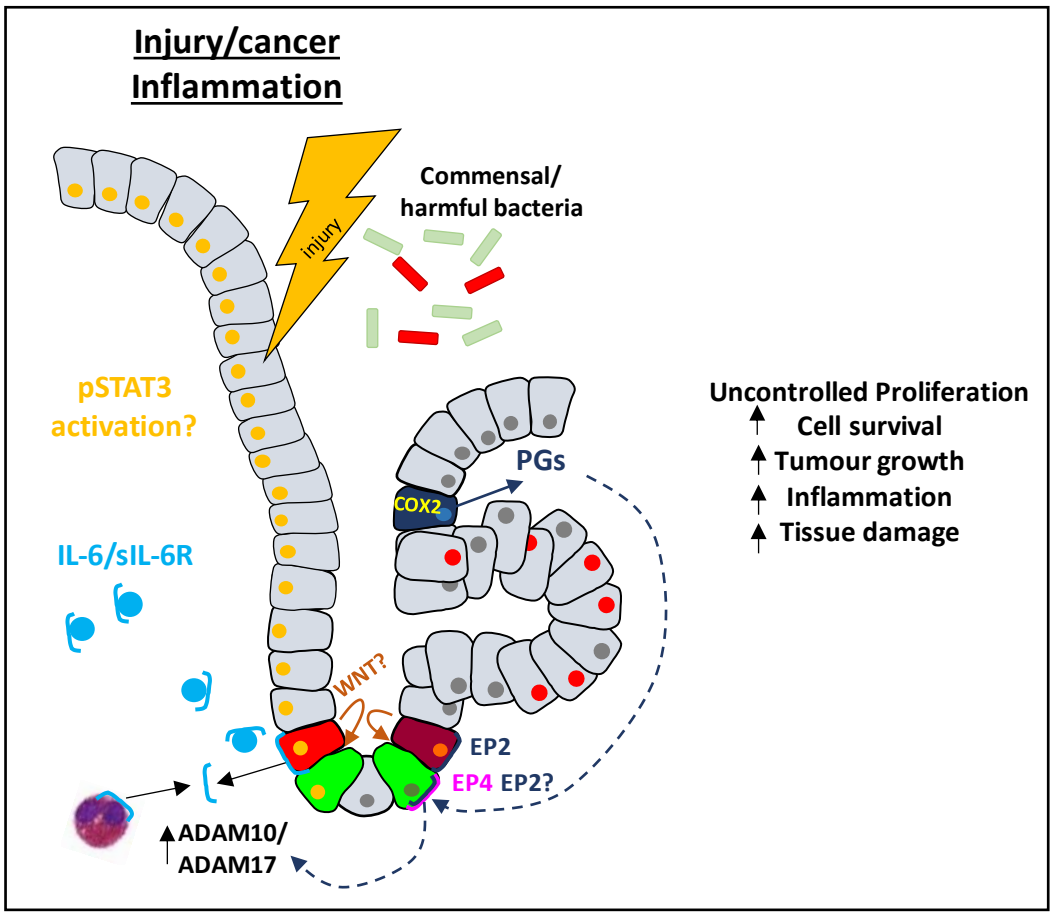


**Figure 6.7. In tissue injury or cancer, IL-6 trans-signalling and PGE2 potentially contribute to inflammation and carcinogenesis**

In cancer, EP4-mediated $PGE_2$ signalling induces upregulation of ADAM protease, which cleave the IL-6R from the Paneth cell and eosinophil membrane. Secreted IL-6 and can then bind and activate downstream signalling in all cells of the epithelium. This induces global pSTAT3 activation to induce activation of genes for proliferation and survival, possibly potentiating carcinogenesis. In tumorigenesis, COX-2 potentially activates the EP2 and EP4 receptor subtypes in GLCs and ISC at the crypt base to induce uncontrolled cell proliferation and carcinogenesis through transactivation of the EGF signalling pathway and IL-6 trans-signalling.

## 6.3 Concluding Remarks

In summary, a reductionist approach has been utilised to investigate a role in intestinal homeostasis for two cytokines previously associated with inflammation and cancer. Novel contributions for IL-6 and $PGE_2$ were demonstrated in the renewal of the intestinal epithelium. Autocrine and paracrine IL-6 (classic) signalling was shown to induce crypt cell proliferation, through activation of pSTAT3 in Paneth cells of the small intestine. A previously unrecognised role for $PGE_2$ signalling, through activation of EP1/3 receptor subtypes was revealed in colonic crypt proliferation during homeostasis. Eosinophils are a potential paracrine source of IL-6 and $PGE_2$, which form a dynamic spatial relationship with the intestinal stem cell niche and shedding zone of the intestinal epithelium. An integrated model for IL-6/$PGE_2$-mediated regulation of epithelial barrier homeostasis has been proposed and future studies will determine the molecular and cellular interactions in these respective pathways and assess the impact on intestinal homeostasis and pathogenesis. It is envisaged that this knowledge will pave the way for the development of novel preventative and therapeutic strategies.

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# Bibliography

Abramovitz, M., Adam, M., Boie, Y., Carriere, M., Denis, D., Godbout, C., Lamontagne, S., Rochette, C., Sawyer, N., Tremblay, N. M., Belley, M., Gallant, M., Dufresne, C., Gareau, Y., Ruel, R., Juteau, H., Labelle, M., Ouimet, N. and Metters, K. M. (2000). "The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs." Biochim Biophys Acta **1483**(2): 285-293.

Abreu, M. T., Thomas, L. S., Arnold, E. T., Lukasek, K., Michelsen, K. S. and Arditi, M. (2003). "TLR signaling at the intestinal epithelial interface." J Endotoxin Res **9**(5): 322-330.

Aden, K., Breuer, A., Rehman, A., Geese, H., Tran, F., Sommer, J., Waetzig, G. H., Reinheimer, T. M., Schreiber, S., Rose-John, S., Scheller, J. and Rosenstiel, P. (2016). "Classic IL-6R signalling is dispensable for intestinal epithelial proliferation and repair." Oncogenesis **5**(11): e270.

af Forselles, K. J., Root, J., Clarke, T., Davey, D., Aughton, K., Dack, K. and Pullen, N. (2011). "In vitro and in vivo characterization of PF-04418948, a novel, potent and selective prostaglandin EP(2) receptor antagonist." Br J Pharmacol **164**(7): 1847-1856.

Agace, W. W., Roberts, A. I., Wu, L., Greineder, C., Ebert, E. C. and Parker, C. M. (2000). "Human intestinal lamina propria and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation." Eur J Immunol **30**(3): 819-826.

Ahrens, R., Waddell, A., Seidu, L., Blanchard, C., Carey, R., Forbes, E., Lampinen, M., Wilson, T., Cohen, E., Stringer, K., Ballard, E., Munitz, A., Xu, H., Lee, N., Lee, J. J., Rothenberg, M. E., Denson, L. and Hogan, S. P. (2008). "Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis." J Immunol **181**(10): 7390-7399.

Akuthota, P., Shamri, R. and Weller, P. F. (2012). "Isolation of human eosinophils." Curr Protoc Immunol **Chapter 7**: Unit 7 31.

Akuthota, P., Ueki, S., Estanislau, J. and Weller, P. F. (2013). "Human eosinophils express functional CCR7." Am J Respir Cell Mol Biol **48**(6): 758-764.

Al-Haddad, S. and Riddell, R. H. (2005). "The role of eosinophils in inflammatory bowel disease." Gut **54**(12): 1674-1675.

Amano, H., Hayashi, I., Endo, H., Kitasato, H., Yamashina, S., Maruyama, T., Kobayashi, M., Satoh, K., Narita, M., Sugimoto, Y., Murata, T., Yoshimura, H., Narumiya, S. and Majima, M. (2003). "Host prostaglandin E(2)-EP3 signaling regulates tumor-associated angiogenesis and tumor growth." J Exp Med **197**(2): 221-232.

Amano, H., Ito, Y., Suzuki, T., Kato, S., Matsui, Y., Ogawa, F., Murata, T., Sugimoto, Y., Senior, R., Kitasato, H., Hayashi, I., Satoh, Y., Narumiya, S. and Majima, M. (2009). "Roles of a prostaglandin E-type receptor, EP3, in upregulation of matrix metalloproteinase-9 and vascular endothelial growth factor during enhancement of tumor metastasis." Cancer Sci **100**(12): 2318-2324.

Andrianifahanana, M., Moniaux, N. and Batra, S. K. (2006). "Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases." Biochim Biophys Acta **1765**(2): 189-222.

Arber, N., Eagle, C. J., Spicak, J., Racz, I., Dite, P., Hajer, J., Zavoral, M., Lechuga, M. J., Gerletti, P., Tang, J., Rosenstein, R. B., Macdonald, K., Bhadra, P., Fowler, R., Wittes, J., Zauber, A. G., Solomon, S. D., Levin, B. and Pre, S. A. P. T. I. (2006). "Celecoxib for the prevention of colorectal adenomatous polyps." N Engl J Med **355**(9): 885-895.

Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E. and Ouellette, A. J. (2000). "Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria." Nat Immunol **1**(2): 113-118.

Bai, X., Wang, J., Zhang, L., Ma, J., Zhang, H., Xia, S., Zhang, M., Ma, X., Guo, Y., Rong, R., Cheng, S., Shu, W., Wang, Y. and Leng, J. (2013). "Prostaglandin E(2) receptor EP1-mediated phosphorylation of focal adhesion kinase enhances cell adhesion and migration in hepatocellular carcinoma cells." Int J Oncol **42**(5): 1833-1841.

Barber, D. L., Andrade, B. B., McBerry, C., Sereti, I. and Sher, A. (2014). "Role of IL-6 in Mycobacterium avium--associated immune reconstitution inflammatory syndrome." J Immunol **192**(2): 676-682.

Barker, N. and Clevers, H. (2007). "Tracking down the stem cells of the intestine: strategies to identify adult stem cells." Gastroenterology **133**(6): 1755-1760.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J. and Clevers, H. (2009). "Crypt stem cells as the cells-of-origin of intestinal cancer." Nature **457**(7229): 608-611.

Barker, N., van Es, J. H., Jaks, V., Kasper, M., Snippert, H., Toftgard, R. and Clevers, H. (2008). "Very long-term self-renewal of small intestine, colon, and hair follicles from cycling Lgr5+ve stem cells." Cold Spring Harb Symp Quant Biol **73**: 351-356.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J. and Clevers, H. (2007). "Identification of stem cells in small intestine and colon by marker gene Lgr5." Nature **449**(7165): 1003-1007.

Bassil, A. K., Borman, R. A., Jarvie, E. M., McArthur-Wilson, R. J., Thangiah, R., Sung, E. Z., Lee, K. and Sanger, G. J. (2008). "Activation of prostaglandin EP receptors by lubiprostone in rat and human stomach and colon." Br J Pharmacol **154**(1): 126-135.

Bates, M. E., Sedgwick, J. B., Zhu, Y., Liu, L. Y., Heuser, R. G., Jarjour, N. N., Kita, H. and Bertics, P. J. (2010). "Human airway eosinophils respond to chemoattractants with greater eosinophil-derived neurotoxin release, adherence to fibronectin, and activation of the Ras-ERK pathway when compared with blood eosinophils." J Immunol **184**(12): 7125-7133.

Baumann, A., Feilhauer, K., Bischoff, S. C., Froy, O. and Lorentz, A. (2015). "IgE-dependent activation of human mast cells and fMLP-mediated activation of human eosinophils is controlled by the circadian clock." Mol Immunol **64**(1): 76-81.

Baumgart, D. C. and Carding, S. R. (2007). "Inflammatory bowel disease: cause and immunobiology." Lancet **369**(9573): 1627-1640.

Becker, C., Fantini, M. C., Schramm, C., Lehr, H. A., Wirtz, S., Nikolaev, A., Burg, J., Strand, S., Kiesslich, R., Huber, S., Ito, H., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Galle, P. R., Blessing, M., Rose-John, S. and Neurath, M. F. (2004). "TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling." Immunity **21**(4): 491-501.

Belnoue, E., Pihlgren, M., McGaha, T. L., Tougne, C., Rochat, A. F., Bossen, C., Schneider, P., Huard, B., Lambert, P. H. and Siegrist, C. A. (2008). "APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells." Blood **111**(5): 2755-2764.

Benevides, L., da Fonseca, D. M., Donate, P. B., Tiezzi, D. G., De Carvalho, D. D., de Andrade, J. M., Martins, G. A. and Silva, J. S. (2015). "IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment." Cancer Res **75**(18): 3788-3799.

Berger, H., Vegran, F., Chikh, M., Gilardi, F., Ladoire, S., Bugaut, H., Mignot, G., Chalmin, F., Bruchard, M., Derangere, V., Chevriaux, A., Rebe, C., Ryffel, B., Pot, C., Hichami, A., Desvergne, B., Ghiringhelli, F. and Apetoh, L. (2013). "SOCS3 transactivation by PPARgamma prevents IL-17-driven cancer growth." Cancer Res **73**(12): 3578-3590.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K. (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature **441**(7090): 235-238.

Beumer, J. and Clevers, H. (2016). "Regulation and plasticity of intestinal stem cells during homeostasis and regeneration." Development **143**(20): 3639-3649.

Bevins, C. L. and Salzman, N. H. (2011). "Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis." Nat Rev Microbiol **9**(5): 356-368.

Billot, X., Chateauneuf, A., Chauret, N., Denis, D., Greig, G., Mathieu, M. C., Metters, K. M., Slipetz, D. M. and Young, R. N. (2003). "Discovery of a potent and selective agonist of the prostaglandin EP4 receptor." Bioorg Med Chem Lett **13**(6): 1129-1132.

Birrell, M. A., Maher, S. A., Buckley, J., Dale, N., Bonvini, S., Raemdonck, K., Pullen, N., Giembycz, M. A. and Belvisi, M. G. (2013). "Selectivity profiling of the novel EP2 receptor antagonist, PF-04418948, in functional bioassay systems: atypical affinity at the guinea pig EP2 receptor." Br J Pharmacol **168**(1): 129-138.

Blanchard, C. and Rothenberg, M. E. (2009). "Biology of the eosinophil." Adv Immunol **101**: 81-121.

Blink, E. J., Light, A., Kallies, A., Nutt, S. L., Hodgkin, P. D. and Tarlinton, D. M. (2005). "Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization." J Exp Med **201**(4): 545-554.

Bollrath, J., Phesse, T. J., von Burstin, V. A., Putoczki, T., Bennecke, M., Bateman, T., Nebelsiek, T., Lundgren-May, T., Canli, O., Schwitalla, S., Matthews, V., Schmid, R. M., Kirchner, T., Arkan, M. C., Ernst, M. and Greten, F. R. (2009). "gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis." Cancer Cell **15**(2): 91-102.

Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A. and Jove, R. (2001). "Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis." Proc Natl Acad Sci U S A **98**(13): 7319-7324.

Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanas, A., Konstam, M. A., Baron, J. A. and Adenomatous Polyp Prevention on Vioxx Trial, I. (2005). "Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial." N Engl J Med **352**(11): 1092-1102.

Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C. and Darnell, J. E., Jr. (1999). "Stat3 as an oncogene." Cell **98**(3): 295-303.

Brown, S. L., Riehl, T. E., Walker, M. R., Geske, M. J., Doherty, J. M., Stenson, W. F. and Stappenbeck, T. S. (2007). "Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury." J Clin Invest **117**(1): 258-269.

Buchanan, F. G., Gorden, D. L., Matta, P., Shi, Q., Matrisian, L. M. and DuBois, R. N. (2006). "Role of beta-arrestin 1 in the metastatic progression of colorectal cancer." Proc Natl Acad Sci U S A **103**(5): 1492-1497.

Buczacki, S. J., Zecchini, H. I., Nicholson, A. M., Russell, R., Vermeulen, L., Kemp, R. and Winton, D. J. (2013). "Intestinal label-retaining cells are secretory precursors expressing Lgr5." Nature **495**(7439): 65-69.

Buffa, R., Capella, C., Fontana, P., Usellini, L. and Solcia, E. (1978). "Types of endocrine cells in the human colon and rectum." Cell Tissue Res **192**(2): 227-240.

Camporeale, A. and Poli, V. (2012). "IL-6, IL-17 and STAT3: a holy trinity in auto-immunity?" Front Biosci (Landmark Ed) **17**: 2306-2326.

Carlens, J., Wahl, B., Ballmaier, M., Bulfone-Paus, S., Forster, R. and Pabst, O. (2009). "Common gamma-chain-dependent signals confer selective survival of eosinophils in the murine small intestine." J Immunol **183**(9): 5600-5607.

Carlson, M., Raab, Y., Peterson, C., Hallgren, R. and Venge, P. (1999). "Increased intraluminal release of eosinophil granule proteins EPO, ECP, EPX, and cytokines in ulcerative colitis and proctitis in segmental perfusion." Am J Gastroenterol **94**(7): 1876-1883.

Cassese, G., Arce, S., Hauser, A. E., Lehnert, K., Moewes, B., Mostarac, M., Muehlinghaus, G., Szyska, M., Radbruch, A. and Manz, R. A. (2003). "Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals." J Immunol **171**(4): 1684-1690.

Chalaris, A., Gewiese, J., Paliga, K., Fleig, L., Schneede, A., Krieger, K., Rose-John, S. and Scheller, J. (2010). "ADAM17-mediated shedding of the IL6R induces cleavage of the membrane stub by gamma-secretase." Biochim Biophys Acta **1803**(2): 234-245.

Chalaris, A., Rabe, B., Paliga, K., Lange, H., Laskay, T., Fielding, C. A., Jones, S. A., Rose-John, S. and Scheller, J. (2007). "Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils." Blood **110**(6): 1748-1755.

Chan, A. T., Giovannucci, E. L., Meyerhardt, J. A., Schernhammer, E. S., Wu, K. and Fuchs, C. S. (2008). "Aspirin dose and duration of use and risk of colorectal cancer in men." Gastroenterology **134**(1): 21-28.

Chan, A. T., Ogino, S. and Fuchs, C. S. (2007). "Aspirin and the risk of colorectal cancer in relation to the expression of COX-2." N Engl J Med **356**(21): 2131-2142.

Chan, A. T., Ogino, S. and Fuchs, C. S. (2009). "Aspirin use and survival after diagnosis of colorectal cancer." JAMA **302**(6): 649-658.

Chang, J., Vacher, J., Yao, B., Fan, X., Zhang, B., Harris, R. C. and Zhang, M. Z. (2015). "Prostaglandin E receptor 4 (EP4) promotes colonic tumorigenesis." Oncotarget **6**(32): 33500-33511.

Chang, S. H., Liu, C. H., Wu, M. T. and Hla, T. (2005). "Regulation of vascular endothelial cell growth factor expression in mouse mammary tumor cells by the EP2 subtype of the prostaglandin E2 receptor." Prostaglandins Other Lipid Mediat **76**(1-4): 48-58.

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., Wei, S., Hao, W., Kilgore, J., Williams, N. S., Roth, M. G., Amatruda, J. F., Chen, C. and Lum, L. (2009). "Small molecule-

mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer." Nat Chem Biol **5**(2): 100-107.

Chen, D., Zhao, J., Wang, H., An, N., Zhou, Y., Fan, J., Luo, J., Su, W., Liu, C. and Li, J. (2015). "Oxytocin evokes a pulsatile PGE2 release from ileum mucosa and is required for repair of intestinal epithelium after injury." Sci Rep **5**: 11731.

Chen, W., Paulus, B., Shu, D., Wilson and Chadwick, V. (2001). "Increased serum levels of eotaxin in patients with inflammatory bowel disease." Scand J Gastroenterol **36**(5): 515-520.

Cheng, H. and Leblond, C. P. (1974). "Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine III. Entero-endocrine cells." American Journal of Anatomy **141**(4): 503-519.

Chomarat, P., Banchereau, J., Davoust, J. and Palucka, A. K. (2000). "IL-6 switches the differentiation of monocytes from dendritic cells to macrophages." Nat Immunol **1**(6): 510-514.

Chu, V. T., Beller, A., Rausch, S., Strandmark, J., Zanker, M., Arbach, O., Kruglov, A. and Berek, C. (2014). "Eosinophils promote generation and maintenance of immunoglobulin-A-expressing plasma cells and contribute to gut immune homeostasis." Immunity **40**(4): 582-593.

Chu, V. T., Frohlich, A., Steinhauser, G., Scheel, T., Roch, T., Fillatreau, S., Lee, J. J., Lohning, M. and Berek, C. (2011). "Eosinophils are required for the maintenance of plasma cells in the bone marrow." Nat Immunol **12**(2): 151-159.

Coeffier, M., Marion, R., Leplingard, A., Lerebours, E., Ducrotte, P. and Dechelotte, P. (2002). "Glutamine decreases interleukin-8 and interleukin-6 but not nitric oxide and prostaglandins e(2) production by human gut in-vitro." Cytokine **18**(2): 92-97.

Conroy, D. M. and Williams, T. J. (2001). "Eotaxin and the attraction of eosinophils to the asthmatic lung." Respir Res **2**(3): 150-156.

Cristina, M. L., Lehy, T., Zeitoun, P. and Dufougeray, F. (1978). "Fine structural classification and comparative distribution of endocrine cells in normal human large intestine." Gastroenterology **75**(1): 20-28.

Davies, P. S., Dismuke, A. D., Powell, A. E., Carroll, K. H. and Wong, M. H. (2008). "Wnt-reporter expression pattern in the mouse intestine during homeostasis." BMC Gastroenterol **8**: 57.

Debock, I., Delbauve, S., Dubois, A., Petein, M., Leo, O., Goldman, M. and Flamand, V. (2012). "Th17 alloimmunity prevents neonatal establishment of lymphoid chimerism in IL-4-deprived mice." Am J Transplant **12**(1): 81-89.

Desreumaux, P., Nutten, S. and Colombel, J. F. (1999). "Activated eosinophils in inflammatory bowel disease: do they matter?" Am J Gastroenterol **94**(12): 3396-3398.

Dey, I., Giembycz, M. A. and Chadee, K. (2009). "Prostaglandin E(2) couples through EP(4) prostanoid receptors to induce IL-8 production in human colonic epithelial cell lines." Br J Pharmacol **156**(3): 475-485.

Dharmani, P., Srivastava, V., Kissoon-Singh, V. and Chadee, K. (2009). "Role of intestinal mucins in innate host defense mechanisms against pathogens." J Innate Immun **1**(2): 123-135.

Di Gregorio, G. B., Hensley, L., Lu, T., Ranganathan, G. and Kern, P. A. (2004). "Lipid and carbohydrate metabolism in mice with a targeted mutation in the IL-6 gene: absence of development of age-related obesity." Am J Physiol Endocrinol Metab **287**(1): E182-187.

Diehl, S. and Rincon, M. (2002). "The two faces of IL-6 on Th1/Th2 differentiation." Mol Immunol **39**(9): 531-536.

Dienz, O., Eaton, S. M., Bond, J. P., Neveu, W., Moquin, D., Noubade, R., Briso, E. M., Charland, C., Leonard, W. J., Ciliberto, G., Teuscher, C., Haynes, L. and Rincon, M. (2009). "The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells." J Exp Med **206**(1): 69-78.

Dignass, A. U. and Sturm, A. (2001). "Peptide growth factors in the intestine." Eur J Gastroenterol Hepatol **13**(7): 763-770.

Dilosa, R. M., Maeda, K., Masuda, A., Szakal, A. K. and Tew, J. G. (1991). "Germinal center B cells and antibody production in the bone marrow." J Immunol **146**(12): 4071-4077.

Dohadwala, M., Batra, R. K., Luo, J., Lin, Y., Krysan, K., Pold, M., Sharma, S. and Dubinett, S. M. (2002). "Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion." J Biol Chem **277**(52): 50828-50833.

Drastich, P., Frolova-Brizova, L., Zanvit, P., Spicak, J. and Tlaskalova-Hogenova, H. (2011). "Spontaneous in vitro IL-6 production in various intestinal segments in patients with inflammatory bowel disease." Folia Microbiol (Praha) **56**(3): 185-190.

Driehuis, E. and Clevers, H. (2017). "CRISPR/Cas 9 genome editing and its applications in organoids." Am J Physiol Gastrointest Liver Physiol **312**(3): G257-G265.

Durand, A., Donahue, B., Peignon, G., Letourneur, F., Cagnard, N., Slomianny, C., Perret, C., Shroyer, N. F. and Romagnolo, B. (2012). "Functional intestinal stem cells after Paneth cell

ablation induced by the loss of transcription factor Math1 (Atoh1)." Proc Natl Acad Sci U S A **109**(23): 8965-8970.

Eddahri, F., Denanglaire, S., Bureau, F., Spolski, R., Leonard, W. J., Leo, O. and Andris, F. (2009). "Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities." Blood **113**(11): 2426-2433.

Emery, P., Keystone, E., Tony, H. P., Cantagrel, A., van Vollenhoven, R., Sanchez, A., Alecock, E., Lee, J. and Kremer, J. (2008). "IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial." Ann Rheum Dis **67**(11): 1516-1523.

Ernst, M., Thiem, S., Nguyen, P. M., Eissmann, M. and Putoczki, T. L. (2014). "Epithelial gp130/Stat3 functions: an intestinal signaling node in health and disease." Semin Immunol **26**(1): 29-37.

Ethier, C., Lacy, P. and Davoine, F. (2014). "Identification of human eosinophils in whole blood by flow cytometry." Methods Mol Biol **1178**: 81-92.

Falcone, D. J., Sakamoto, K., Steenport, M. L., Faisal Khan, K. M., Du, B. and Dannenberg, A. J. (2007). "Interleukin 6 stimulates macrophage MMP-9 expression via COX-2-dependent induction of PGE2 synthesis and engagement of the EP4 receptor." FASEB J **21**((Meeting Abstract Supplement) A193).

Fanning, A. S., Jameson, B. J., Jesaitis, L. A. and Anderson, J. M. (1998). "The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton." J Biol Chem **273**(45): 29745-29753.

Farahi, N., Cowburn, A. S., Upton, P. D., Deighton, J., Sobolewski, A., Gherardi, E., Morrell, N. W. and Chilvers, E. R. (2007). "Eotaxin-1/CC chemokine ligand 11: a novel eosinophil survival factor secreted by human pulmonary artery endothelial cells." J Immunol **179**(2): 1264-1273.

Farin, H. F., Jordens, I., Mosa, M. H., Basak, O., Korving, J., Tauriello, D. V., de Punder, K., Angers, S., Peters, P. J., Maurice, M. M. and Clevers, H. (2016). "Visualization of a short-range Wnt gradient in the intestinal stem-cell niche." Nature **530**(7590): 340-343.

Fischer, M., Goldschmitt, J., Peschel, C., Brakenhoff, J. P., Kallen, K. J., Wollmer, A., Grotzinger, J. and Rose-John, S. (1997). "I. A bioactive designer cytokine for human hematopoietic progenitor cell expansion." Nat Biotechnol **15**(2): 142-145.

Fischkoff, S. A. (1988). "Graded increase in probability of eosinophilic differentiation of HL-60 promyelocytic leukemia cells induced by culture under alkaline conditions." Leuk Res **12**(8): 679-686.

Fischkoff, S. A., Brown, G. E. and Pollak, A. (1986). "Synthesis of eosinophil-associated enzymes in HL-60 promyelocytic leukemia cells." Blood **68**(1): 185-192.

Flint, H. J., Scott, K. P., Louis, P. and Duncan, S. H. (2012). "The role of the gut microbiota in nutrition and health." Nat Rev Gastroenterol Hepatol **9**(10): 577-589.

Flossmann, E., Rothwell, P. M., British Doctors Aspirin, T. and the, U. K. T. I. A. A. T. (2007). "Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies." Lancet **369**(9573): 1603-1613.

Fu, C. H., Tsai, W. C., Lee, T. J., Huang, C. C., Chang, P. H. and Su Pang, J. H. (2016). "Simvastatin Inhibits IL-5-Induced Chemotaxis and CCR3 Expression of HL-60-Derived and Human Primary Eosinophils." PLoS One **11**(6): e0157186.

Fujino, H., West, K. A. and Regan, J. W. (2002). "Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2." J Biol Chem **277**(4): 2614-2619.

Fujisawa, T., Kato, Y., Atsuta, J., Terada, A., Iguchi, K., Kamiya, H., Yamada, H., Nakajima, T., Miyamasu, M. and Hirai, K. (2000). "Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines." J Allergy Clin Immunol **105**(1 Pt 1): 126-133.

Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., Nast, C. C., Lechago, J., Xu, R., Naiki, Y., Soliman, A., Arditi, M. and Abreu, M. T. (2005). "Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis." Am J Physiol Gastrointest Liver Physiol **288**(5): G1055-1065.

Fukuyo, S., Yamaoka, K., Sonomoto, K., Oshita, K., Okada, Y., Saito, K., Yoshida, Y., Kanazawa, T., Minami, Y. and Tanaka, Y. (2014). "IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent." Rheumatology (Oxford) **53**(7): 1282-1290.

Funk, C. D., Furci, L., FitzGerald, G. A., Grygorczyk, R., Rochette, C., Bayne, M. A., Abramovitz, M., Adam, M. and Metters, K. M. (1993). "Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype." J Biol Chem **268**(35): 26767-26772.

Furuta, G. T., Nieuwenhuis, E. E., Karhausen, J., Gleich, G., Blumberg, R. S., Lee, J. J. and Ackerman, S. J. (2005). "Eosinophils alter colonic epithelial barrier function: role for major basic protein." Am J Physiol Gastrointest Liver Physiol **289**(5): G890-897.

Gan, L., Qiu, Z., Huang, J., Li, Y., Huang, H., Xiang, T., Wan, J., Hui, T., Lin, Y., Li, H. and Ren, G. (2016). "Cyclooxygenase-2 in tumor-associated macrophages promotes metastatic potential of breast cancer cells through Akt pathway." Int J Biol Sci **12**(12): 1533-1543.

Gao, S. P., Mark, K. G., Leslie, K., Pao, W., Motoi, N., Gerald, W. L., Travis, W. D., Bornmann, W., Veach, D., Clarkson, B. and Bromberg, J. F. (2007). "Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas." J Clin Invest **117**(12): 3846-3856.

Garay, P. A. and McAllister, A. K. (2010). "Novel roles for immune molecules in neural development: implications for neurodevelopmental disorders." Front Synaptic Neurosci **2**: 136.

Garbers, C., Aparicio-Siegmund, S. and Rose-John, S. (2015). "The IL-6/gp130/STAT3 signaling axis: recent advances towards specific inhibition." Curr Opin Immunol **34**: 75-82.

Garrod, D. and Chidgey, M. (2008). "Desmosome structure, composition and function." Biochim Biophys Acta **1778**(3): 572-587.

Gerbe, F., Legraverend, C. and Jay, P. (2012). "The intestinal epithelium tuft cells: specification and function." Cell Mol Life Sci **69**(17): 2907-2917.

Gerbe, F., van Es, J. H., Makrini, L., Brulin, B., Mellitzer, G., Robine, S., Romagnolo, B., Shroyer, N. F., Bourgaux, J. F., Pignodel, C., Clevers, H. and Jay, P. (2011). "Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium." J Cell Biol **192**(5): 767-780.

Gil, D. W., Cheevers, C. V. and Donello, J. E. (2008). "Transient allodynia pain models in mice for early assessment of analgesic activity." Br J Pharmacol **153**(4): 769-774.

Giraud, A. S., Jackson, C., Menheniott, T. R. and Judd, L. M. (2007). "Differentiation of the Gastric Mucosa IV. Role of trefoil peptides and IL-6 cytokine family signaling in gastric homeostasis." Am J Physiol Gastrointest Liver Physiol **292**(1): G1-5.

Gleich, G. J., Frigas, E., Loegering, D. A., Wassom, D. L. and Steinmuller, D. (1979). "Cytotoxic properties of the eosinophil major basic protein." J Immunol **123**(6): 2925-2927.

Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-Cooper, C. L., Weidinger, G., Puder, M., Daley, G. Q., Moon, R. T. and Zon, L. I. (2009). "Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration." Cell **136**(6): 1136-1147.

Gounni, A. S., Gregory, B., Nutku, E., Aris, F., Latifa, K., Minshall, E., North, J., Tavernier, J., Levit, R., Nicolaides, N., Robinson, D. and Hamid, Q. (2000). "Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils." Blood **96**(6): 2163-2171.

Gouon-Evans, V., Rothenberg, M. E. and Pollard, J. W. (2000). "Postnatal mammary gland development requires macrophages and eosinophils." Development **127**(11): 2269-2282.

Gout, T., Ostor, A. J. and Nisar, M. K. (2011). "Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: a systematic literature review." Clin Rheumatol **30**(11): 1471-1474.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G. Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L. and Karin, M. (2009). "IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer." Cancer Cell **15**(2): 103-113.

Gross, J. C., Chaudhary, V., Bartscherer, K. and Boutros, M. (2012). "Active Wnt proteins are secreted on exosomes." Nat Cell Biol **14**(10): 1036-1045.

Gum, J. R., Jr., Hicks, J. W., Toribara, N. W., Siddiki, B. and Kim, Y. S. (1994). "Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor." J Biol Chem **269**(4): 2440-2446.

Gunawardene, A. R., Corfe, B. M. and Staton, C. A. (2011). "Classification and functions of enteroendocrine cells of the lower gastrointestinal tract." Int J Exp Pathol **92**(4): 219-231.

Gustafsson, A., Hansson, E., Kressner, U., Nordgren, S., Andersson, M., Wang, W., Lonnroth, C. and Lundholm, K. (2007). "EP1-4 subtype, COX and PPAR gamma receptor expression in colorectal cancer in prediction of disease-specific mortality." Int J Cancer **121**(2): 232-240.

Hansson, G. C. and Johansson, M. E. (2010). "The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria." Gut Microbes **1**(1): 51-54.

Hao, W. L. and Lee, Y. K. (2004). "Microflora of the gastrointestinal tract: a review." Methods Mol Biol **268**: 491-502.

Hara, M., Nagasaki, T., Shiga, K., Takahashi, H. and Takeyama, H. (2017). "High serum levels of interleukin-6 in patients with advanced or metastatic colorectal cancer: the effect on the outcome and the response to chemotherapy plus bevacizumab." Surg Today **47**(4): 483-489.

Haramis, A. P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G. J. and Clevers, H. (2004). "De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine." Science **303**(5664): 1684-1686.

Hargreaves, D. C., Hyman, P. L., Lu, T. T., Ngo, V. N., Bidgol, A., Suzuki, G., Zou, Y. R., Littman, D. R. and Cyster, J. G. (2001). "A coordinated change in chemokine responsiveness guides plasma cell movements." J Exp Med **194**(1): 45-56.

Harris, S. G., Padilla, J., Koumas, L., Ray, D. and Phipps, R. P. (2002). "Prostaglandins as modulators of immunity." Trends Immunol **23**(3): 144-150.

Hartman, Z. C., Yang, X. Y., Glass, O., Lei, G., Osada, T., Dave, S. S., Morse, M. A., Clay, T. M. and Lyerly, H. K. (2011). "HER2 overexpression elicits a proinflammatory IL-6 autocrine signaling loop that is critical for tumorigenesis." Cancer Res **71**(13): 4380-4391.

Haskell, M. D., Moy, J. N., Gleich, G. J. and Thomas, L. L. (1995). "Analysis of signaling events associated with activation of neutrophil superoxide anion production by eosinophil granule major basic protein." Blood **86**(12): 4627-4637.

Hauser, A. E., Debes, G. F., Arce, S., Cassese, G., Hamann, A., Radbruch, A. and Manz, R. A. (2002). "Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response." J Immunol **169**(3): 1277-1282.

Hawcroft, G., Ko, C. W. and Hull, M. A. (2007). "Prostaglandin E2-EP4 receptor signalling promotes tumorigenic behaviour of HT-29 human colorectal cancer cells." Oncogene **26**(21): 3006-3019.

He, X. C., Zhang, J., Tong, W. G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M., Mishina, Y. and Li, L. (2004). "BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling." Nat Genet **36**(10): 1117-1121.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K. H. and Kishimoto, T. (1985). "Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2)." Proc Natl Acad Sci U S A **82**(16): 5490-5494.

Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S. and Ichikawa, A. (1999). "Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2)." Proc Natl Acad Sci U S A **96**(18): 10501-10506.

Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P., Kay, A. B. and Rothenberg, M. E. (2008). "Eosinophils: biological properties and role in health and disease." Clin Exp Allergy **38**(5): 709-750.

Hunter, C. A. and Jones, S. A. (2015). "IL-6 as a keystone cytokine in health and disease." Nat Immunol **16**(5): 448-457.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998). "Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin." EMBO J **17**(5): 1371-1384.

Ikegami, R., Sugimoto, Y., Segi, E., Katsuyama, M., Karahashi, H., Amano, F., Maruyama, T., Yamane, H., Tsuchiya, S. and Ichikawa, A. (2001). "The expression of prostaglandin E receptors EP2 and EP4 and their different regulation by lipopolysaccharide in C3H/HeN peritoneal macrophages." J Immunol **166**(7): 4689-4696.

Ishikawa, T. O., Oshima, M. and Herschman, H. R. (2011). "Cox-2 deletion in myeloid and endothelial cells, but not in epithelial cells, exacerbates murine colitis." Carcinogenesis **32**(3): 417-426.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K. C., Santee, C. A., Lynch, S. V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K. and Littman, D. R. (2009). "Induction of intestinal Th17 cells by segmented filamentous bacteria." Cell **139**(3): 485-498.

Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R. (2006). "The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells." Cell **126**(6): 1121-1133.

Jacobsen, E. A., Ochkur, S. I., Pero, R. S., Taranova, A. G., Protheroe, C. A., Colbert, D. C., Lee, N. A. and Lee, J. J. (2008). "Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells." J Exp Med **205**(3): 699-710.

Jarnicki, A., Putoczki, T. and Ernst, M. (2010). "Stat3: linking inflammation to epithelial cancer - more than a "gut" feeling?" Cell Div **5**: 14.

Jawien, J., Chlopicki, S., Olszanecki, R., Lorkowska, B. and Gryglewski, R. J. (2002). "Eosinophil-epithelial cell interaction augments cysteinyl leukotrienes synthesis." J Physiol Pharmacol **53**(1): 127-132.

Jedrzkiewicz, S., Nakamura, H., Silverman, E. S., Luster, A. D., Mansharamani, N., In, K. H., Tamura, G. and Lilly, C. M. (2000). "IL-1beta induces eotaxin gene transcription in A549 airway epithelial cells through NF-kappaB." Am J Physiol Lung Cell Mol Physiol **279**(6): L1058-1065.

Jiang, H., Patel, P. H., Kohlmaier, A., Grenley, M. O., McEwen, D. G. and Edgar, B. A. (2009). "Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut." Cell **137**(7): 1343-1355.

Jin, X., Zimmers, T. A., Zhang, Z., Pierce, R. H. and Koniaris, L. G. (2010). "Interleukin-6 is an important in vivo inhibitor of intestinal epithelial cell death in mice." Gut **59**(2): 186-196.

Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L. and Hansson, G. C. (2008). "The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria." Proc Natl Acad Sci U S A **105**(39): 15064-15069.

Johansson, M. E., Sjovall, H. and Hansson, G. C. (2013). "The gastrointestinal mucus system in health and disease." Nat Rev Gastroenterol Hepatol **10**(6): 352-361.

Johnson, L. (2013). Gastrointestinal Physiology.

Jones, G. W., McLoughlin, R. M., Hammond, V. J., Parker, C. R., Williams, J. D., Malhotra, R., Scheller, J., Williams, A. S., Rose-John, S., Topley, N. and Jones, S. A. (2010). "Loss of CD4+ T cell IL-6R expression during inflammation underlines a role for IL-6 trans signaling in the local maintenance of Th17 cells." J Immunol **184**(4): 2130-2139.

Jones, S. A., Richards, P. J., Scheller, J. and Rose-John, S. (2005). "IL-6 transsignaling: the in vivo consequences." J Interferon Cytokine Res **25**(5): 241-253.

Jostock, T., Mullberg, J., Ozbek, S., Atreya, R., Blinn, G., Voltz, N., Fischer, M., Neurath, M. F. and Rose-John, S. (2001). "Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses." Eur J Biochem **268**(1): 160-167.

Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. and Kagnoff, M. F. (1995). "A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion." J Clin Invest **95**(1): 55-65.

Jung, P., Sato, T., Merlos-Suarez, A., Barriga, F. M., Iglesias, M., Rossell, D., Auer, H., Gallardo, M., Blasco, M. A., Sancho, E., Clevers, H. and Batlle, E. (2011). "Isolation and in vitro expansion of human colonic stem cells." Nat Med **17**(10): 1225-1227.

Juteau, H., Gareau, Y., Labelle, M., Sturino, C. F., Sawyer, N., Tremblay, N., Lamontagne, S., Carriere, M. C., Denis, D. and Metters, K. M. (2001). "Structure-activity relationship of cinnamic acylsulfonamide analogues on the human EP3 prostanoid receptor." Bioorg Med Chem **9**(8): 1977-1984.

Kabashima, K., Saji, T., Murata, T., Nagamachi, M., Matsuoka, T., Segi, E., Tsuboi, K., Sugimoto, Y., Kobayashi, T., Miyachi, Y., Ichikawa, A. and Narumiya, S. (2002). "The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut." J Clin Invest **109**(7): 883-893.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K. and Perrimon, N. (1996). "The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing." Genes Dev **10**(24): 3116-3128.

Kalinski, P. (2012). "Regulation of immune responses by prostaglandin E2." J Immunol **188**(1): 21-28.

Kamiyama, M., Pozzi, A., Yang, L., DeBusk, L. M., Breyer, R. M. and Lin, P. C. (2006). "EP2, a receptor for PGE2, regulates tumor angiogenesis through direct effects on endothelial cell motility and survival." Oncogene **25**(53): 7019-7028.

Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A. and Farnarier, C. (2003). "IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation." Trends Immunol **24**(1): 25-29.

Kato, M., Ishioka, T., Kita, H., Kozawa, K., Hayashi, Y. and Kimura, H. (2012). "Eosinophil granular proteins damage bronchial epithelial cells infected with respiratory syncytial virus." Int Arch Allergy Immunol **158 Suppl 1**: 11-18.

Katsuyama, M., Nishigaki, N., Sugimoto, Y., Morimoto, K., Negishi, M., Narumiya, S. and Ichikawa, A. (1995). "The mouse prostaglandin E receptor EP2 subtype: cloning, expression, and northern blot analysis." FEBS Lett **372**(2-3): 151-156.

Kawamori, T., Kitamura, T., Watanabe, K., Uchiya, N., Maruyama, T., Narumiya, S., Sugimura, T. and Wakabayashi, K. (2005). "Prostaglandin E receptor subtype EP(1) deficiency inhibits colon cancer development." Carcinogenesis **26**(2): 353-357.

Keysselt, K., Kreutzmann, T., Rother, K., Kerner, C., Krohn, K., Przybilla, J., Buske, P., Loffler-Wirth, H., Loeffler, M., Galle, J. and Aust, G. (2017). "Different in vivo and in vitro transformation of intestinal stem cells in mismatch repair deficiency." Oncogene **36**(19): 2750-2761.

Khmaladze, I., Kelkka, T., Guerard, S., Wing, K., Pizzolla, A., Saxena, A., Lundqvist, K., Holmdahl, M., Nandakumar, K. S. and Holmdahl, R. (2014). "Mannan induces ROS-regulated, IL-17A-dependent psoriasis arthritis-like disease in mice." Proc Natl Acad Sci U S A **111**(35): E3669-3678.

Kiesslich, R., Goetz, M., Angus, E. M., Hu, Q., Guan, Y., Potten, C., Allen, T., Neurath, M. F., Shroyer, N. F., Montrose, M. H. and Watson, A. J. (2007). "Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy." Gastroenterology **133**(6): 1769-1778.

Kim, H. J., Alonzo, E. S., Dorothee, G., Pollard, J. W. and Sant'Angelo, D. B. (2010). "Selective depletion of eosinophils or neutrophils in mice impacts the efficiency of apoptotic cell clearance in the thymus." PLoS One **5**(7): e11439.

Kim, K. A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P., Funk, W. D. and Tomizuka, K. (2005). "Mitogenic influence of human R-spondin1 on the intestinal epithelium." Science **309**(5738): 1256-1259.

Kim, S. H., Park, Y. Y., Kim, S. W., Lee, J. S., Wang, D. and DuBois, R. N. (2011). "ANGPTL4 induction by prostaglandin E2 under hypoxic conditions promotes colorectal cancer progression." Cancer Res **71**(22): 7010-7020.

Kim, T. H., Escudero, S. and Shivdasani, R. A. (2012). "Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells." Proc Natl Acad Sci U S A **109**(10): 3932-3937.

Kim, Y. S. and Ho, S. B. (2010). "Intestinal goblet cells and mucins in health and disease: recent insights and progress." Curr Gastroenterol Rep **12**(5): 319-330.

King, J. B., von Furstenberg, R. J., Smith, B. J., McNaughton, K. K., Galanko, J. A. and Henning, S. J. (2012). "CD24 can be used to isolate Lgr5+ putative colonic epithelial stem cells in mice." Am J Physiol Gastrointest Liver Physiol **303**(4): G443-452.

Kinzler, K. W. and Vogelstein, B. (1996). "Lessons from hereditary colorectal cancer." Cell **87**(2): 159-170.

Kiriyama, M., Ushikubi, F., Kobayashi, T., Hirata, M., Sugimoto, Y. and Narumiya, S. (1997). "Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells." Br J Pharmacol **122**(2): 217-224.

Kitamura, T., Itoh, M., Noda, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., Ohuchida, S., Sugimura, T. and Wakabayashi, K. (2003). "Combined effects of prostaglandin E receptor subtype EP1 and subtype EP4 antagonists on intestinal tumorigenesis in adenomatous polyposis coli gene knockout mice." Cancer Sci **94**(7): 618-621.

Kleinewietfeld, M. and Hafler, D. A. (2014). "Regulatory T cells in autoimmune neuroinflammation." Immunol Rev **259**(1): 231-244.

Knupfer, H. and Preiss, R. (2010). "Serum interleukin-6 levels in colorectal cancer patients--a summary of published results." Int J Colorectal Dis **25**(2): 135-140.

Komiya, Y. and Habas, R. (2008). "Wnt signal transduction pathways." Organogenesis **4**(2): 68-75.

Koo, B. K., Spit, M., Jordens, I., Low, T. Y., Stange, D. E., van de Wetering, M., van Es, J. H., Mohammed, S., Heck, A. J., Maurice, M. M. and Clevers, H. (2012). "Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors." Nature **488**(7413): 665-669.

Koo, B. K., Stange, D. E., Sato, T., Karthaus, W., Farin, H. F., Huch, M., van Es, J. H. and Clevers, H. (2011). "Controlled gene expression in primary Lgr5 organoid cultures." Nat Methods **9**(1): 81-83.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. and Clevers, H. (1998). "Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4." Nat Genet **19**(4): 379-383.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). "Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma." Science **275**(5307): 1784-1787.

Krausova, M. and Korinek, V. (2014). "Wnt signaling in adult intestinal stem cells and cancer." Cell Signal **26**(3): 570-579.

Kugler, D. G., Mittelstadt, P. R., Ashwell, J. D., Sher, A. and Jankovic, D. (2013). "CD4+ T cells are trigger and target of the glucocorticoid response that prevents lethal immunopathology in toxoplasma infection." J Exp Med **210**(10): 1919-1927.

Kuhn, K. A., Manieri, N. A., Liu, T. C. and Stappenbeck, T. S. (2014). "IL-6 stimulates intestinal epithelial proliferation and repair after injury." PLoS One **9**(12): e114195.

Kuhnert, F., Davis, C. R., Wang, H. T., Chu, P., Lee, M., Yuan, J., Nusse, R. and Kuo, C. J. (2004). "Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1." Proc Natl Acad Sci U S A **101**(1): 266-271.

Laiosa, C. V., Stadtfeld, M. and Graf, T. (2006). "Determinants of lymphoid-myeloid lineage diversification." Annu Rev Immunol **24**: 705-738.

Lamkhioued, B., Renzi, P. M., Abi-Younes, S., Garcia-Zepada, E. A., Allakhverdi, Z., Ghaffar, O., Rothenberg, M. D., Luster, A. D. and Hamid, Q. (1997). "Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation." J Immunol **159**(9): 4593-4601.

Lamouse-Smith, E. S. and Furuta, G. T. (2006). "Eosinophils in the gastrointestinal tract." Curr Gastroenterol Rep **8**(5): 390-395.

Lampinen, M., Ronnblom, A., Amin, K., Kristjansson, G., Rorsman, F., Sangfelt, P., Safsten, B., Wagner, M., Wanders, A., Winqvist, O. and Carlson, M. (2005). "Eosinophil granulocytes are activated during the remission phase of ulcerative colitis." Gut **54**(12): 1714-1720.

Lee, B. C., Kim, H. S., Shin, T. H., Kang, I., Lee, J. Y., Kim, J. J., Kang, H. K., Seo, Y., Lee, S., Yu, K. R., Choi, S. W. and Kang, K. S. (2016). "PGE2 maintains self-renewal of human adult stem cells via EP2-mediated autocrine signaling and its production is regulated by cell-to-cell contact." Sci Rep **6**: 26298.

Lee, J. J., Jacobsen, E. A., McGarry, M. P., Schleimer, R. P. and Lee, N. A. (2010). "Eosinophils in health and disease: the LIAR hypothesis." Clin Exp Allergy **40**(4): 563-575.

Lee, M. J., Lee, J. K., Choi, J. W., Lee, C. S., Sim, J. H., Cho, C. H., Lee, K. H., Cho, I. H., Chung, M. H., Kim, H. R. and Ye, S. K. (2012). "Interleukin-6 induces S100A9 expression in colonic

epithelial cells through STAT3 activation in experimental ulcerative colitis." PLoS One **7**(9): e38801.

Lejeune, M., Moreau, F. and Chadee, K. (2014). "Loss of EP2 receptor subtype in colonic cells compromise epithelial barrier integrity by altering claudin-4." PLoS One **9**(11): e113270.

Levy, A. M., Yamazaki, K., Van Keulen, V. P., Burgart, L. J., Sandborn, W. J., Phillips, S. F., Kephart, G. M., Gleich, G. J. and Leiferman, K. M. (2001). "Increased eosinophil infiltration and degranulation in colonic tissue from patients with collagenous colitis." Am J Gastroenterol **96**(5): 1522-1528.

Li, H., Yang, B., Huang, J., Lin, Y., Xiang, T., Wan, J., Li, H., Chouaib, S. and Ren, G. (2015). "Cyclooxygenase-2 in tumor-associated macrophages promotes breast cancer cell survival by triggering a positive-feedback loop between macrophages and cancer cells." Oncotarget **6**(30): 29637-29650.

Li, Y. Y., Hsieh, L. L., Tang, R. P., Liao, S. K. and Yeh, K. Y. (2009). "Macrophage-derived interleukin-6 up-regulates MUC1, but down-regulates MUC2 expression in the human colon cancer HT-29 cell line." Cell Immunol **256**(1-2): 19-26.

Lievin-Le Moal, V. and Servin, A. L. (2006). "The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota." Clin Microbiol Rev **19**(2): 315-337.

Lilly, C. M., Nakamura, H., Kesselman, H., Nagler-Anderson, C., Asano, K., Garcia-Zepeda, E. A., Rothenberg, M. E., Drazen, J. M. and Luster, A. D. (1997). "Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids." J Clin Invest **99**(7): 1767-1773.

Lindemans, C. A., Calafiore, M., Mertelsmann, A. M., O'Connor, M. H., Dudakov, J. A., Jenq, R. R., Velardi, E., Young, L. F., Smith, O. M., Lawrence, G., Ivanov, J. A., Fu, Y. Y., Takashima, S., Hua, G., Martin, M. L., O'Rourke, K. P., Lo, Y. H., Mokry, M., Romera-Hernandez, M., Cupedo, T., Dow, L. E., Nieuwenhuis, E. E., Shroyer, N. F., Liu, C., Kolesnick, R., van den Brink, M. R. and Hanash, A. M. (2015). "Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration." Nature **528**(7583): 560-564.

Linnskog, R., Jonsson, G., Axelsson, L., Prasad, C. P. and Andersson, T. (2014). "Interleukin-6 drives melanoma cell motility through p38alpha-MAPK-dependent up-regulation of WNT5A expression." Mol Oncol **8**(8): 1365-1378.

Little, F. F., Cruikshank, W. W. and Center, D. M. (2001). "Il-9 stimulates release of chemotactic factors from human bronchial epithelial cells." Am J Respir Cell Mol Biol **25**(3): 347-352.

Liu, J. F., Fong, Y. C., Chang, C. S., Huang, C. Y., Chen, H. T., Yang, W. H., Hsu, C. J., Jeng, L. B., Chen, C. Y. and Tang, C. H. (2010). "Cyclooxygenase-2 enhances alpha2beta1 integrin

expression and cell migration via EP1 dependent signaling pathway in human chondrosarcoma cells." Mol Cancer **9**: 43.

Liu, X., Ji, Q., Ye, N., Sui, H., Zhou, L., Zhu, H., Fan, Z., Cai, J. and Li, Q. (2015). "Berberine Inhibits Invasion and Metastasis of Colorectal Cancer Cells via COX-2/PGE2 Mediated JAK2/STAT3 Signaling Pathway." PLoS One **10**(5): e0123478.

Lotfi, R. and Lotze, M. T. (2008). "Eosinophils induce DC maturation, regulating immunity." J Leukoc Biol **83**(3): 456-460.

Maaser, C. and Kagnoff, M. F. (2002). "Role of the intestinal epithelium in orchestrating innate and adaptive mucosal immunity." Z Gastroenterol **40**(7): 525-529.

Macias-Perez, I. M., Zent, R., Carmosino, M., Breyer, M. D., Breyer, R. M. and Pozzi, A. (2008). "Mouse EP3 alpha, beta, and gamma receptor variants reduce tumor cell proliferation and tumorigenesis in vivo." J Biol Chem **283**(18): 12538-12545.

Mahnke, Y. D., Brodie, T. M., Sallusto, F., Roederer, M. and Lugli, E. (2013). "The who's who of T-cell differentiation: human memory T-cell subsets." Eur J Immunol **43**(11): 2797-2809.

Malysheva, K., de Rooij, K., Lowik, C. W., Baeten, D. L., Rose-John, S., Stoika, R. and Korchynskyi, O. (2016). "Interleukin 6/Wnt interactions in rheumatoid arthritis: interleukin 6 inhibits Wnt signaling in synovial fibroblasts and osteoblasts." Croat Med J **57**(2): 89-98.

Manieri, N. A., Drylewicz, M. R., Miyoshi, H. and Stappenbeck, T. S. (2012). "Igf2bp1 is required for full induction of Ptgs2 mRNA in colonic mesenchymal stem cells in mice." Gastroenterology **143**(1): 110-121 e110.

Manieri, N. A., Mack, M. R., Himmelrich, M. D., Worthley, D. L., Hanson, E. M., Eckmann, L., Wang, T. C. and Stappenbeck, T. S. (2015). "Mucosally transplanted mesenchymal stem cells stimulate intestinal healing by promoting angiogenesis." J Clin Invest **125**(9): 3606-3618.

Marchiando, A. M., Shen, L., Graham, W. V., Edelblum, K. L., Duckworth, C. A., Guan, Y., Montrose, M. H., Turner, J. R. and Watson, A. J. (2011). "The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding." Gastroenterology **140**(4): 1208-1218 e1201-1202.

Marshall, N. B. and Swain, S. L. (2011). "Cytotoxic CD4 T cells in antiviral immunity." J Biomed Biotechnol **2011**: 954602.

Masuda, M., Suzui, M., Yasumatu, R., Nakashima, T., Kuratomi, Y., Azuma, K., Tomita, K., Komiyama, S. and Weinstein, I. B. (2002). "Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma." Cancer Res **62**(12): 3351-3355.

Matsumoto, S., Hara, T., Mitsuyama, K., Yamamoto, M., Tsuruta, O., Sata, M., Scheller, J., Rose-John, S., Kado, S. and Takada, T. (2010). "Essential roles of IL-6 trans-signaling in colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model." J Immunol **184**(3): 1543-1551.

Matsuoka, Y., Furuyashiki, T., Bito, H., Ushikubi, F., Tanaka, Y., Kobayashi, T., Muro, S., Satoh, N., Kayahara, T., Higashi, M., Mizoguchi, A., Shichi, H., Fukuda, Y., Nakao, K. and Narumiya, S. (2003). "Impaired adrenocorticotropic hormone response to bacterial endotoxin in mice deficient in prostaglandin E receptor EP1 and EP3 subtypes." Proc Natl Acad Sci U S A **100**(7): 4132-4137.

Matsuoka, Y., Furuyashiki, T., Yamada, K., Nagai, T., Bito, H., Tanaka, Y., Kitaoka, S., Ushikubi, F., Nabeshima, T. and Narumiya, S. (2005). "Prostaglandin E receptor EP1 controls impulsive behavior under stress." Proc Natl Acad Sci U S A **102**(44): 16066-16071.

Matsushita, T., Maruyama, R., Ishikawa, N., Harada, Y., Araki, A., Chen, D., Tauchi-Nishi, P., Yuki, T. and Kinoshita, Y. (2015). "The number and distribution of eosinophils in the adult human gastrointestinal tract: a study and comparison of racial and environmental factors." Am J Surg Pathol **39**(4): 521-527.

Matthews, J. R., Sansom, O. J. and Clarke, A. R. (2011). "Absolute requirement for STAT3 function in small-intestine crypt stem cell survival." Cell Death Differ **18**(12): 1934-1943.

Matthews, V., Schuster, B., Schutze, S., Bussmeyer, I., Ludwig, A., Hundhausen, C., Sadowski, T., Saftig, P., Hartmann, D., Kallen, K. J. and Rose-John, S. (2003). "Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE)." J Biol Chem **278**(40): 38829-38839.

Matthews, V. B., Allen, T. L., Risis, S., Chan, M. H., Henstridge, D. C., Watson, N., Zaffino, L. A., Babb, J. R., Boon, J., Meikle, P. J., Jowett, J. B., Watt, M. J., Jansson, J. O., Bruce, C. R. and Febbraio, M. A. (2010). "Interleukin-6-deficient mice develop hepatic inflammation and systemic insulin resistance." Diabetologia **53**(11): 2431-2441.

McGuckin, M. A., Eri, R., Simms, L. A., Florin, T. H. and Radford-Smith, G. (2009). "Intestinal barrier dysfunction in inflammatory bowel diseases." Inflamm Bowel Dis **15**(1): 100-113.

Medzhitov, R. and Janeway, C., Jr. (2000). "Innate immune recognition: mechanisms and pathways." Immunol Rev **173**: 89-97.

Melo, R. C., Liu, L., Xenakis, J. J. and Spencer, L. A. (2013). "Eosinophil-derived cytokines in health and disease: unraveling novel mechanisms of selective secretion." Allergy **68**(3): 274-284.

Mihara, M., Hashizume, M., Yoshida, H., Suzuki, M. and Shiina, M. (2012). "IL-6/IL-6 receptor system and its role in physiological and pathological conditions." Clin Sci (Lond) **122**(4): 143-159.

Minami, T., Matsumura, S., Mabuchi, T., Kobayashi, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S. and Ito, S. (2003). "Functional evidence for interaction between prostaglandin EP3 and kappa-opioid receptor pathways in tactile pain induced by human immunodeficiency virus type-1 (HIV-1) glycoprotein gp120." Neuropharmacology **45**(1): 96-105.

Mir, A., Minguez, M., Tatay, J., Pascual, I., Pena, A., Sanchiz, V., Almela, P., Mora, F. and Benages, A. (2002). "Elevated serum eotaxin levels in patients with inflammatory bowel disease." Am J Gastroenterol **97**(6): 1452-1457.

Miyoshi, H., Ajima, R., Luo, C. T., Yamaguchi, T. P. and Stappenbeck, T. S. (2012). "Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury." Science **338**(6103): 108-113.

Miyoshi, H., VanDussen, K. L., Malvin, N. P., Ryu, S. H., Wang, Y., Sonnek, N. M., Lai, C. W. and Stappenbeck, T. S. (2017). "Prostaglandin E2 promotes intestinal repair through an adaptive cellular response of the epithelium." EMBO J **36**(1): 5-24.

Mnich, S. J., Veenhuizen, A. W., Monahan, J. B., Sheehan, K. C., Lynch, K. R., Isakson, P. C. and Portanova, J. P. (1995). "Characterization of a monoclonal antibody that neutralizes the activity of prostaglandin E2." J Immunol **155**(9): 4437-4444.

Morimoto, K., Shirata, N., Taketomi, Y., Tsuchiya, S., Segi-Nishida, E., Inazumi, T., Kabashima, K., Tanaka, S., Murakami, M., Narumiya, S. and Sugimoto, Y. (2014). "Prostaglandin E2-EP3 signaling induces inflammatory swelling by mast cell activation." J Immunol **192**(3): 1130-1137.

Motojima, S., Adachi, T., Manaka, K., Arima, M., Fukuda, T. and Makino, S. (1996). "Eosinophil peroxidase stimulates the release of granulocyte-macrophage colony-stimulating factor from bronchial epithelial cells." J Allergy Clin Immunol **98**(6 Pt 2): S216-223.

Mowat, A. M. and Agace, W. W. (2014). "Regional specialization within the intestinal immune system." Nat Rev Immunol **14**(10): 667-685.

Mudter, J. and Neurath, M. F. (2007). "Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance." Inflamm Bowel Dis **13**(8): 1016-1023.

Munitz, A. and Levi-Schaffer, F. (2004). "Eosinophils: 'new' roles for 'old' cells." Allergy **59**(3): 268-275.

Mutoh, M., Watanabe, K., Kitamura, T., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., Ohuchida, S., Sugimoto, Y., Narumiya, S., Sugimura, T. and Wakabayashi, K. (2002). "Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis." Cancer Res **62**(1): 28-32.

Myung, S. J., Rerko, R. M., Yan, M., Platzer, P., Guda, K., Dotson, A., Lawrence, E., Dannenberg, A. J., Lovgren, A. K., Luo, G., Pretlow, T. P., Newman, R. A., Willis, J., Dawson, D. and Markowitz, S. D. (2006). "15-Hydroxyprostaglandin dehydrogenase is an in vivo suppressor of colon tumorigenesis." Proc Natl Acad Sci U S A **103**(32): 12098-12102.

Nagalakshmi, M. L., Rascle, A., Zurawski, S., Menon, S. and de Waal Malefyt, R. (2004). "Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells." Int Immunopharmacol **4**(5): 679-691.

Najdi, R., Proffitt, K., Sprowl, S., Kaur, S., Yu, J., Covey, T. M., Virshup, D. M. and Waterman, M. L. (2012). "A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities." Differentiation **84**(2): 203-213.

Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A. and Narumiya, S. (1993). "Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity." Nature **365**(6442): 166-170.

Nie, Y., Waite, J., Brewer, F., Sunshine, M. J., Littman, D. R. and Zou, Y. R. (2004). "The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity." J Exp Med **200**(9): 1145-1156.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S. and Hedge, P. (1991). "Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients." Science **253**(5020): 665-669.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., Carotenuto, A., De Feo, G., Caponigro, F. and Salomon, D. S. (2006). "Epidermal growth factor receptor (EGFR) signaling in cancer." Gene **366**(1): 2-16.

Nusse, R. (2012). "Wnt signaling." Cold Spring Harb Perspect Biol **4**(5).

Nusse, R. and Clevers, H. (2017). "Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities." Cell **169**(6): 985-999.

O'Callaghan, G. and Houston, A. (2015). "Prostaglandin E2 and the EP receptors in malignancy: possible therapeutic targets?" Br J Pharmacol **172**(22): 5239-5250.

Obermajer, N. and Kalinski, P. (2012). "Generation of myeloid-derived suppressor cells using prostaglandin E2." Transplant Res **1**(1): 15.

Ogawa, Y., Suzuki, T., Oikawa, A., Hosono, K., Kubo, H., Amano, H., Ito, Y., Kitasato, H., Hayashi, I., Kato, T., Sugimoto, Y., Narumiya, S., Watanabe, M. and Majima, M. (2009). "Bone marrow-derived EP3-expressing stromal cells enhance tumor-associated angiogenesis and tumor growth." Biochem Biophys Res Commun **382**(4): 720-725.

Oguma, K., Oshima, H., Aoki, M., Uchio, R., Naka, K., Nakamura, S., Hirao, A., Saya, H., Taketo, M. M. and Oshima, M. (2008). "Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells." EMBO J **27**(12): 1671-1681.

Oheim, M., Michael, D. J., Geisbauer, M., Madsen, D. and Chow, R. H. (2006). "Principles of two-photon excitation fluorescence microscopy and other nonlinear imaging approaches." Adv Drug Deliv Rev **58**(7): 788-808.

Olsen Hult, L. T., Kleiveland, C. R., Fosnes, K., Jacobsen, M. and Lea, T. (2011). "EP receptor expression in human intestinal epithelium and localization relative to the stem cell zone of the crypts." PLoS One **6**(10): e26816.

Oshima, H., Popivanova, B. K., Oguma, K., Kong, D., Ishikawa, T. O. and Oshima, M. (2011). "Activation of epidermal growth factor receptor signaling by the prostaglandin E(2) receptor EP4 pathway during gastric tumorigenesis." Cancer Sci **102**(4): 713-719.

Osman, D., Buchon, N., Chakrabarti, S., Huang, Y. T., Su, W. C., Poidevin, M., Tsai, Y. C. and Lemaitre, B. (2012). "Autocrine and paracrine unpaired signaling regulate intestinal stem cell maintenance and division." J Cell Sci **125**(Pt 24): 5944-5949.

Otero, K., Vecchi, A., Hirsch, E., Kearley, J., Vermi, W., Del Prete, A., Gonzalvo-Feo, S., Garlanda, C., Azzolino, O., Salogni, L., Lloyd, C. M., Facchetti, F., Mantovani, A. and Sozzani, S. (2010). "Nonredundant role of CCRL2 in lung dendritic cell trafficking." Blood **116**(16): 2942-2949.

Parikh, A. A., Salzman, A. L., Kane, C. D., Fischer, J. E. and Hasselgren, P. O. (1997). "IL-6 production in human intestinal epithelial cells following stimulation with IL-1 beta is associated with activation of the transcription factor NF-kappa B." J Surg Res **69**(1): 139-144.

Parris, A. and Williams, M. R. (2015). "A human colonic crypt culture system to study regulation of stem cell-driven tissue renewal and physiological function." Methods Mol Biol **1212**: 141-161.

Pastorelli, L., De Salvo, C., Mercado, J. R., Vecchi, M. and Pizarro, T. T. (2013). "Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics." Front Immunol **4**: 280.

Pedersen, B. K. and Febbraio, M. A. (2008). "Muscle as an endocrine organ: focus on muscle-derived interleukin-6." Physiol Rev **88**(4): 1379-1406.

Perez-Moreno, M. and Fuchs, E. (2006). "Catenins: keeping cells from getting their signals crossed." Dev Cell **11**(5): 601-612.

Peters, M., Schirmacher, P., Goldschmitt, J., Odenthal, M., Peschel, C., Fattori, E., Ciliberto, G., Dienes, H. P., Meyer zum Buschenfelde, K. H. and Rose-John, S. (1997). "Extramedullary expansion of hematopoietic progenitor cells in interleukin (IL)-6-sIL-6R double transgenic mice." J Exp Med **185**(4): 755-766.

Peterson, L. W. and Artis, D. (2014). "Intestinal epithelial cells: regulators of barrier function and immune homeostasis." Nat Rev Immunol **14**(3): 141-153.

Peugh, J. L. (2010). "A practical guide to multilevel modeling." J Sch Psychol **48**(1): 85-112.

Philpott, A. and Winton, D. J. (2014). "Lineage selection and plasticity in the intestinal crypt." Curr Opin Cell Biol **31**: 39-45.

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H. A., Hirth, S., Weigmann, B., Wirtz, S., Ouyang, W., Neurath, M. F. and Becker, C. (2009). "STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing." J Exp Med **206**(7): 1465-1472.

Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003). "Canonical Wnt signals are essential for homeostasis of the intestinal epithelium." Genes Dev **17**(14): 1709-1713.

Potten, C. S., Owen, G. and Booth, D. (2002). "Intestinal stem cells protect their genome by selective segregation of template DNA strands." J Cell Sci **115**(Pt 11): 2381-2388.

Pull, S. L., Doherty, J. M., Mills, J. C., Gordon, J. I. and Stappenbeck, T. S. (2005). "Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury." Proc Natl Acad Sci U S A **102**(1): 99-104.

Putoczki, T. L., Thiem, S., Loving, A., Busuttil, R. A., Wilson, N. J., Ziegler, P. K., Nguyen, P. M., Preaudet, A., Farid, R., Edwards, K. M., Boglev, Y., Luwor, R. B., Jarnicki, A., Horst, D., Boussioutas, A., Heath, J. K., Sieber, O. M., Pleines, I., Kile, B. T., Nash, A., Greten, F. R., McKenzie, B. S. and Ernst, M. (2013). "Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically." Cancer Cell **24**(2): 257-271.

Quyn, A. J., Appleton, P. L., Carey, F. A., Steele, R. J., Barker, N., Clevers, H., Ridgway, R. A., Sansom, O. J. and Nathke, I. S. (2010). "Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue." Cell Stem Cell **6**(2): 175-181.

Raab, Y., Fredens, K., Gerdin, B. and Hallgren, R. (1998). "Eosinophil activation in ulcerative colitis: studies on mucosal release and localization of eosinophil granule constituents." Dig Dis Sci **43**(5): 1061-1070.

Rabe, B., Chalaris, A., May, U., Waetzig, G. H., Seegert, D., Williams, A. S., Jones, S. A., Rose-John, S. and Scheller, J. (2008). "Transgenic blockade of interleukin 6 transsignaling abrogates inflammation." Blood **111**(3): 1021-1028.

Radbruch, A., Muehlinghaus, G., Luger, E. O., Inamine, A., Smith, K. G., Dorner, T. and Hiepe, F. (2006). "Competence and competition: the challenge of becoming a long-lived plasma cell." Nat Rev Immunol **6**(10): 741-750.

Raetz, C. R. and Whitfield, C. (2002). "Lipopolysaccharide endotoxins." Annu Rev Biochem **71**: 635-700.

Rehman, M. Q., Beal, D., Liang, Y., Noronha, A., Winter, H., Farraye, F. A. and Ganley-Leal, L. (2013). "B cells secrete eotaxin-1 in human inflammatory bowel disease." Inflamm Bowel Dis **19**(5): 922-933.

Rescigno, M. (2011). "The intestinal epithelial barrier in the control of homeostasis and immunity." Trends Immunol **32**(6): 256-264.

Reya, T. and Clevers, H. (2005). "Wnt signalling in stem cells and cancer." Nature **434**(7035): 843-850.

Reynolds, A., Parris, A., Evans, L. A., Lindqvist, S., Sharp, P., Lewis, M., Tighe, R. and Williams, M. R. (2007). "Dynamic and differential regulation of NKCC1 by calcium and cAMP in the native human colonic epithelium." J Physiol **582**(Pt 2): 507-524.

Reynolds, A., Wharton, N., Parris, A., Mitchell, E., Sobolewski, A., Kam, C., Bigwood, L., El Hadi, A., Munsterberg, A., Lewis, M., Speakman, C., Stebbings, W., Wharton, R., Sargen, K., Tighe, R., Jamieson, C., Hernon, J., Kapur, S., Oue, N., Yasui, W. and Williams, M. R. (2014). "Canonical Wnt signals combined with suppressed TGFbeta/BMP pathways promote renewal of the native human colonic epithelium." Gut **63**(4): 610-621.

Roderick, H. L. and Cook, S. J. (2008). "Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival." Nat Rev Cancer **8**(5): 361-375.

Rodriguez-Lagunas, M. J., Martin-Venegas, R., Moreno, J. J. and Ferrer, R. (2010). "PGE2 promotes Ca2+-mediated epithelial barrier disruption through EP1 and EP4 receptors in Caco-2 cell monolayers." Am J Physiol Cell Physiol **299**(2): C324-334.

Rose-John, S. (2012). "IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6." Int J Biol Sci **8**(9): 1237-1247.

Rosenberg, H. F., Dyer, K. D. and Foster, P. S. (2013). "Eosinophils: changing perspectives in health and disease." Nat Rev Immunol **13**(1): 9-22.

Rostom, A., Dube, C., Lewin, G., Tsertsvadze, A., Barrowman, N., Code, C., Sampson, M., Moher, D. and Force, U. S. P. S. T. (2007). "Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force." Ann Intern Med **146**(5): 376-389.

Roth, S., Franken, P., Sacchetti, A., Kremer, A., Anderson, K., Sansom, O. and Fodde, R. (2012). "Paneth cells in intestinal homeostasis and tissue injury." PLoS One **7**(6): e38965.

Rothenberg, M. E., Nusse, Y., Kalisky, T., Lee, J. J., Dalerba, P., Scheeren, F., Lobo, N., Kulkarni, S., Sim, S., Qian, D., Beachy, P. A., Pasricha, P. J., Quake, S. R. and Clarke, M. F. (2012). "Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice." Gastroenterology **142**(5): 1195-1205 e1196.

Roulis, M., Armaka, M., Manoloukos, M., Apostolaki, M. and Kollias, G. (2011). "Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology." Proc Natl Acad Sci U S A **108**(13): 5396-5401.

Roulis, M., Nikolaou, C., Kotsaki, E., Kaffe, E., Karagianni, N., Koliaraki, V., Salpea, K., Ragoussis, J., Aidinis, V., Martini, E., Becker, C., Herschman, H. R., Vetrano, S., Danese, S. and Kollias, G. (2014). "Intestinal myofibroblast-specific Tpl2-Cox-2-PGE2 pathway links innate sensing to epithelial homeostasis." Proc Natl Acad Sci U S A.

Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. and Polakis, P. (1993). "Association of the APC gene product with beta-catenin." Science **262**(5140): 1731-1734.

Saad, A. G. (2011). "Normal quantity and distribution of mast cells and eosinophils in the pediatric colon." Pediatr Dev Pathol **14**(4): 294-300.

Sales, K. J., Maudsley, S. and Jabbour, H. N. (2004). "Elevated prostaglandin EP2 receptor in endometrial adenocarcinoma cells promotes vascular endothelial growth factor expression via cyclic 3',5'-adenosine monophosphate-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 signaling pathways." Mol Endocrinol **18**(6): 1533-1545.

Salvi, S., Semper, A., Blomberg, A., Holloway, J., Jaffar, Z., Papi, A., Teran, L., Polosa, R., Kelly, F., Sandstrom, T., Holgate, S. and Frew, A. (1999). "Interleukin-5 production by human airway epithelial cells." Am J Respir Cell Mol Biol **20**(5): 984-991.

Sangiorgi, E. and Capecchi, M. R. (2008). "Bmi1 is expressed in vivo in intestinal stem cells." Nat Genet **40**(7): 915-920.

Sartor, R. B. (2008). "Microbial influences in inflammatory bowel diseases." Gastroenterology **134**(2): 577-594.

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G., Van Es, J. H., Van den Brink, S., Van Houdt, W. J., Pronk, A., Van Gorp, J., Siersema, P. D. and Clevers, H. (2011). "Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium." Gastroenterology **141**(5): 1762-1772.

Sato, T., van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., van den Born, M., Barker, N., Shroyer, N. F., van de Wetering, M. and Clevers, H. (2011). "Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts." Nature **469**(7330): 415-418.

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kujala, P., Peters, P. J. and Clevers, H. (2009). "Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche." Nature **459**(7244): 262-265.

Schaper, F. and Rose-John, S. (2015). "Interleukin-6: Biology, signaling and strategies of blockade." Cytokine Growth Factor Rev **26**(5): 475-487.

Scheller, J., Chalaris, A., Schmidt-Arras, D. and Rose-John, S. (2011). "The pro- and anti-inflammatory properties of the cytokine interleukin-6." Biochim Biophys Acta **1813**(5): 878-888.

Schepers, A. and Clevers, H. (2012). "Wnt signaling, stem cells, and cancer of the gastrointestinal tract." Cold Spring Harb Perspect Biol **4**(4): a007989.

Schust, J., Sperl, B., Hollis, A., Mayer, T. U. and Berg, T. (2006). "Stattic: a small-molecule inhibitor of STAT3 activation and dimerization." Chem Biol **13**(11): 1235-1242.

Schwank, G., Andersson-Rolf, A., Koo, B. K., Sasaki, N. and Clevers, H. (2013). "Generation of BAC transgenic epithelial organoids." PLoS One **8**(10): e76871.

Sedgwick, J. B., Shikama, Y., Nagata, M., Brener, K. and Busse, W. W. (1996). "Effect of isolation protocol on eosinophil function: Percoll gradients versus immunomagnetic beads." J Immunol Methods **198**(1): 15-24.

Seno, H., Miyoshi, H., Brown, S. L., Geske, M. J., Colonna, M. and Stappenbeck, T. S. (2009). "Efficient colonic mucosal wound repair requires Trem2 signaling." Proc Natl Acad Sci U S A **106**(1): 256-261.

Sexton, D. W., Blaylock, M. G. and Walsh, G. M. (2001). "Human alveolar epithelial cells engulf apoptotic eosinophils by means of integrin- and phosphatidylserine receptor-dependent mechanisms: a process upregulated by dexamethasone." J Allergy Clin Immunol **108**(6): 962-969.

Sexton, D. W. and Walsh, G. M. (2002). "Eosinophil-epithelial cell interactions: an important facet of asthmatic inflammation." Clin Exp Allergy **32**(6): 811-813.

Sharma, S., Yang, S. C., Zhu, L., Reckamp, K., Gardner, B., Baratelli, F., Huang, M., Batra, R. K. and Dubinett, S. M. (2005). "Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer." Cancer Res **65**(12): 5211-5220.

Shoji, Y., Takahashi, M., Kitamura, T., Watanabe, K., Kawamori, T., Maruyama, T., Sugimoto, Y., Negishi, M., Narumiya, S., Sugimura, T. and Wakabayashi, K. (2004). "Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development." Gut **53**(8): 1151-1158.

Siegfried, E., Wilder, E. L. and Perrimon, N. (1994). "Components of wingless signalling in Drosophila." Nature **367**(6458): 76-80.

Simmonds, N., Furman, M., Karanika, E., Phillips, A. and Bates, A. W. (2014). "Paneth cell metaplasia in newly diagnosed inflammatory bowel disease in children." BMC Gastroenterol **14**: 93.

Sinha, P., Clements, V. K., Fulton, A. M. and Ostrand-Rosenberg, S. (2007). "Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells." Cancer Res **67**(9): 4507-4513.

Sjolund, K., Sanden, G., Hakanson, R. and Sundler, F. (1983). "Endocrine cells in human intestine: an immunocytochemical study." Gastroenterology **85**(5): 1120-1130.

Skoczek, D. A., Walczysko, P., Horn, N., Parris, A., Clare, S., Williams, M. R. and Sobolewski, A. (2014). "Luminal microbes promote monocyte-stem cell interactions across a healthy colonic epithelium." J Immunol **193**(1): 439-451.

Skurk, T., Alberti-Huber, C., Herder, C. and Hauner, H. (2007). "Relationship between adipocyte size and adipokine expression and secretion." J Clin Endocrinol Metab **92**(3): 1023-1033.

Smith, W. L., DeWitt, D. L. and Garavito, R. M. (2000). "Cyclooxygenases: structural, cellular, and molecular biology." Annu Rev Biochem **69**: 145-182.

Smyth, C. M., Akasheh, N., Woods, S., Kay, E., Morgan, R. K., Thornton, M. A., O'Grady, A., Cummins, R., Sheils, O., Smyth, P., Gleich, G. J., Murray, F. M. and Costello, R. W. (2013). "Activated eosinophils in association with enteric nerves in inflammatory bowel disease." PLoS One **8**(5): e64216.

Snippert, H. J. and Clevers, H. (2011). "Tracking adult stem cells." EMBO Rep **12**(2): 113-122.

Snippert, H. J., van der Flier, L. G., Sato, T., van Es, J. H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A. M., van Rheenen, J., Simons, B. D. and Clevers, H. (2010). "Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells." Cell **143**(1): 134-144.

Snoeck, V., Goddeeris, B. and Cox, E. (2005). "The role of enterocytes in the intestinal barrier function and antigen uptake." Microbes and Infection **7**(7–8): 997-1004.

Solomon, S. D., McMurray, J. J., Pfeffer, M. A., Wittes, J., Fowler, R., Finn, P., Anderson, W. F., Zauber, A., Hawk, E., Bertagnolli, M. and Adenoma Prevention with Celecoxib Study, I. (2005). "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention." N Engl J Med **352**(11): 1071-1080.

Sommer, J., Engelowski, E., Baran, P., Garbers, C., Floss, D. M. and Scheller, J. (2014). "Interleukin-6, but not the interleukin-6 receptor plays a role in recovery from dextran sodium sulfate-induced colitis." Int J Mol Med **34**(3): 651-660.

Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M. and Taketo, M. M. (2001). "Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice." Nat Med **7**(9): 1048-1051.

Sosinsky, G. E. and Nicholson, B. J. (2005). "Structural organization of gap junction channels." Biochim Biophys Acta **1711**(2): 99-125.

Steinbach, K. H., Schick, P., Trepel, F., Raffler, H., Dohrmann, J., Heilgeist, G., Heltzel, W., Li, K., Past, W., van der Woerd-de Lange, J. A., Theml, H., Fliedner, T. M. and Begemann, H. (1979). "Estimation of kinetic parameters of neutrophilic, eosinophilic, and basophilic granulocytes in human blood." Blut **39**(1): 27-38.

Stellato, C., Brummet, M. E., Plitt, J. R., Shahabuddin, S., Baroody, F. M., Liu, M. C., Ponath, P. D. and Beck, L. A. (2001). "Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells." J Immunol **166**(3): 1457-1461.

Stephanou, A., Brar, B. K., Knight, R. A. and Latchman, D. S. (2000). "Opposing actions of STAT-1 and STAT-3 on the Bcl-2 and Bcl-x promoters." Cell Death Differ **7**(3): 329-330.

Stockinger, B. and Veldhoen, M. (2007). "Differentiation and function of Th17 T cells." Curr Opin Immunol **19**(3): 281-286.

Strange, P. G. (2008). "Agonist binding, agonist affinity and agonist efficacy at G protein-coupled receptors." Br J Pharmacol **153**(7): 1353-1363.

Sugimoto, Y., Namba, T., Honda, A., Hayashi, Y., Negishi, M., Ichikawa, A. and Narumiya, S. (1992). "Cloning and expression of a cDNA for mouse prostaglandin E receptor EP3 subtype." J Biol Chem **267**(10): 6463-6466.

Sugimoto, Y. and Narumiya, S. (2007). "Prostaglandin E receptors." J Biol Chem **282**(16): 11613-11617.

Tai, H. H., Ensor, C. M., Tong, M., Zhou, H. and Yan, F. (2002). "Prostaglandin catabolizing enzymes." Prostaglandins Other Lipid Mediat **68-69**: 483-493.

Takasaki, I., Nojima, H., Shiraki, K., Sugimoto, Y., Ichikawa, A., Ushikubi, F., Narumiya, S. and Kuraishi, Y. (2005). "Involvement of cyclooxygenase-2 and EP3 prostaglandin receptor in acute herpetic but not postherpetic pain in mice." Neuropharmacology **49**(3): 283-292.

Tanaka, M., Saito, H., Kusumi, T., Fukuda, S., Shimoyama, T., Sasaki, Y., Suto, K., Munakata, A. and Kudo, H. (2001). "Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease." J Gastroenterol Hepatol **16**(12): 1353-1359.

Tetteh, P. W., Basak, O., Farin, H. F., Wiebrands, K., Kretzschmar, K., Begthel, H., van den Born, M., Korving, J., de Sauvage, F., van Es, J. H., van Oudenaarden, A. and Clevers, H. (2016). "Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters." Cell Stem Cell **18**(2): 203-213.

Tian, H., Biehs, B., Warming, S., Leong, K. G., Rangell, L., Klein, O. D. and de Sauvage, F. J. (2011). "A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable." Nature **478**(7368): 255-259.

Tortora GJ, D. B. (2008). "Principles of Anatomy and Physiology." **Version 12**: 922-926.

Tsukamoto, H., Senju, S., Matsumura, K., Swain, S. L. and Nishimura, Y. (2015). "IL-6-mediated environmental conditioning of defective Th1 differentiation dampens antitumour immune responses in old age." Nat Commun **6**: 6702.

Turner, J. R. (2009). "Intestinal mucosal barrier function in health and disease." Nat Rev Immunol **9**(11): 799-809.

Uller, L., Persson, C. G., Kallstrom, L. and Erjefalt, J. S. (2001). "Lung tissue eosinophils may be cleared through luminal entry rather than apoptosis: effects of steroid treatment." Am J Respir Crit Care Med **164**(10 Pt 1): 1948-1956.

Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M. and Roy, N. C. (2011). "Regulation of tight junction permeability by intestinal bacteria and dietary components." J Nutr **141**(5): 769-776.

Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N. and Narumiya, S. (1998). "Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3." Nature **395**(6699): 281-284.

van Amerongen, R. (2012). "Alternative Wnt pathways and receptors." Cold Spring Harb Perspect Biol **4**(10).

van Dam, M., Mullberg, J., Schooltink, H., Stoyan, T., Brakenhoff, J. P., Graeve, L., Heinrich, P. C. and Rose-John, S. (1993). "Structure-function analysis of interleukin-6 utilizing human/murine chimeric molecules. Involvement of two separate domains in receptor binding." J Biol Chem **268**(20): 15285-15290.

van Es, J. H., Sato, T., van de Wetering, M., Lyubimova, A., Nee, A. N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J., Martens, A. C., Barker, N., van Oudenaarden, A. and Clevers, H. (2012). "Dll1+ secretory progenitor cells revert to stem cells upon crypt damage." Nat Cell Biol **14**(10): 1099-1104.

Vanuytsel, T., Senger, S., Fasano, A. and Shea-Donohue, T. (2013). "Major signaling pathways in intestinal stem cells." Biochim Biophys Acta **1830**(2): 2410-2426.

Vetrano, S., Rescigno, M., Cera, M. R., Correale, C., Rumio, C., Doni, A., Fantini, M., Sturm, A., Borroni, E., Repici, A., Locati, M., Malesci, A., Dejana, E. and Danese, S. (2008). "Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease." Gastroenterology **135**(1): 173-184.

Villarino, A. V., Kanno, Y., Ferdinand, J. R. and O'Shea, J. J. (2015). "Mechanisms of Jak/STAT signaling in immunity and disease." J Immunol **194**(1): 21-27.

Vora, P., Youdim, A., Thomas, L. S., Fukata, M., Tesfay, S. Y., Lukasek, K., Michelsen, K. S., Wada, A., Hirayama, T., Arditi, M. and Abreu, M. T. (2004). "Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells." J Immunol **173**(9): 5398-5405.

Waddell, A., Ahrens, R., Steinbrecher, K., Donovan, B., Rothenberg, M. E., Munitz, A. and Hogan, S. P. (2011). "Colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C(high) CCR2(+) inflammatory monocyte/macrophage-derived CCL11." J Immunol **186**(10): 5993-6003.

Waldner, M. J. and Neurath, M. F. (2014). "Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development." Semin Immunol **26**(1): 75-79.

Wallenius, V., Wallenius, K., Ahren, B., Rudling, M., Carlsten, H., Dickson, S. L., Ohlsson, C. and Jansson, J. O. (2002). "Interleukin-6-deficient mice develop mature-onset obesity." Nat Med **8**(1): 75-79.

Wallon, C., Persborn, M., Jonsson, M., Wang, A., Phan, V., Lampinen, M., Vicario, M., Santos, J., Sherman, P. M., Carlson, M., Ericson, A. C., McKay, D. M. and Soderholm, J. D. (2011). "Eosinophils express muscarinic receptors and corticotropin-releasing factor to disrupt the mucosal barrier in ulcerative colitis." Gastroenterology **140**(5): 1597-1607.

Walsh, G. M. (2001). "Eosinophil-epithelial cell interactions: a special relationship?" Clin Exp Allergy **31**(3): 351-354.

Walsh, G. M., Sexton, D. W., Blaylock, M. G. and Convery, C. M. (1999). "Resting and cytokine-stimulated human small airway epithelial cells recognize and engulf apoptotic eosinophils." Blood **94**(8): 2827-2835.

Walsh, R. E. and Gaginella, T. S. (1991). "The eosinophil in inflammatory bowel disease." Scand J Gastroenterol **26**(12): 1217-1224.

Wang, D. and Dubois, R. N. (2006). "Prostaglandins and cancer." Gut **55**(1): 115-122.

Wang, D. and Dubois, R. N. (2010). "Eicosanoids and cancer." Nat Rev Cancer **10**(3): 181-193.

Wang, D. and Dubois, R. N. (2010). "The role of COX-2 in intestinal inflammation and colorectal cancer." Oncogene **29**(6): 781-788.

Wang, H. B., Ghiran, I., Matthaei, K. and Weller, P. F. (2007). "Airway eosinophils: allergic inflammation recruited professional antigen-presenting cells." J Immunol **179**(11): 7585-7592.

Wang, S. W. and Sun, Y. M. (2014). "The IL-6/JAK/STAT3 pathway: potential therapeutic strategies in treating colorectal cancer (Review)." Int J Oncol **44**(4): 1032-1040.

Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Ito, S., Narumiya, S. and Ichikawa, A. (1993). "Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor." J Biol Chem **268**(27): 20175-20178.

Watson, A. J., Chu, S., Sieck, L., Gerasimenko, O., Bullen, T., Campbell, F., McKenna, M., Rose, T. and Montrose, M. H. (2005). "Epithelial barrier function in vivo is sustained despite gaps in epithelial layers." Gastroenterology **129**(3): 902-912.

Wilson, R. P., Ives, M. L., Rao, G., Lau, A., Payne, K., Kobayashi, M., Arkwright, P. D., Peake, J., Wong, M., Adelstein, S., Smart, J. M., French, M. A., Fulcher, D. A., Picard, C., Bustamante, J., Boisson-Dupuis, S., Gray, P., Stepensky, P., Warnatz, K., Freeman, A. F., Rossjohn, J., McCluskey, J., Holland, S. M., Casanova, J. L., Uzel, G., Ma, C. S., Tangye, S. G. and Deenick, E. K. (2015). "STAT3 is a critical cell-intrinsic regulator of human unconventional T cell numbers and function." J Exp Med **212**(6): 855-864.

Wolk, K., Kunz, S., Witte, E., Friedrich, M., Asadullah, K. and Sabat, R. (2004). "IL-22 increases the innate immunity of tissues." Immunity **21**(2): 241-254.

Woolley, K. L., Gibson, P. G., Carty, K., Wilson, A. J., Twaddell, S. H. and Woolley, M. J. (1996). "Eosinophil apoptosis and the resolution of airway inflammation in asthma." Am J Respir Crit Care Med **154**(1): 237-243.

Wu, D., Molofsky, A. B., Liang, H. E., Ricardo-Gonzalez, R. R., Jouihan, H. A., Bando, J. K., Chawla, A. and Locksley, R. M. (2011). "Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis." Science **332**(6026): 243-247.

Wunderlich, F. T., Strohle, P., Konner, A. C., Gruber, S., Tovar, S., Bronneke, H. S., Juntti-Berggren, L., Li, L. S., van Rooijen, N., Libert, C., Berggren, P. O. and Bruning, J. C. (2010). "Interleukin-6 signaling in liver-parenchymal cells suppresses hepatic inflammation and improves systemic insulin action." Cell Metab **12**(3): 237-249.

Yamamoto, K. and Rose-John, S. (2012). "Therapeutic blockade of interleukin-6 in chronic inflammatory disease." Clin Pharmacol Ther **91**(4): 574-576.

Yan, K. S., Janda, C. Y., Chang, J., Zheng, G. X. Y., Larkin, K. A., Luca, V. C., Chia, L. A., Mah, A. T., Han, A., Terry, J. M., Ootani, A., Roelf, K., Lee, M., Yuan, J., Li, X., Bolen, C. R., Wilhelmy, J., Davies, P. S., Ueno, H., von Furstenberg, R. J., Belgrader, P., Ziraldo, S. B., Ordonez, H., Henning, S. J., Wong, M. H., Snyder, M. P., Weissman, I. L., Hsueh, A. J., Mikkelsen, T. S., Garcia, K. C. and Kuo, C. J. (2017). "Non-equivalence of Wnt and R-spondin ligands during Lgr5+ intestinal stem-cell self-renewal." Nature **545**(7653): 238-242.

Yang, D., Chen, Q., Su, S. B., Zhang, P., Kurosaka, K., Caspi, R. R., Michalek, S. M., Rosenberg, H. F., Zhang, N. and Oppenheim, J. J. (2008). "Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses." J Exp Med **205**(1): 79-90.

Yang, D., Rosenberg, H. F., Chen, Q., Dyer, K. D., Kurosaka, K. and Oppenheim, J. J. (2003). "Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells." Blood **102**(9): 3396-3403.

Yang, S. F., Chen, M. K., Hsieh, Y. S., Chung, T. T., Hsieh, Y. H., Lin, C. W., Su, J. L., Tsai, M. H. and Tang, C. H. (2010). "Prostaglandin E2/EP1 signaling pathway enhances intercellular adhesion molecule 1 (ICAM-1) expression and cell motility in oral cancer cells." J Biol Chem **285**(39): 29808-29816.

Yao, C., Hirata, T., Soontrapa, K., Ma, X., Takemori, H. and Narumiya, S. (2013). "Prostaglandin E(2) promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase." Nat Commun **4**: 1685.

Yokomuro, S., Lunz, J. G., 3rd, Sakamoto, T., Ezure, T., Murase, N. and Demetris, A. J. (2000). "The effect of interleukin-6 (IL-6)/gp130 signalling on biliary epithelial cell growth, in vitro." Cytokine **12**(6): 727-730.

Yousefi, S., Gold, J. A., Andina, N., Lee, J. J., Kelly, A. M., Kozlowski, E., Schmid, I., Straumann, A., Reichenbach, J., Gleich, G. J. and Simon, H. U. (2008). "Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense." Nat Med **14**(9): 949-953.

Yousefi, S., Simon, D. and Simon, H. U. (2012). "Eosinophil extracellular DNA traps: molecular mechanisms and potential roles in disease." Curr Opin Immunol **24**(6): 736-739.

Yu, H., Lee, H., Herrmann, A., Buettner, R. and Jove, R. (2014). "Revisiting STAT3 signalling in cancer: new and unexpected biological functions." Nat Rev Cancer **14**(11): 736-746.

Yu, H., Pardoll, D. and Jove, R. (2009). "STATs in cancer inflammation and immunity: a leading role for STAT3." Nat Rev Cancer **9**(11): 798-809.

Zhang, H., Cheng, S., Zhang, M., Ma, X., Zhang, L., Wang, Y., Rong, R., Ma, J., Xia, S., Du, M., Shi, F., Wang, J., Yang, Q., Bai, X. and Leng, J. (2014). "Prostaglandin E2 promotes hepatocellular carcinoma cell invasion through upregulation of YB-1 protein expression." Int J Oncol **44**(3): 769-780.

Zhang, Y., Desai, A., Yang, S. Y., Bae, K. B., Antczak, M. I., Fink, S. P., Tiwari, S., Willis, J. E., Williams, N. S., Dawson, D. M., Wald, D., Chen, W. D., Wang, Z., Kasturi, L., Larusch, G. A., He, L., Cominelli, F., Di Martino, L., Djuric, Z., Milne, G. L., Chance, M., Sanabria, J., Dealwis, C., Mikkola, D., Naidoo, J., Wei, S., Tai, H. H., Gerson, S. L., Ready, J. M., Posner, B., Willson, J. K. and Markowitz, S. D. (2015). "TISSUE REGENERATION. Inhibition of the prostaglandin-degrading enzyme 15-PGDH potentiates tissue regeneration." Science **348**(6240): aaa2340.

Zhang, Y., Liu, Q., Zhang, M., Yu, Y., Liu, X. and Cao, X. (2009). "Fas signal promotes lung cancer growth by recruiting myeloid-derived suppressor cells via cancer cell-derived PGE2." J Immunol **182**(6): 3801-3808.

Zushi, S., Shinomura, Y., Kiyohara, T., Miyazaki, Y., Kondo, S., Sugimachi, M., Higashimoto, Y., Kanayama, S. and Matsuzawa, Y. (1998). "STAT3 mediates the survival signal in oncogenic ras-transfected intestinal epithelial cells." Int J Cancer **78**(3): 326-330.

# Appendix A. EP Receptor Gradient Intensity Analysis

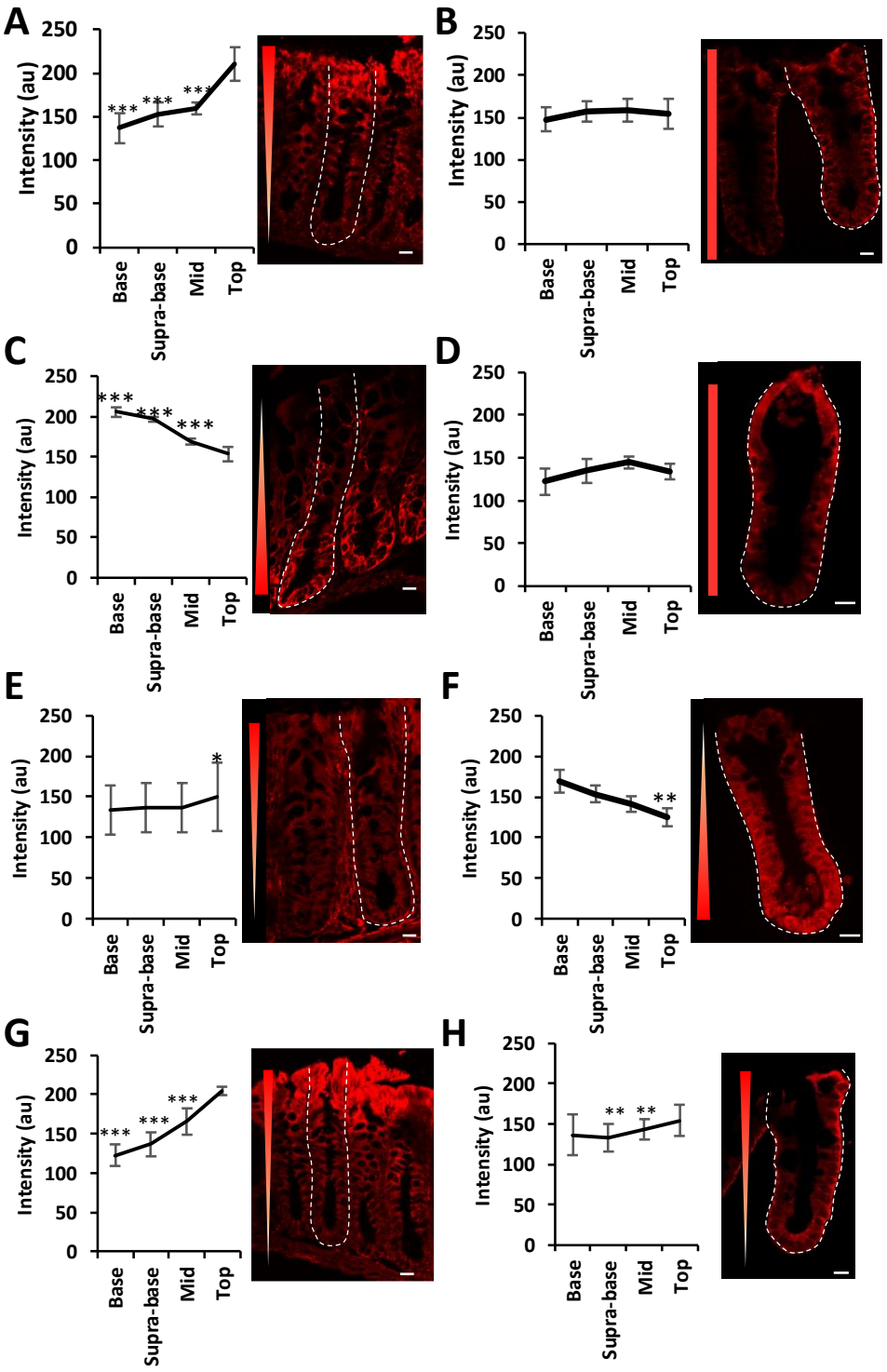


**Figure A.0.1. Analysis of gradient intensity of EP receptor labelling along crypt-axis in vivo and in vitro.**

Histograms (LEFT) and representative fluorescence images (RIGHT) of EP receptor fluorescence intensity of: (**A**) EP1 *in vivo*, (B) EP1 *in vitro*, (C) EP2 *in vitro*, (D) EP2 *in vitro*, (E) EP3 *in vivo*, (F) EP3 *in vitro*, (G) EP4 *in vitro*, (H) EP4 *in vivo*. EP receptor labelling shown in red, white dash denotes crypt outline. (n=3, *P<0.05, **P<0.005, ***P<0.001) scale bar 10µm.

# Appendix B. Chemoattractant markers expressed by isolated immune cells

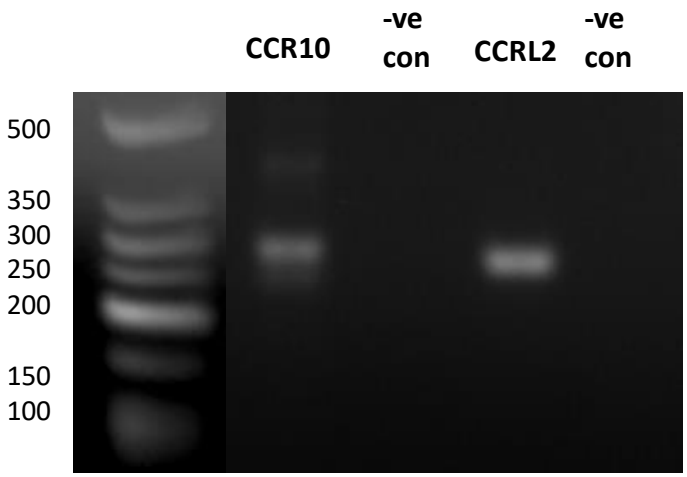


**Figure B.0.1. Immune cells isolated from the colonic lamina propria express markers characteristic of immune cells.**

Expression of immune cell characteristic mRNA by RT-PCR using cDNA from isolated tissue resident immune cells, eosinophil purity was between 55 and 65% for all samples used. Expected PCR band sizes: CCR10=285bp, CCRL2=272bp.

# Appendix C. Monocyte distribution along the crypt axis in vivo

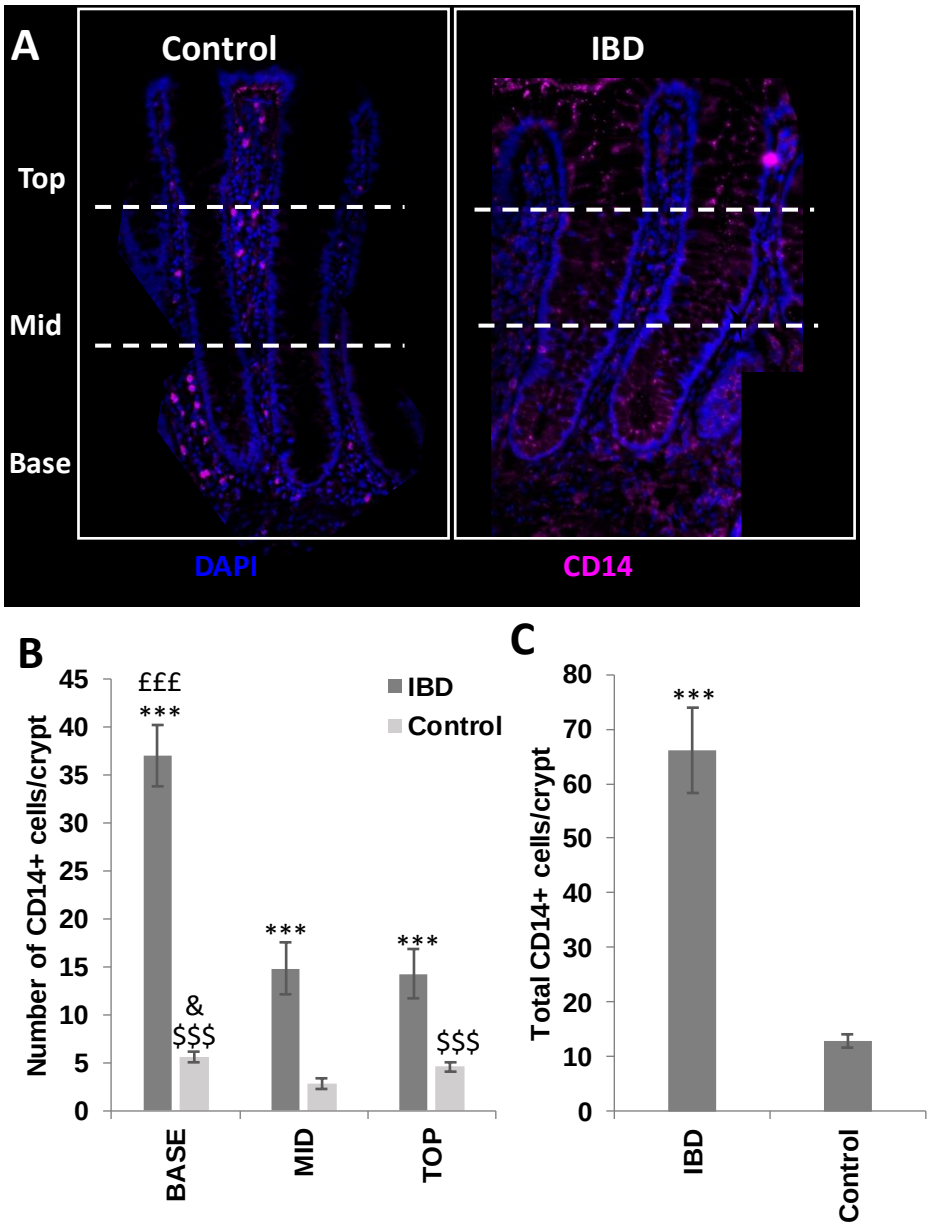


**Figure C.0.1. Characterisation of tissue resident CD14+ cells in the colonic mucosa of healthy and IBD patients.**

(**A**) Immunofluorescent labelling of CD14+ cells (pink) and nuclei (DAPI-blue) in the colonic mucosa of tissue obtained from healthy (control, left) and IBD (right) patients, white dashes represent base, mid and top regions. Images representative of n=3 experiments. (**B**) Histogram showing the number of CD14+ cells per crypt in the base, mid and top regions of IBD and control sections, mean values ± SEM were plotted for each crypt region (n=3, ***P<0.001 compared to indicated crypt regions, $$$P<0.001 compared to mid region of control crypts, &P<0.05 compared to top region of control crypts). (**C**) Histogram showing the total number of CD14+ cells per crypt in IBD samples compared to control (n=3,***P<0.001). Scale bar 50µm.

# Appendix D. Monocyte distribution along the crypt-axis in vitro

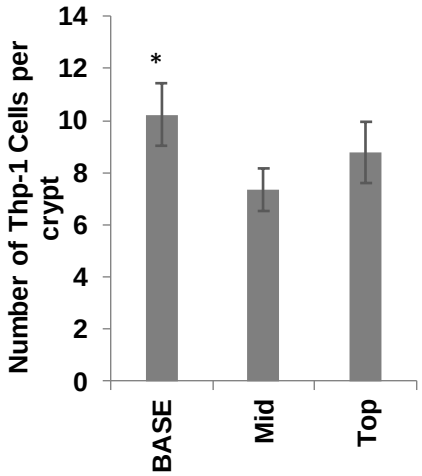


**Figure D.0.1. A similar spatial distribution to that observed in vivo is observed between monocytes and the epithelium in vitro.**

Histogram showing a significant increase in the number of THP-1 cells surrounding the base of human colonic crypts in co-culture compared to mid and top region (n=3, *P<0.01).

# Appendix E. Interleukin-6 Signaling Regulates Small Intestinal Crypt Homeostasis

Chapter 3 includes work from the following jointly authored publication in The Journal of Immunology:

Jeffery, V., A. J. Goldson, J. R. Dainty, M. Chieppa and A. Sobolewski (2017). "IL-6 Signaling Regulates Small Intestinal Crypt Homeostasis." J Immunol 199(1): 304-311.

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# IL-6 Signaling Regulates Small Intestinal Crypt Homeostasis

**Victoria Jeffery,*,† Andrew J. Goldson,‡ Jack R. Dainty,§ Marcello Chieppa,¶ and Anastasia Sobolewski*,†,‡**

**Gut homeostasis is a tightly regulated process requiring finely tuned complex interactions between different cell types, growth factors, or cytokines and their receptors. Previous work has implicated a role for IL-6 and mucosal immune cells in intestinal regeneration following injury and in promoting inflammation and cancer. We hypothesized that IL-6 signaling could also modulate crypt homeostasis. Using mouse in vitro crypt organoid and in vivo models, this study first demonstrated that exogenous IL-6 promoted crypt organoid proliferation and increased stem cell numbers through pSTAT3 activation in Paneth cells. Immunolabeling studies showed that the IL-6 receptor was restricted to the basal membrane of Paneth cells both in vitro and in vivo and that the crypt epithelium also expressed IL-6. Either a blocking Ab to the IL-6 receptor or a neutralizing Ab to IL-6 significantly reduced in vitro basal crypt organoid proliferation and budding, and in vivo significantly reduced the number of nuclei and the number of Lgr5EGFP-positive stem cells per crypt compared with IgG-treated mice, with the number of Paneth cells per crypt also significantly reduced. Functional studies demonstrated that IL-6–induced in vitro crypt organoid proliferation and crypt budding was abrogated by the Wnt inhibitor IWP2. This work demonstrates that autocrine IL-6 signaling in the gut epithelium regulates crypt homeostasis through the Paneth cells and the Wnt signaling pathway.** ***The Journal of Immunology*****, 2017, 199: 000–000.**

The intestinal epithelium is the most rapidly renewing tissue in the body, with the entire epithelium being replaced every 5–7 d. This renewal takes place by way of Lgr5-positive stem cells located at the base of intestinal crypts; stem cells proliferate, migrate along the crypt-villus axis, differentiate (into Tuft cells, enteroendocrine cells, Paneth cells, enterocytes, and goblet cells), and are shed into the gut lumen (1). Through the secretion of Wnts, epithelial Paneth cells play a major role in the maintenance of the crypt stem cell niche (2). Previous work has also shown that other growth factors and cytokines, as well as immune cells are key in modulating epithelial stem cell–driven tissue renewal during homeostasis (3–8). Understanding the mechanisms by which these pathways are regulated in the epithelium through autocrine (and paracrine) signaling is not fully understood.

Seminal work in the *Drosophila* gut has shown regenerative responses following infection are regulated by JAK/STAT signaling in gut epithelial stem cells through the release of enterocyte-derived Upd3, an IL-6–like cytokine (9, 10). In the mammalian gut both IL-6 and STAT3 have been shown to play a role in the proliferation of the colonic epithelium following injury and to promote the survival of epithelial cells (11–14) during inflammation and inflammatory bowel disease (15, 16).

IL-6 is a pleiotropic cytokine involved in a plethora of cellular and immune responses in health, disease, and cancer (17–19). IL-6 signaling involves the convergence of a number of signaling components (20). IL-6 first binds to a membrane-bound non-signaling α-receptor IL-6 (mbIL-6R) located on the target cell. Next, this IL-6R/IL-6 complex binds to the ubiquitously expressed type I transmembrane transducer protein gp130, which results in activation of downstream signaling components JAK/STAT, ERK, and PI3K signaling pathways. Cells that express both the IL-6R and gp130 are responsive to IL-6; this is termed classic signaling and is traditionally associated with homeostasis. IL-6 can also signal via *trans* signaling, where the soluble IL-6 receptor, shed from the cell membrane via proteolytic cleavage of a membrane-bound precursor, binds to IL-6. This IL-6/soluble IL-6 receptor complex can then activate IL-6 signaling in any cell expressing gp130; this *trans*-signaling pathway is associated with inflammation and cancer (21).

The aim of this study was to determine whether IL-6 classic signaling could modulate small intestinal crypt homeostasis. This work demonstrates a previously unidentified role for autocrine IL-6 signaling in the maintenance of the crypt stem cell niche, through the differential expression of the IL-6 receptor and downstream STAT3 signaling in Paneth cells and the Wnt signaling pathway.

## Materials and Methods

### *Mice and in vivo studies*

LGR5-EGFP-Ires-CreERT2 (Jackson Laboratory) or C57BL/6 mice aged 8–12 wk were used. Generation and genotyping of the LGR5-EGFP-Ires-CreERT2 allele has been described previously (1). All animal experiments were conducted in accordance with the Home Office Animals Scientific Procedures Act of 1986 with approval of the University of East Anglia Ethical Review Committee, Norwich, U.K. and under Home Office project

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Abbreviations used in this article: MCM, mouse crypt culture medium; UEA-1, *Ulex europaeus* lectin.

license number 80/2545. Blocking Abs for IL-6 and IL-6 receptor or IgG controls (Bio X Cell) were administered to mice three times on alternate days by i.p. injection at a concentration of 58 μg/g. Animals were euthanized by Schedule One approved methods on day 6, and tissue processed immediately. In addition, small intestinal tissue from IL-6 knockout mice (B6.129S2-Il6tm1Kopf/J; Jackson Laboratory) was used to count the number of lysozyme-positive cells per small intestinal crypt.

### *Reagents*

*Crypt culture media and supplements.* Advanced DMEM/F12, GlutaMAX, B27, and N2 were purchased from Invitrogen. Murine recombinant epidermal growth factor, noggin, IL-6, and IL-22 were all obtained from PeproTech and mouse recombinant R-spondin 1 from R&D Systems. Growth factor–reduced Matrigel was purchased from VWR International.

*Immunolabeling.* Primary and secondary Abs were as follows: rat anti-BrdU (Abcam), mouse anti-lysozyme (Abcam), goat anti–E-cadherin (R&D), rabbit IgG, rabbit anti–IL-6 receptor, anti–IL-6 (Bio X Cell), rabbit anti-pSTAT3 Tyr705, pSTAT3 Y705 blocking peptide, and rabbit anti–cleaved caspase-3 (Cell Signaling). Immunolabeling was visualized by using an appropriate combination of species-specific Alexa Fluor–conjugated secondary Abs (488, 568, and 647 nm), raised in mouse, donkey, or goat (Invitrogen). FITC-conjugated *Ulex europaeus* lectin (UEA-1) was purchased from Sigma. Vectashield-mounting medium with DAPI was purchased from Vector Laboratories; STATTIC and IWP2 were purchased from Tocris Bioscience.

### *Crypt isolation and culture*

Small intestinal crypts were isolated from the proximal small intestine of LGR5EGFP mice as previously described (2). Briefly, the mouse small intestine was opened longitudinally, washed with PBS, cut into 2–4 mm pieces and incubated with 1 mM EDTA in PBS (pH 7.4) for 30 min at 4˚C. Crypts were liberated by serial rounds of pipetting in ice cold PBS and removal of the crypt enriched supernatant; the solution was then filtered through a 70 μm cell strainer followed by centrifugation. Next, 50–100 crypts were embedded in a 200 μl droplet of growth factor–reduced Matrigel (VWR) and seeded on No. 0 coverslips (VWR) contained within a 12-well plate (Nunc). After polymerization at 37˚C for 5–10 min, crypts were flooded with 0.5 ml of mouse crypt culture medium (MCM): advanced F12/DMEM containing B27, N2, *n*-acetylcysteine (1 mM), HEPES (10 mM), penicillin/streptomycin (100 U/ml), GlutaMAX (2 mM), epidermal growth factor (50 ng/ml), noggin (100 ng/ml), and R-spondin 1 (1 μg/ml).

In IL-6–neutralizing or IL-6 receptor–blocking Ab experiments, crypts were incubated for the entire 48 h period in MCM and appropriate Ab or respective IgG control before addition of BrdU (1 μM) for 17 h.

Following 48 h culture with MCM, crypts were stimulated with IL-6 at concentrations of 10 ng/ml to 1 μg/ml for 15 min to 17 h for pSTAT3 experiments, and for proliferation experiments placed into 10% MCM for 5 h, after which BrdU (1 μM) was added for 17 h. For STAT3 inhibition studies, crypts were preincubated with STATTIC (20 μM) or IWP2 (5 μM) for 1 h prior to addition of IL-6 (100 ng/ml) stimulation for 17 h (as described previously).

For pSTAT3 activation studies, the IL-6–neutralizing Ab was preincubated with IL-6 (100 ng/ml) for 1 h before addition to the crypts for 1 h. For receptor Ab studies, crypts were preincubated for 1 h with the IL-6 receptor–blocking Ab before addition of IL-6 (100 ng/ml) for 1 h. The same protocol was used for proliferation experiments, prior to the addition of IL-6 and BrdU (1 μM) 17 h.

For chronic IL-6 stimulation, crypts were incubated for 5 d with MCM containing 100 ng/ml IL-6 before addition of BrdU (1 μM) for 17 h; medium was changed every 2 d.

### *Immunolabeling*

Mouse Lgr5EGFP small intestines were fixed in 4% paraformaldehyde, frozen in isopentane, and stored at −20˚C. Following the culture period, small intestinal crypts were fixed with 4% paraformaldehyde. Next, 8–20 μm cryosections of small intestinal tissue (CM 1100C cryostat; Leica) or small intestinal crypts were permeabilized with 0.5% Triton–X (Sigma), blocked with 10% FBS, and stained with primary Abs diluted (1:100) in PBS overnight at 4˚C, followed by the corresponding secondary fluorescence-conjugated Abs (1:200 in PBS) for 2 h as previously described (3, 4, 22).

### *Microscopy*

Following immunofluorescent staining, samples were visualized by laser scanning confocal (510 META; Zeiss) or epifluorescence (Nikon Ti) microscopy. For confocal microscopy a ×40 1.3 NA oil objective or ×63 1.4 NA 0.75 mm WD oil immersion objective was used to obtain confocal images. Image stacks were taken at 1–3 μm intervals, which allowed selection of precise focal planes. A ×40 1.1 NA oil objective was used to image samples on the epifluorescent microscope.

### *Image analysis*

For all experiments, between 20 and 100 crypts (in vivo) or organoids (in vitro) were counted per condition and each experiment was performed at least three separate times (i.e., at least three different mice were used).

*In vitro.* ImageJ software was used to count the total number of DAPI-labeled cell nuclei in the crypt equatorial plane. The number of cells that were positive for BrdU or Lgr5EGFP was then expressed as a percentage of the total nuclei number. For semiquantitative analysis of pSTAT3 activation, regions of interest were drawn around the entire Paneth cell nuclei and the average fluorescence intensity of the region calculated.

*In vivo.* Lgr5EGFP stem cells, lysozyme-, or UEA-1–positive Paneth cells were counted by taking a z-stack across the equatorial plane of the crypt and counting the number of nuclei in this stack that were positive for each marker. To determine the crypt length and villus height in vivo, at least 25 well-orientated crypts and villi were measured using the segmented line tool (Fiji 1.50e) from three animals in each treatment group.

### *mRNA isolation and quantification*

*Isolation.* RNA was isolated from freshly liberated or cultured small intestinal crypts using the Isolate II RNA mini kit (Bioline). Total RNA yield was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and purity assessed by the ratio of absorbance at 260–280 nm. cDNA was generated from 0.5 μg RNA using the $RT^2$ First Strand cDNA synthesis kit (Qiagen).

*Quantification.* End-point PCRs were performed in a final volume of 25 μl, comprising 200 nm forward and reverse primers, 200 μM dNTP, 0.5 U Taq polymerase, PCR buffer (Roche), 2.5 mM $MgCl_2$, 0.5 μl cDNA using a Tprofessional TRIO thermal cycler (Biometra) with the following thermal profile: one cycle at 94˚C for 3 min, 30 cycles at 94˚C for 25 s, 58˚C for 30 s, and 72˚C for 50 s, and 1 cycle at 72˚C for 5 min. The primer sequences used are as follows; IL-6 forward primer: 5′-GCTACCAAACTGGATATAATCAGGA-3′; reverse primer: 5′-CCAGGTAGCTATGGTACTCCAGAA-3′. PCR products were run on a 2% agarose gel visualized by ethidium bromide staining.

### *Statistics*

Experiments were performed at least three times. Data are expressed as mean ± SE and significance determined by a Student *t* test or by one-way ANOVA with post hoc Tukey analysis, with $p$ values $< 0.05$ considered significant.

## Results

### *IL-6 stimulates proliferation of small intestinal organoids via a STAT3-mediated signaling pathway*

First we determined the effect of exogenous IL-6 on the BrdU incorporation of small intestinal organoids through immunofluorescence microscopy (Fig. 1A). IL-6 caused a significant increase in the incorporation of BrdU at 10 and 100 ng/ml IL-6, but exhibited a dome-shaped response curve at higher concentrations (1000 ng/ml) (Fig. 1B). Using the specific STAT3 inhibitor STATTIC, we demonstrated that the increased BrdU incorporation induced by IL-6 was significantly reduced compared with IL-6 alone (Fig. 1C). There was no effect of STATTIC on basal proliferation, which was in contrast with the effects of chronic treatment with STATTIC, where a significant decrease in BrdU incorporation compared with control was observed (Supplemental Fig. 1A). Organoid survival following STATTIC treatment in the presence or absence of IL-6 was comparable to control (DMSO control 74.9 ± 3.9; STATTIC 73.7 ± 4.5; IL-6 63.1 ± 4.9; STATTIC + IL-6 69.5 ± 4.7). However, there was a small but significant ($n = 3$, $^{**}p < 0.01$) effect of STATTIC on the percentage of caspase 3–positive cells per crypt compared with control (DMSO control 3.6 ± 0.4; **STATTIC 6.0 ± 0.7; IL-6 2.2 ± 0.4; STATTIC + IL-6 4.8 ± 0.7). A significant increase in

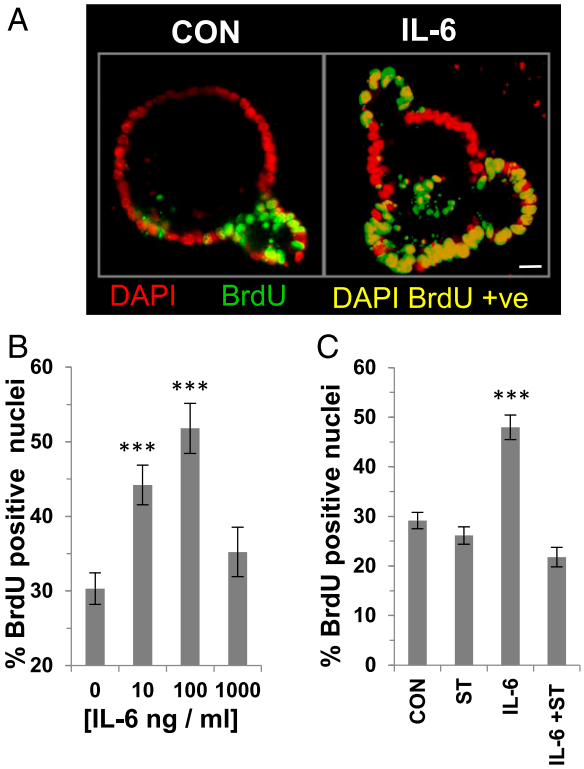


**FIGURE 1.** IL-6 stimulates proliferation of small intestinal organoids via a STAT3-mediated signaling pathway. (**A**) Representative confocal images of BrdU incorporation (green) into the nuclei (DAPI; red) of small intestinal organoids cultured in the absence or presence of IL-6 (100 ng/ml). DAPI BrdU positive cells (yellow). Scale bar, 10 μm. (**B**) Histogram showing the percentage of BrdU-positive nuclei in mouse small intestinal organoids following 24 h IL-6 (10–1000 ng/ml) stimulation compared with control. (**C**) Histogram showing percentage of BrdU-positive nuclei in mouse small intestinal organoids following 24 h IL-6 stimulation (100 ng/ml) in the presence or absence of STATTIC and compared with control. $n = 3$. $^{***}p < 0.001$.

BrdU incorporation was observed following chronic IL-6 stimulation (Supplemental Fig. 1B).

### *IL-6 induces activation of nuclear pSTAT3 in Paneth cells of the small intestine via differential expression of the IL-6 receptor*

We next investigated the effect of IL-6 on pSTAT3 activation in the small intestinal epithelium using immunolabeling and visualization of pSTAT3 activation by confocal microscopy. Following 1 h stimulation with IL-6 (100 ng/ml), we observed pSTAT3 (green) in the nucleus (red) of UEA-1– (pink) and lysozyme-positive (blue) cells, which was abrogated by a blocking peptide to pSTAT3 (Fig. 2A). No fluorescent labeling was observed in IgG controls for the lysozyme Ab (Supplemental Fig. 1C). Following IL-6 stimulation, all UEA-1 lysozyme-positive cells in a crypt domain were positive for nuclear pSTAT3 (see projection Fig. 2A). All lysozyme-positive cells were UEA-1 positive and characteristic of Paneth cells (23). Although the majority of control organoids expressed no pSTAT3 (Fig. 2B), nuclear pSTAT3 activation in UEA-1–positive cells was observed in 9 out of 113 control organoids counted. Time course studies showed that maximal activation of nuclear (red) pSTAT3 (green) in UEA-1–positive (pink) Paneth cells occurred at 30 min, and persisted at 1 and 3 h poststimulation (Fig. 2B). Semiquantitative analysis showed the level of nuclear pSTAT3 activation remained significantly higher than control levels 1 and 3 h after IL-6 stimulation. Results also showed that pSTAT3 phosphorylation levels at 1 h were the same for 100 or 1000 ng/ml IL-6 (Fig. 2C).

The localization of the IL-6 receptor (red) in organoids was restricted to the basal membrane of lysozyme-positive (green) cells of the small intestine in vitro (Fig. 2D). Furthermore, in vivo labeling of the IL-6 receptor displayed the same distribution as in the in vitro crypt organoid cultures (Fig. 2E), with basal epithelial IL-6 receptor expression constrained to the lysozyme-positive cells (green) and on the membrane of their intracellular granules (Fig. 2F) with no fluorescence observed in the IgG control for lysozyme (Supplemental Fig. 2D). No IL-6R was expressed on Lgr5EGFP-positive stem cells (cyan). The small intestinal crypt in vitro model was therefore considered a relevant in vitro model to study IL-6 signaling in the small intestinal crypt epithelium.

Next, we demonstrated the ability to modulate the IL-6 STAT3 signaling axis in small intestinal organoids utilizing a receptor-blocking Ab to IL-6 or neutralizing Ab to IL-6. Either of these Abs in combination with IL-6 abrogated the IL-6–induced phosphorylation of STAT3 (Supplemental Fig. 1J–M).

### *Autocrine IL-6 signaling in the small intestinal epithelium modulates crypt cell proliferation in vitro*

The next step was to determine whether autocrine IL-6 signaling played a role in crypt homeostasis. We first used conventional PCR to demonstrate the expression of IL-6 mRNA in crypts in vivo using freshly isolated (0 h) and in vitro cultured (48 h) small intestinal crypts (Fig. 3A). Immunofluorescent studies showed that IL-6 protein (red) was localized inside epithelial cells toward the basal pole as well as extracellularly in discrete pools around the crypt basal membrane (white arrows, Fig. 3B). We next used a blocking Ab to the IL-6 receptor or a neutralizing Ab to the IL-6 cytokine in crypt organoid cultures and assessed basal BrdU incorporation (Fig. 3C, 3D respectively), and observed a significant decrease in the percentage of crypt nuclei incorporating BrdU compared with the respective IgG controls with both Abs.

We also confirmed that the Abs could functionally block IL-6 signaling in organoids. Incubation of either an IL-6 receptor–blocking or an IL-6 neutralizing–Ab with IL-6 significantly decreased BrdU incorporation of organoids compared with IL-6 stimulation alone (Supplemental Fig. 1D, 1E). Caspase 3 (used as a measure of cell death), and organoid survival was unaffected by either of these Ab treatments compared with IgG control organoids (Supplemental Fig. 1F–I).

### *Lack of gut phenotype in IL-6 knockout mice*

To understand the effects of perturbation of the IL-6 signaling pathway in vivo, tissue was obtained from IL-6 knockout mice. No difference was observed in the number of lysozyme-positive cells per crypt, crypt length, and the number of nuclei per crypt in IL-6 knockout tissue compared with wild-type tissue (Supplemental Fig. 2A–C). Furthermore, using immunolabeling, low levels of pSTAT3 were detected in some UEA-1–positive cells at the base of the crypts in both wild-type and IL-6 KO small intestine (Supplemental Fig. 2J, 2K). We confirmed the specificity of the pSTAT3 Ab labeling by using a blocking peptide to pSTAT3, which abrogated the pSTAT3 signal in the UEA-1–positive cells (Supplemental Fig. 2I).

### *IL-6 signaling axis modulates Lgr5EGFP stem cell numbers in vitro and in vivo*

As intestinal stem cells drive the renewal of the crypt epithelium, we examined the effects of modifying the IL-6 signaling axis on crypt stem cells both in vitro and in vivo. In addition to the observed increase in the small intestinal organoid proliferation in vitro, we

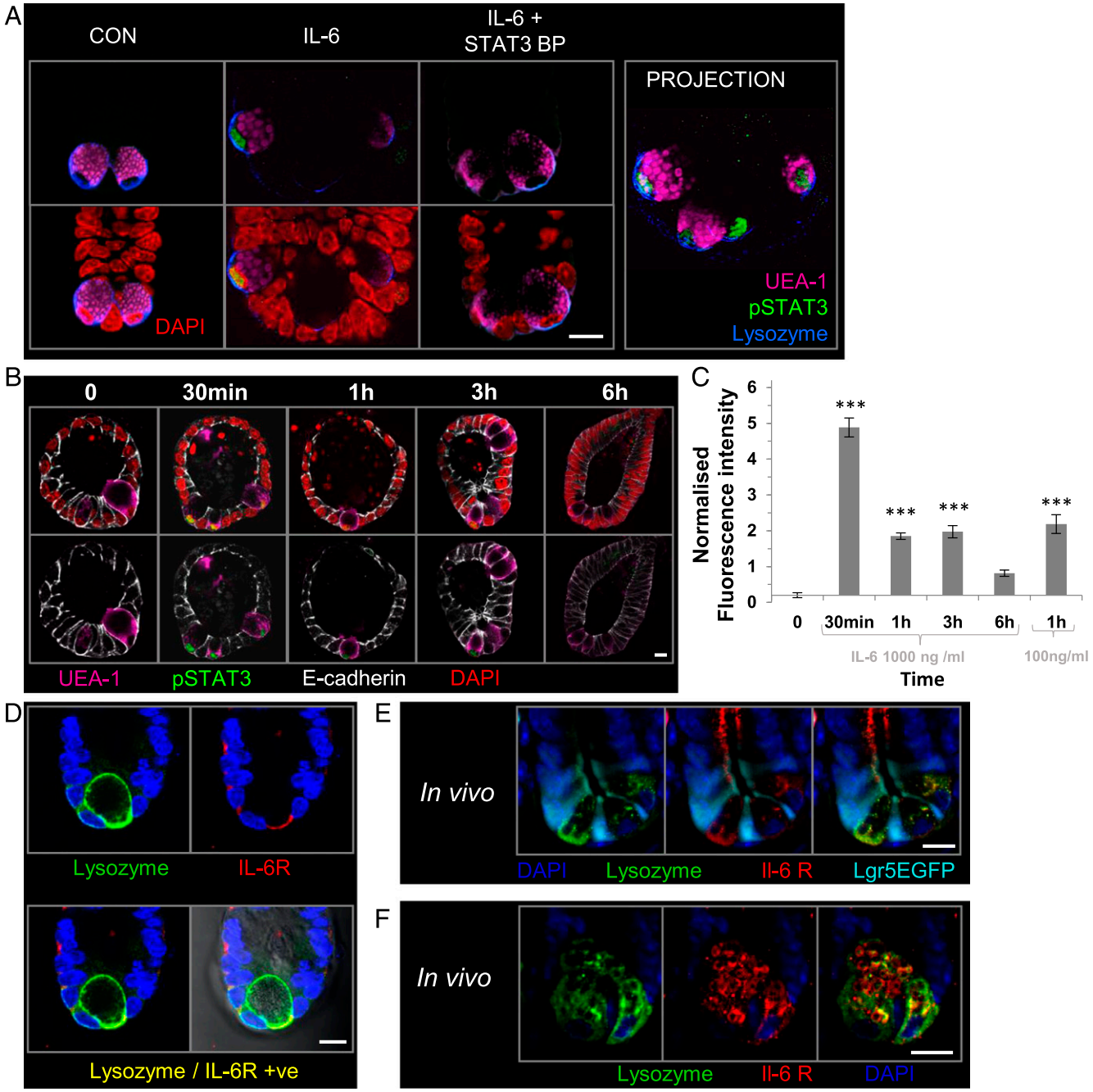


**FIGURE 2.** IL-6 induces activation of nuclear pSTAT3 in Paneth cells of the small intestine through differential expression of the IL-6 receptor. (**A**) Representative confocal images showing the presence of pSTAT3 immunofluorescent labeling (green) in the nuclei (DAPI; red) of UEA-1– (pink) and lysozyme-positive (blue) Paneth cells in organoids following 1 h IL-6 (100 ng/ml) stimulation in the presence or absence of a pSTAT3-blocking peptide. (**B**) Representative confocal images of pSTAT3 (green) in the nuclei (DAPI; red) of UEA-1–positive (pink) cells in organoids stimulated with IL-6 (1000 ng/ml) for 0–6 h. E-cadherin-white. (**C**) Histogram showing the time course of IL-6–induced pSTAT3 activation in UEA-1–positive cells from 0 to 6 h post-stimulation using 1000 or 100 ng/ml. Data expressed as fluorescence intensity (arbitrary units) and normalized to control UEA-1–positive nuclei ($n$ = 3, $^{***}p < 0.001$). (**D**) Representative confocal images showing immunofluorescent labeling of IL-6 receptor localization (red) in lysozyme-positive (green) cells in small intestinal organoid culture. (**E** and **F**) Representative confocal images of small intestine taken from Lgr5EGFP mice showing IL-6 receptor localization (red) on the basal membrane of lysozyme-positive cells (E) and intracellular granules (F); Lgr5EGFP- (cyan) and lysozyme-positive cells (green). Confocal imaging data are representative of $n$ = 3 independent experiments. Scale bar, 10 μm.

also demonstrated that the percentage of Lgr5EGFP-positive nuclei per organoid (Fig. 4A) was significantly increased compared with control. Furthermore, the average number of new buds per small intestinal organoid, also a measure of stem cell numbers, was significantly increased compared with control, an effect that was abrogated by STAT3 inhibition with STATTIC (Fig. 4B). The use of an IL-6R blocking Ab also caused a significant reduction in the budding of small intestinal crypts (Fig. 4C). Complementary in vivo studies using Lgr5EGFP mice treated with the IL-6 receptor–blocking Ab caused a significant reduction in the number of Lgr5EGFP cells per crypt, the number of crypt nuclei, crypt length, and villus height compared with IgG-treated mice (Fig. 4D–G respectively). Similar findings were obtained after mice were treated with an IL-6–neutralizing Ab with a significant

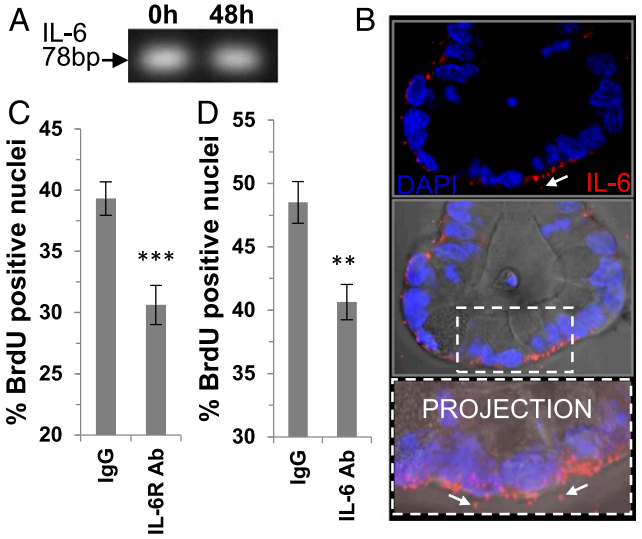


**FIGURE 3.** Autocrine IL-6 signaling regulates small intestinal organoid proliferation. (**A**) PCR gel showing IL-6 transcript expression in in vivo and freshly isolated (0 h) and cultured (48 h) small intestinal crypts (**B**) Representative confocal image showing immunofluorescent labeling of IL-6 (red), nuclei (DAPI blue) of small intestinal organoids with the differential interference contrast image overlaid and associated projection image in vitro. White arrows indicate extracellular pools of IL-6. Scale bar, 10 μm. (**C**) Histogram showing percentage of BrdU-positive nuclei in mouse small intestinal organoids in the presence of an IL-6 receptor–blocking Ab and (**D**) an IL-6 neutralizing–Ab compared with respective IgG control. Data are represented as mean ± SEM. $n = 3$. $^{**}p < 0.01$, $^{***}p < 0.001$.

reduction in the number of Lgr5EGFP cells per crypt, the number of crypt nuclei, and villus height compared with IgG-treated mice (Fig. 4H, 4I, 4K). No significant difference was observed in crypt length between mice treated with a IL-6–neutralizing Ab or control IgG (Fig. 4J). Furthermore, both the IL-6 receptor–blocking Ab and the neutralizing IL-6 Ab caused a significant reduction in the number of lysozyme- (Fig. 4L, 4N) and UEA-1–positive (Fig. 4M, 4O) cells per crypt compared with the respective IgG-treated mice. Representative confocal images from mouse in vivo Ab experiments including Lgr5EGFP, lysozyme, UEA-1, Ki67, and caspase 3 labeling are included in Supplemental Fig. 2E–H.

### *IL-6 induces crypt cell proliferation through a Wnt signaling pathway*

Paneth cells have been shown to be a major source of Wnts and the Wnt signaling pathway is a master regulator of epithelial renewal. We next determined whether IL-6 was affecting epithelial renewal or proliferation and stem cell numbers in vitro through this pathway by utilizing the Wnt inhibitor IWP2. We show that IWP2 abrogates the IL-6–induced increase in both proliferation and the average number of buds per organoid compared with control (Fig. 5). The percentage of caspase 3–positive cells and organoid survival in the presence of IWP2 and/or IL-6 was comparable to control (Supplemental Fig. 4).

## Discussion

This work highlights a previously unrecognized role for autocrine IL-6 signaling during homeostasis in the small intestine. IL-6 stimulation was shown to increase in vitro crypt cell proliferation and stem cell numbers through the STAT3 signaling pathway. IL-6 activated pSTAT3 specifically in the nucleus of the Paneth cell through differential expression of the IL-6 receptor located on the Paneth cell basal membrane. IL-6 was also expressed in the crypt epithelium. Neutralizing IL-6 or blocking the IL-6 receptor with Abs lowered basal organoid proliferation and crypt budding in vitro and reduced crypt stem and Paneth cell numbers in vivo. Use of the Wnt inhibitor IWP2 abrogated the IL-6–induced increase in organoid proliferation and crypt budding. These data suggest a role of autocrine IL-6 signaling and the Wnt signaling pathway in the regulation of crypt homeostasis.

Previous work using IL-6 receptors or IL-6 knockout mice demonstrated a role for IL-6 signaling in mouse models of inflammation and crypt epithelial cell survival and regeneration rather than homeostasis (11, 12, 14, 16, 24, 25). In agreement with other studies, we also showed that gut tissue from healthy IL-6 knockout mice was similar to tissue from wild type mice (Supplemental Fig. 2A–C). However, using a blocking Ab approach we demonstrated a previously unrecognized role for autocrine IL-6 signaling in the maintenance of crypt stem cell numbers (and Paneth cell numbers; see below) in vivo. These differences may be explained by redundancy effects known to occur in knockout mouse models, and highlights the importance of interrogating this pathway using a variety of methods as has been shown in other studies (26).

Other groups have also implicated a role for IL-6 in maintaining homeostasis (27–29) as well as ageing (30–32). Our findings suggest that classic signaling is involved in crypt homeostasis; we show that addition of IL-6 to the basal side of crypts in vitro causes pSTAT3 activation in Paneth cells only, which correlates with the expression of the IL-6 receptor on the Paneth cell basal membrane. It has been suggested that anti-inflammatory and regenerative properties of IL-6 most likely depend on IL-6 classic rather than *trans* signaling (20, 33). Our studies concur with this; if binding of soluble IL-6 receptor to the ubiquitously expressed gp130 was occurring in our system then this would permit all cells of the crypt epithelium to express nuclear pSTAT3 in response to IL-6, an effect that we did not observe. The relative contribution of classic IL-6 signaling versus *trans* signaling in crypt homeostasis versus inflammation remains the focus of future work.

Our findings demonstrated that IL-6 mediated its in vitro effects on crypt cell proliferation and stem cells through the pSTAT3 pathway. Crypt cell proliferation in response to IL-6 exhibited a bell-shaped concentration response curve, which has previously been described (34). The epithelial STAT3 signaling pathway has shown to be key for small intestinal stem cell survival (35) and regeneration during intestinal mucosal wound healing (9, 13). Using full growth factor media and chronic STAT3 inhibition, we also showed that epithelial STAT3 was important for in vitro crypt regeneration even in the absence of exogenous IL-6 (Supplemental Fig. 1A). Interestingly, we showed in vivo that low levels of nuclear pSTAT3 activation (Supplemental Fig. 2J) were present in most UEA-1–positive Paneth cells at the base of small intestinal crypts; there was also evidence of a small number of crypts expressing high pSTAT3 activation in Paneth cells (Supplemental Fig. 2K). These findings support the hypothesis that pSTAT3 signaling is dynamic and transient during homeostasis. In the future the development of fluorescent reporter systems would be required to study dynamic pSTAT3 signaling in real time.

The STAT3 signaling pathway can be activated by a number of cytokines including IL-6 and IL-22 (36, 37). Interestingly, activation of IL-22 rather than IL-6 was shown to be key in regulating epithelial STAT3 wound healing following dextran sodium sulfate–induced experimental colitis in mice (13) and in ameliorating gut inflammation (38). This highlights the complexity of STAT3 signaling and it is likely that finetuning of this pathway through different cytokines exerts different functional responses depending on the context. Indeed, we showed that IL-22 stimulation of

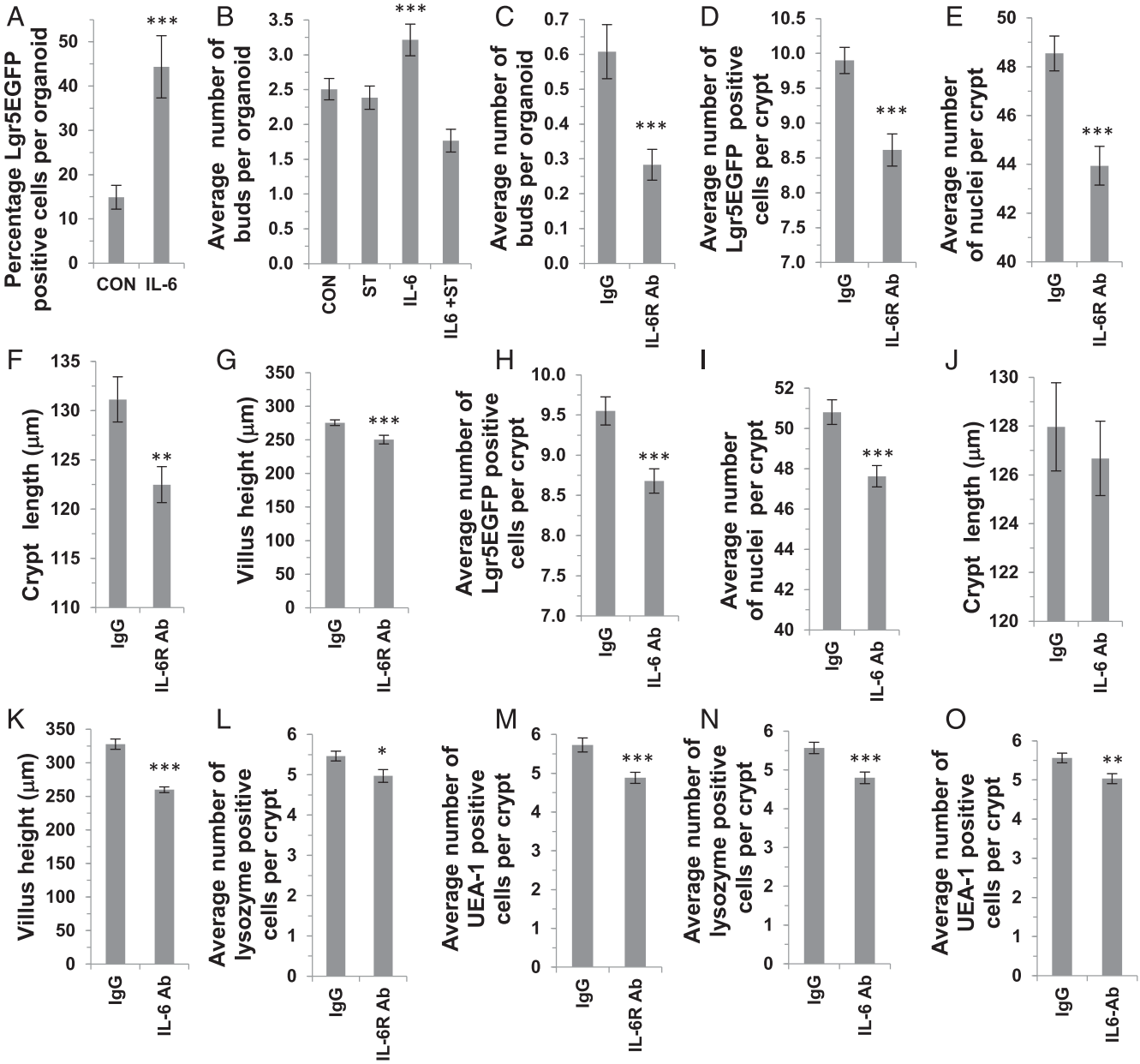


**FIGURE 4.** IL-6 receptor signaling modulates Lgr5EGFP$^+$ crypt stem cell numbers in vitro and in vivo. (**A**) Histogram showing the effect of IL-6 (100 ng/ml) treatment on the percentage of Lgr5EGFP-positive cells per crypt organoid compared with control. (**B**) Histogram showing the average number of buds per crypt organoid following IL-6 (100 ng/ml) treatment, or treatment with STATTIC (20 μM). (**C**) Histogram showing the average number of buds per organoid in vitro when cultured in the presence of an IL-6 receptor–blocking Ab compared with IgG control. (**D**) Histogram showing the average number of Lgr5EGFP-positive stem cells per crypt, (**E**) the average number of nuclei per crypt, (**F**) the crypt length, and (**G**) the villus height in the small intestine of mice treated with a IL-6 receptor–blocking Ab compared with respective IgG controls. (**H**) Histogram showing the average number of Lgr5EGFP-positive stem cells per crypt, (**I**) the average number of nuclei per crypt, (**J**) the average crypt length, and (**K**) the average villus height in the small intestine of mice treated with a IL-6–neutralizing Ab. Histograms showing the in vivo effect of an IL-6 receptor–blocking Ab on the number of (**L**) lysozyme- or (**M**) UEA-1–positive cells per crypt compared with IgG controls. Histograms showing the effect of an IL-6–neutralizing Ab on the number of (**N**) lysozyme- or (**O**) UEA-1–positive cells per small intestinal crypt compared with IgG controls. Data are represented as mean ± SEM. $n = 3$. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

organoids resulted in global epithelial pSTAT3 activation (Supplemental Fig. 3A), which was in contrast to the restricted activation in Paneth cells following IL-6 exposure. We also concur with previous findings (39) that IL-22 induces proliferation of both large and small intestinal organoids and affects organoid budding (Supplemental Fig. 3), and we show that IL-6 can cause proliferation of colonic organoids. Whether the same mechanisms exist in the human gut epithelium remains to be elucidated. The relative contribution of different cytokines in regulating the STAT3 pathway in gut homeostasis versus inflammation and in the small intestine versus the colon is the subject of future investigation.

Although Paneth cells are not an absolute requirement for survival, proliferation, and stem cell activity (40, 41), previous work has shown that they provide important regenerative signals for modulation of the crypt stem cell niche (2, 42). Future work will elucidate the identity of the Wnt pathway(s) involved in IL-6 and STAT3 signaling in small intestinal crypt homeostasis. This

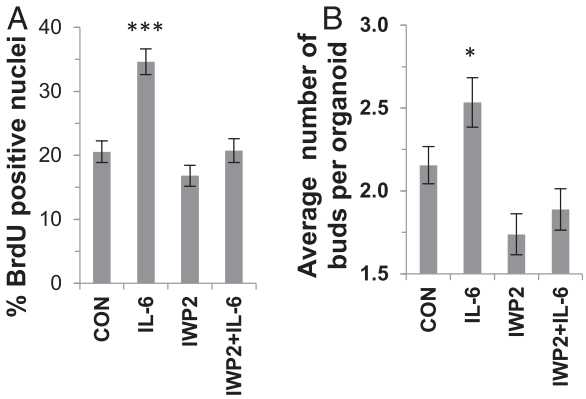


**FIGURE 5.** Wnt inhibition abrogates the IL-6–induced proliferation and budding of small intestinal organoids. Histograms showing the effect of IWP2 on (**A**) the percentage of BrdU-positive crypt nuclei ($n = 3$, $^{***}p < 0.001$) and (**B**) the average number of buds per crypt organoid following IL-6 stimulation ($n = 3$, $^{*}p < 0.05$). Data are represented as mean ± SEM.

study has also identified a novel role for the IL-6 signaling axis in regulating Paneth cell numbers in vivo. Paneth cell metaplasia in the colon is a feature of inflammatory bowel disease (43, 44), in addition to elevated levels of IL-6 in the serum and tissues (45, 46). We speculate that alterations in the IL-6 signaling axis may be a contributing factor to this phenotype. Furthermore, in the clinic, some rheumatoid arthritis patients treated with the IL-6 Ab tocilizumab display adverse side effects (47, 48); the mechanism by which this occurs is unknown.

IL-6 can also activate other signaling pathways not studied in this paper (17). This highlights the importance of understanding the complex biology of IL-6 during health and disease. In this study, we show a previously unrecognized role for IL-6 signaling in health. Understanding the mechanisms of IL-6 regulation of crypt cell renewal has wider implications for the prevention or development of future treatments for inflammatory bowel disease. More targeted treatments for inflammatory disorders that retain classic IL-6 signaling and homeostatic levels of epithelial crypt renewal but inhibit *trans* IL-6 signaling (49) through specific cell types appears to be the ideal scenario for the future (50, 51).

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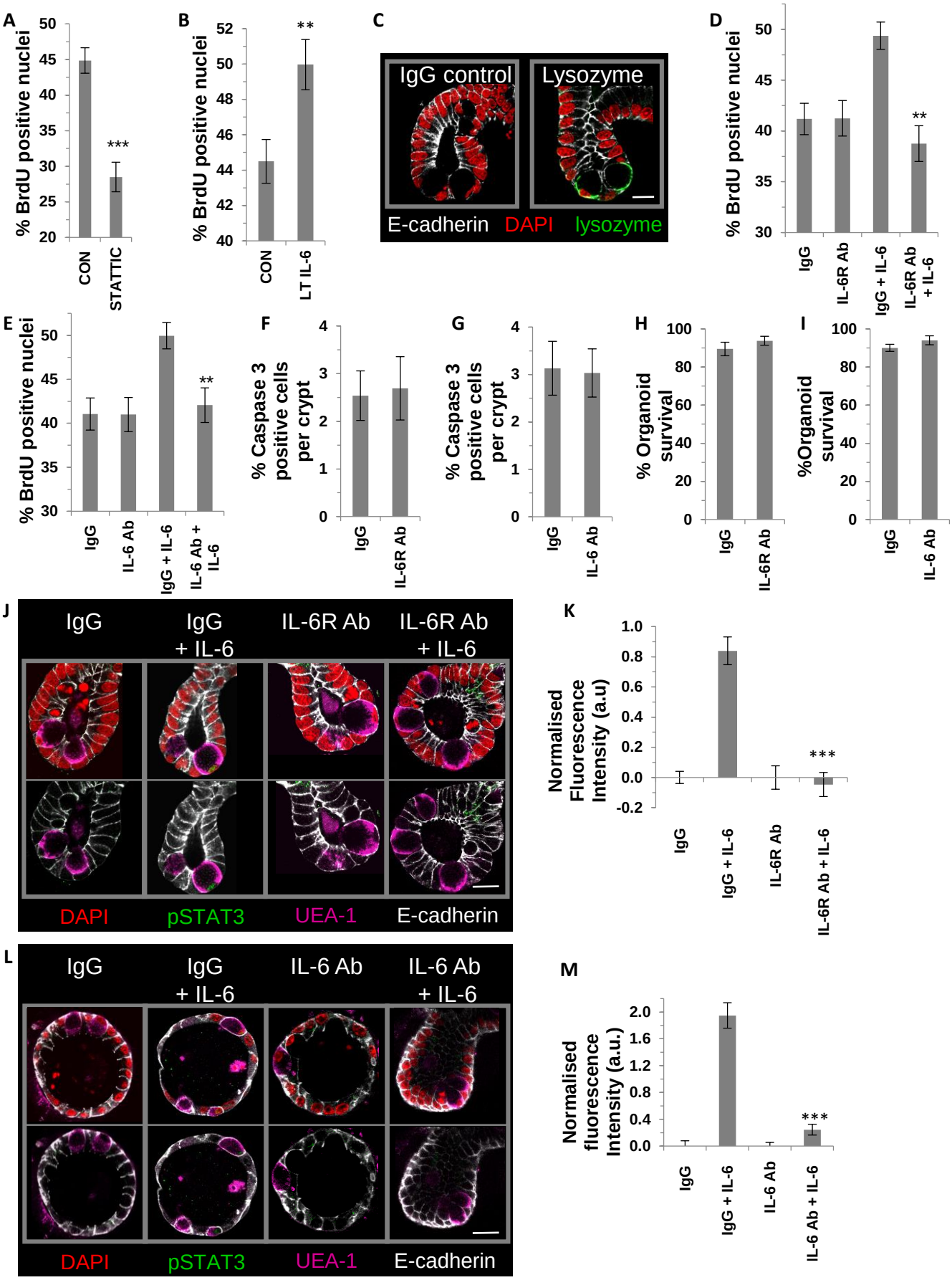
## Disclosures

The authors have no financial conflicts of interest.

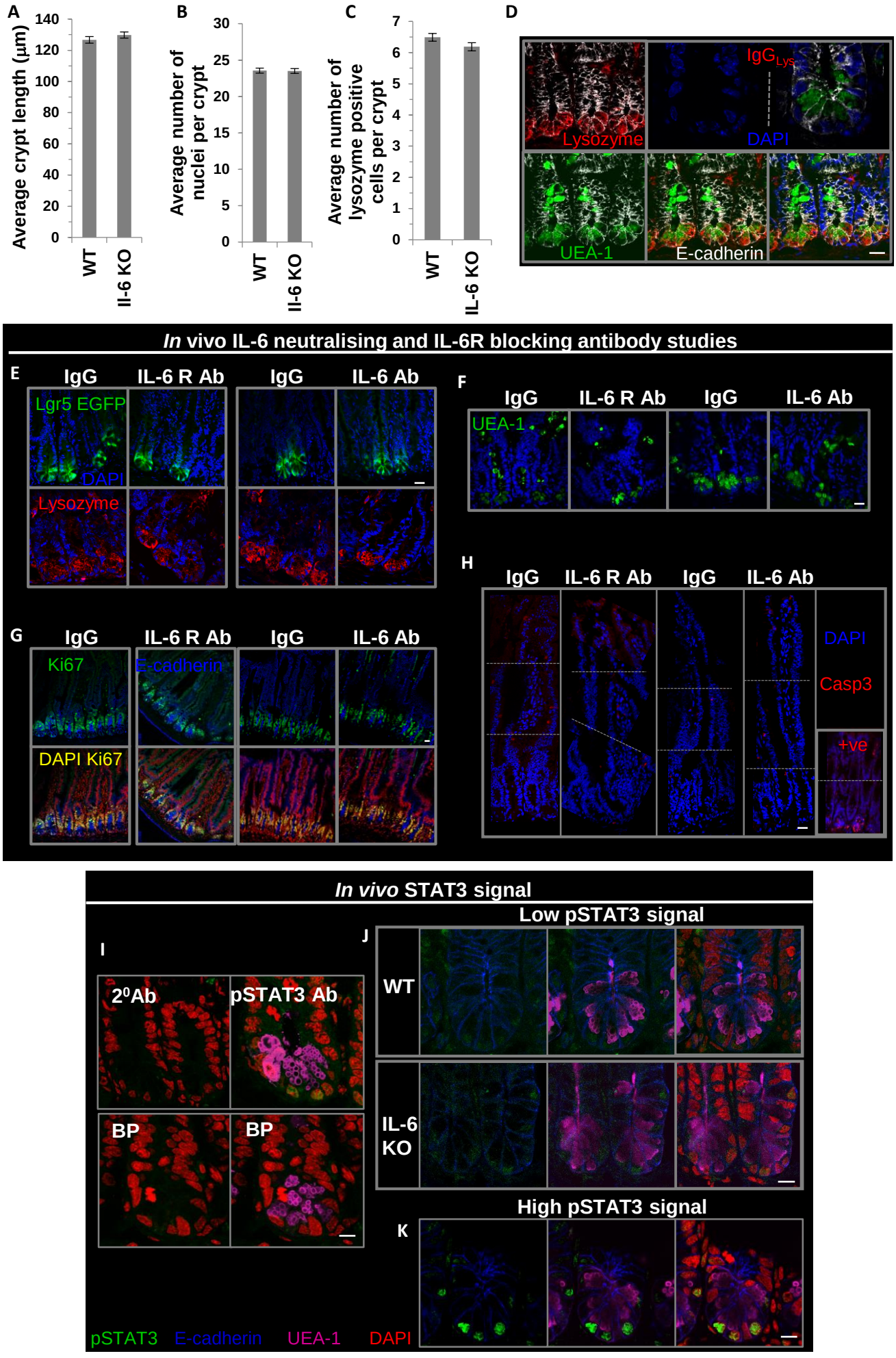
## References

1. Barker, N., J. H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters, and H. Clevers. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003–1007.
2. Sato, T., J. H. van Es, H. J. Snippert, D. E. Stange, R. G. Vries, M. van den Born, N. Barker, N. F. Shroyer, M. van de Wetering, and H. Clevers. 2011. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 469: 415–418.
3. Reynolds, A., N. Wharton, A. Parris, E. Mitchell, A. Sobolewski, C. Kam, L. Bigwood, A. El Hadi, A. Münsterberg, M. Lewis, et al. 2014. Canonical Wnt signals combined with suppressed TGFβ/BMP pathways promote renewal of the native human colonic epithelium. *Gut* 63: 610–621.
4. Skoczek, D. A., P. Walczysko, N. Horn, A. Parris, S. Clare, M. R. Williams, and A. Sobolewski. 2014. Luminal microbes promote monocyte-stem cell interactions across a healthy colonic epithelium. *J. Immunol.* 193: 439–451.
5. Brown, S. L., T. E. Riehl, M. R. Walker, M. J. Geske, J. M. Doherty, W. F. Stenson, and T. S. Stappenbeck. 2007. Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury. *J. Clin. Invest.* 117: 258–269.
6. Pull, S. L., J. M. Doherty, J. C. Mills, J. I. Gordon, and T. S. Stappenbeck. 2005. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc. Natl. Acad. Sci. USA* 102: 99–104.
7. Seno, H., H. Miyoshi, S. L. Brown, M. J. Geske, M. Colonna, and T. S. Stappenbeck. 2009. Efficient colonic mucosal wound repair requires Trem2 signaling. *Proc. Natl. Acad. Sci. USA* 106: 256–261.
8. Ayyaz, A., H. Li, and H. Jasper. 2015. Haemocytes control stem cell activity in the Drosophila intestine. *Nat. Cell Biol.* 17: 736–748.
9. Jiang, H., P. H. Patel, A. Kohlmaier, M. O. Grenley, D. G. McEwen, and B. A. Edgar. 2009. Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. *Cell* 137: 1343–1355.
10. Osman, D., N. Buchon, S. Chakrabarti, Y. T. Huang, W. C. Su, M. Poidevin, Y. C. Tsai, and B. Lemaitre. 2012. Autocrine and paracrine unpaired signaling regulate intestinal stem cell maintenance and division. *J. Cell Sci.* 125: 5944–5949.
11. Grivennikov, S., E. Karin, J. Terzic, D. Mucida, G. Y. Yu, S. Vallabhapurapu, J. Scheller, S. Rose-John, H. Cheroutre, L. Eckmann, and M. Karin. 2009. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 15: 103–113.
12. Jin, X., T. A. Zimmers, Z. Zhang, R. H. Pierce, and L. G. Koniaris. 2010. Interleukin-6 is an important in vivo inhibitor of intestinal epithelial cell death in mice. *Gut* 59: 186–196.
13. Pickert, G., C. Neufert, M. Leppkes, Y. Zheng, N. Wittkopf, M. Warntjen, H. A. Lehr, S. Hirth, B. Weigmann, S. Wirtz, et al. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J. Exp. Med.* 206: 1465–1472.
14. Kuhn, K. A., N. A. Manieri, T. C. Liu, and T. S. Stappenbeck. 2014. IL-6 stimulates intestinal epithelial proliferation and repair after injury. *PLoS One* 9: e114195.
15. Bollrath, J., T. J. Phesse, V. A. von Burstin, T. Putoczki, M. Bennecke, T. Bateman, T. Nebelsiek, T. Lundgren-May, O. Canli, S. Schwitalla, et al. 2009. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell* 15: 91–102.
16. Lee, M. J., J. K. Lee, J. W. Choi, C. S. Lee, J. H. Sim, C. H. Cho, K. H. Lee, I. H. Cho, M. H. Chung, H. R. Kim, and S. K. Ye. 2012. Interleukin-6 induces S100A9 expression in colonic epithelial cells through STAT3 activation in experimental ulcerative colitis. *PLoS One* 7: e38801.
17. Mihara, M., M. Hashizume, H. Yoshida, M. Suzuki, and M. Shiina. 2012. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. *Clin. Sci.* 122: 143–159.
18. Hunter, C. A., and S. A. Jones. 2015. IL-6 as a keystone cytokine in health and disease. *Nat. Immunol.* 16: 448–457.
19. Garbers, C., S. Aparicio-Siegmund, and S. Rose-John. 2015. The IL-6/gp130/STAT3 signaling axis: recent advances towards specific inhibition. *Curr. Opin. Immunol.* 34: 75–82.
20. Scheller, J., A. Chalaris, D. Schmidt-Arras, and S. Rose-John. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta* 1813: 878–888.
21. Jones, S. A., P. J. Richards, J. Scheller, and S. Rose-John. 2005. IL-6 transsignaling: the in vivo consequences. *J. Interferon Cytokine Res.* 25: 241–253.
22. Sato, T., R. G. Vries, H. J. Snippert, M. van de Wetering, N. Barker, D. E. Stange, J. H. van Es, A. Abo, P. Kujala, P. J. Peters, and H. Clevers. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262–265.
23. Farin, H. F., W. R. Karthaus, P. Kujala, M. Rakhshandehroo, G. Schwank, R. G. Vries, E. Kalkhoven, E. E. Nieuwenhuis, and H. Clevers. 2014. Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-γ. *J. Exp. Med.* 211: 1393–1405.
24. François, M., E. Birman, K. A. Forner, L. Gaboury, and J. Galipeau. 2012. Adoptive transfer of mesenchymal stromal cells accelerates intestinal epithelium recovery of irradiated mice in an interleukin-6-dependent manner. *Cytotherapy* 14: 1164–1170.
25. Bernardo, D., S. Vallejo-Díez, E. R. Mann, H. O. Al-Hassi, B. Martínez-Abad, E. Montalvillo, C. T. Tee, A. U. Murugananthan, H. Núñez, S. T. Peake, et al. 2012. IL-6 promotes immune responses in human ulcerative colitis and induces a skin-homing phenotype in the dendritic cells and Tcells they stimulate. *Eur. J. Immunol.* 42: 1337–1353.
26. Sommer, J., E. Engelowski, P. Baran, C. Garbers, D. M. Floss, and J. Scheller. 2014. Interleukin-6, but not the interleukin-6 receptor plays a role in recovery from dextran sodium sulfate-induced colitis. *Int. J. Mol. Med.* 34: 651–660.
27. Giraud, A. S., C. Jackson, T. R. Menheniott, and L. M. Judd. 2007. Differentiation of the gastric mucosa IV. Role of trefoil peptides and IL-6 cytokine family signaling in gastric homeostasis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292: G1–G5.
28. Sims, N. A., and N. C. Walsh. 2010. GP130 cytokines and bone remodelling in health and disease. *BMB Rep.* 43: 513–523.
29. Jain, U., Q. Cao, N. A. Thomas, T. M. Woodruff, W. J. Schwaeble, C. M. Stover, and A. W. Stadnyk. 2015. Properdin provides protection from *Citrobacter* rodentium-induced intestinal inflammation in a C5a/IL-6-dependent manner. *J. Immunol.* 194: 3414–3421.
30. Palin, K., M. L. Moreau, H. Orcel, A. Duvoid-Guillou, A. Rabié, K. W. Kelley, and F. Moos. 2009. Age-impaired fluid homeostasis depends on the balance of IL-6/IGF-I in the rat supraoptic nuclei. *Neurobiol. Aging* 30: 1677–1692.

31. Man, A. L., N. Gicheva, and C. Nicoletti. 2014. The impact of ageing on the intestinal epithelial barrier and immune system. *Cell. Immunol.* 289: 112–118.
32. Man, A. L., E. Bertelli, S. Rentini, M. Regoli, G. Briars, M. Marini, A. J. Watson, and C. Nicoletti. 2015. Age-associated modifications of intestinal permeability and innate immunity in human small intestine. *Clin. Sci.* 129: 515–527.
33. Becker, C., M. C. Fantini, C. Schramm, H. A. Lehr, S. Wirtz, A. Nikolaev, J. Burg, S. Strand, R. Kiesslich, S. Huber, et al. 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 21: 491–501.
34. van Dam, M., J. Müllberg, H. Schooltink, T. Stoyan, J. P. Brakenhoff, L. Graeve, P. C. Heinrich, and S. Rose-John. 1993. Structure-function analysis of interleukin-6 utilizing human/murine chimeric molecules. Involvement of two separate domains in receptor binding. *J. Biol. Chem.* 268: 15285–15290.
35. Matthews, J. R., O. J. Sansom, and A. R. Clarke. 2011. Absolute requirement for STAT3 function in small-intestine crypt stem cell survival. *Cell Death Differ.* 18: 1934–1943.
36. Nguyen, P. M., T. L. Putoczki, and M. Ernst. 2015. STAT3-activating cytokines: a therapeutic opportunity for inflammatory bowel disease? *J. Interferon Cytokine Res.* 35: 340–350.
37. Aden, K., A. Breuer, A. Rehman, H. Geese, F. Tran, J. Sommer, G. H. Waetzig, T. M. Reinheimer, S. Schreiber, S. Rose-John, et al. 2016. Classic IL-6R signalling is dispensable for intestinal epithelial proliferation and repair. *Oncogenesis* 5: e270.
38. Sugimoto, K., A. Ogawa, E. Mizoguchi, Y. Shimomura, A. Andoh, A. K. Bhan, R. S. Blumberg, R. J. Xavier, and A. Mizoguchi. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J. Clin. Invest.* 118: 534–544.
39. Lindemans, C. A., M. Calafiore, A. M. Mertelsmann, M. H. O'Connor, J. A. Dudakov, R. R. Jenq, E. Velardi, L. F. Young, O. M. Smith, G. Lawrence, et al. 2015. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. *Nature* 528: 560–564.
40. Kim, T. H., S. Escudero, and R. A. Shivdasani. 2012. Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. *Proc. Natl. Acad. Sci. USA* 109: 3932–3937.
41. Durand, A., B. Donahue, G. Peignon, F. Letourneur, N. Cagnard, C. Slomianny, C. Perret, N. F. Shroyer, and B. Romagnolo. 2012. Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1). *Proc. Natl. Acad. Sci. USA* 109: 8965–8970.
42. Farin, H. F., I. Jordens, M. H. Mosa, O. Basak, J. Korving, D. V. Tauriello, K. de Punder, S. Angers, P. J. Peters, M. M. Maurice, and H. Clevers. 2016. Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature* 530: 340–343.
43. Simmonds, N., M. Furman, E. Karanika, A. Phillips, and A. W. Bates. 2014. Paneth cell metaplasia in newly diagnosed inflammatory bowel disease in children. *BMC Gastroenterol.* 14: 93.
44. Tanaka, M., H. Saito, T. Kusumi, S. Fukuda, T. Shimoyama, Y. Sasaki, K. Suto, A. Munakata, and H. Kudo. 2001. Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease. *J. Gastroenterol. Hepatol.* 16: 1353–1359.
45. Mudter, J., and M. F. Neurath. 2007. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm. Bowel Dis.* 13: 1016–1023.
46. Waldner, M. J., and M. F. Neurath. 2014. Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. *Semin. Immunol.* 26: 75–79.
47. Gout, T., A. J. Ostör, and M. K. Nisar. 2011. Lower gastrointestinal perforation in rheumatoid arthritis patients treated with conventional DMARDs or tocilizumab: a systematic literature review. *Clin. Rheumatol.* 30: 1471–1474.
48. Emery, P., E. Keystone, H. P. Tony, A. Cantagrel, R. van Vollenhoven, A. Sanchez, E. Alecock, J. Lee, and J. Kremer. 2008. IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial. *Ann. Rheum. Dis.* 67: 1516–1523.
49. Rose-John, S. 2012. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *Int. J. Biol. Sci.* 8: 1237–1247.
50. Matsumoto, S., T. Hara, K. Mitsuyama, M. Yamamoto, O. Tsuruta, M. Sata, J. Scheller, S. Rose-John, S. Kado, and T. Takada. 2010. Essential roles of IL-6 trans-signaling in colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model. *J. Immunol.* 184: 1543–1551.
51. Schaper, F., and S. Rose-John. 2015. Interleukin-6: biology, signaling and strategies of blockade. *Cytokine Growth Factor Rev.* 26: 475–487.

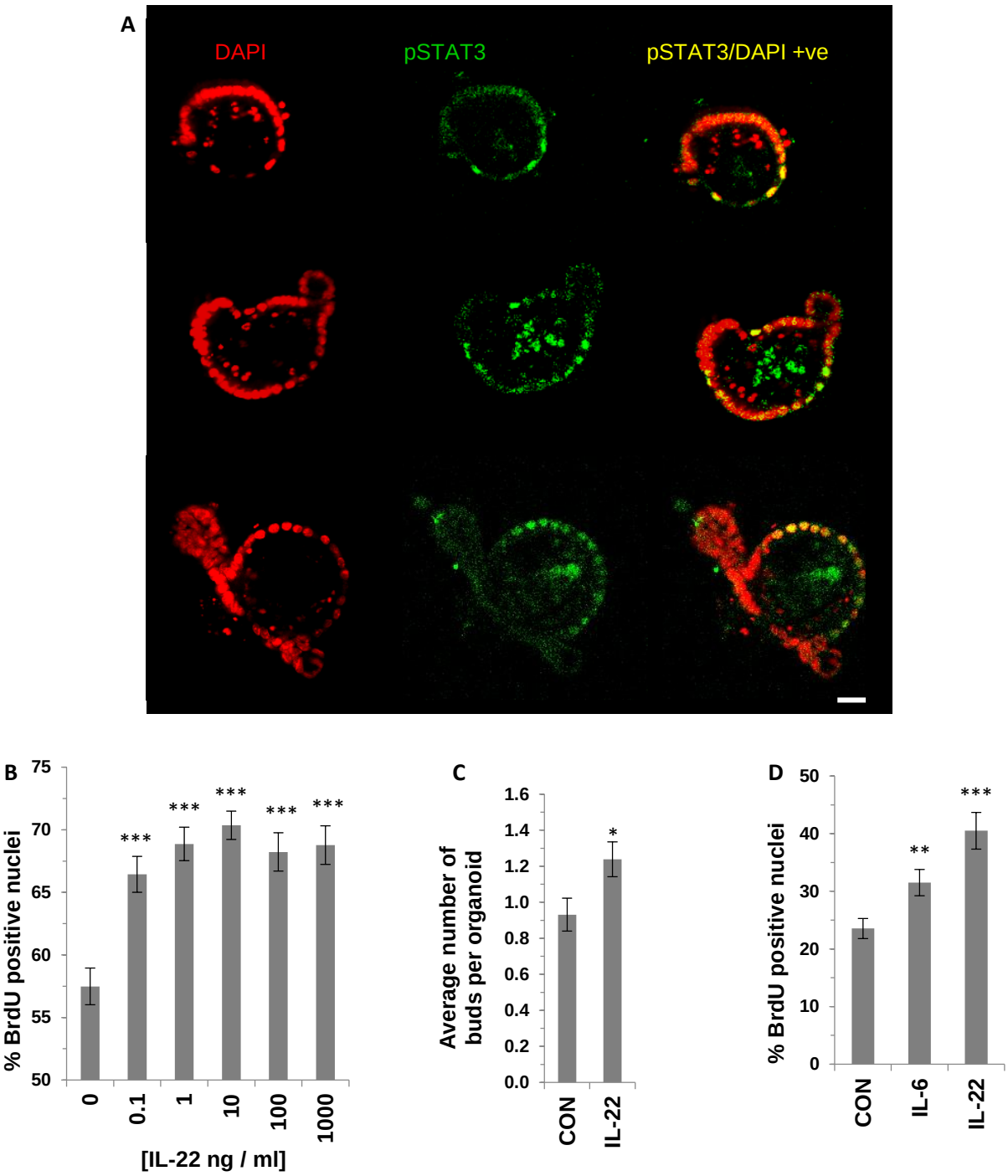



**Supplemental Figure 1. Modulation of the STAT3-IL-6 signalling-axis in small intestinal organoids.**
(**A**) Histogram showing a significant reduction in the percentage of BrdU positive nuclei in small intestinal organoids following 48 hour culture with STATTIC (20μM) in full growth factor media compared to control organoids (n=3, ***P<0.001). (**B**) Histogram showing a significant increase in BrdU incorporation following exposure to IL-6 (100ng / ml) for 5 days, media was changed every 2 days. BrdU was added for the last 18 hours of culture (n=3, **P<0.01) (**C**) Representative confocal images showing labelling of lysozyme (green) with a primary mouse anti-lysozyme antibody followed by a secondary donkey anti-mouse Alexafluor 568 antibody and absence of labelling with the mouse IgG antibody control. E-cadherin white. (**D-E**) Histograms showing a significant reduction in the percentage of BrdU positive nuclei in small intestinal organoids treated for 18 hours with IL-6 (100 ng / ml) in the presence of (**D**) an IL-6 receptor blocking (n=4, **P<0.01) or (**E**) an IL-6 neutralising antibody compared to respective IgG + IL-6 treated crypts (n=3, **P<0.01). (**F**) Histogram showing the percentage of caspase 3 positive cells in organoids following culture with an IL-6 receptor antibody or a (**G**) IL-6 neutralising antibody compared to respective IgG controls (n=3). Histograms showing the organoid survival after treatment with (**H**) an IL-6 receptor antibody or (**I**) IL-6 neutralising antibody compared to respective IgG controls (n=3) (**J**) Representative confocal images showing abrogation of IL-6-induced pSTAT3 labelling (green) in the nucleus (DAPI-red) of UEA-1 positive cells (pink) in organoids treated with an IL-6 receptor blocking antibody compared to IL-6 + IgG treated crypts. E-cadherin white. (n=3). (**K**) Histogram showing a significant reduction in IL-6-induced pSTAT3 fluorescence intensity (a.u.) in organoids treated with an IL-6 receptor blocking antibody compared to IL-6 + IgG treated crypts. Fluorescence intensity was normalised to control (n=3, ***p<0.001). (**L**) Representative confocal images showing abrogation of IL-6-induced pSTAT3 labelling (green) in the nucleus (DAPI-red) of UEA-1 positive cells (pink) in organoids treated with an IL-6 neutralising antibody compared to IL-6 + IgG treated crypts. E-cadherin white. (n=3). (**M**) Histogram showing a significant reduction in IL-6-induced pSTAT3 fluorescence intensity (a.u.) in organoids treated with an IL-6 neutralising antibody compared to IgG + IL-6 treated crypts. Fluorescence intensity was normalised to control (n=3, ***p<0.001). Data are represented as mean +/- SEM. Scale bar 10μm.

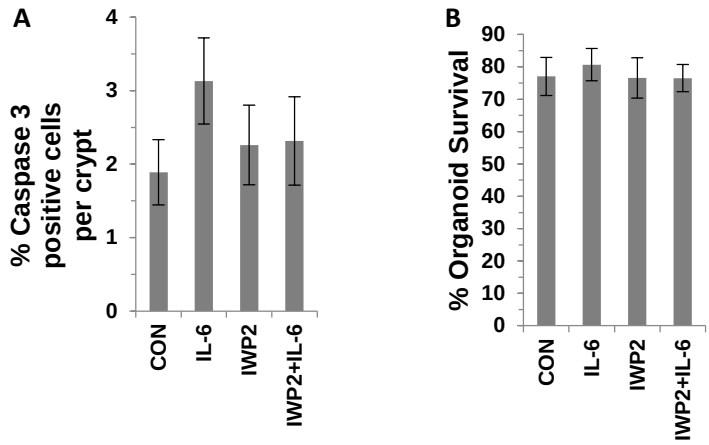



**Supplemental Figure 2. Crypt morphology and pSTAT3 labelling of IL6KO tissue is the same as WT. IL-6 receptor blocking or neutralising antibodies alter crypt stem cell and Paneth cell status.**

Histogram showing (**A**) the average crypt length (**B**) the average number of crypt nuclei and (**C**) the average number of lysozyme positive cells per crypt in the small intestine of IL-6 knockout mice and WT (n=3). Data are represented as mean +/- SEM. (**D**) Representative confocal images demonstrating *in vivo* labelling of lysozyme (red) and absence of labelling with a corresponding IgG to the mouse lysozyme antibody followed by a secondary anti-mouse IgG Alexafluor 568 antibody (red). Lysozyme positive cells (red) were also UEA-1 positive (green). E-cadherin; white and DAPI / nuclei blue. (**E-H**) Sections from IL-6 neutralising antibody, IL-6 receptor antibody and IgG treated mice. Representative confocal images showing a decrease in (**E**) Lgr5EGFP (green) , lysozyme (red) and (**F**) UEA-1 (green) positive cells in antibody treated mice compared to IgG controls. (**G**) Representative confocal images of Ki67 labelling (green). DAPI / nuclei-red, E-cadherin-blue. (**H**) Caspase 3 (red) labelling (+ve; caspase 3 positive control tissue) DAPI / nuclei-blue. (**I-K**) Representative confocal images of *in vivo* WT and IL-6KO small intestine. (**I**) A blocking peptide (BP) to pSTAT3 pre-incubated with the pSTAT3 antibody abrogated the pSTAT3 labelling in the nucleus of UEA-1 positive cells (pink) compared to tissue treated with pSTAT3 antibody (green) alone. No nuclear labelling was observed utilizing the secondary antibody ($2^0$Ab) alone. (**J**) Representative confocal images demonstrating low level pSTAT3 (green) labelling in UEA-1 (pink) positive cells of both WT and IL-6 KO mice. Images are projections of several focal planes formed using image analysis software. (**K**) Representative confocal image of high pSTAT3 fluorescent labelling observed in the mouse small intestine. Experiments were repeated on three different mice. Scale bar 10µm.




**Supplemental Figure 3. IL-22 induces global nuclear pSTAT3 activation, BrdU incorporation and crypt budding in small intestinal organoids. IL-6 and IL-22 induces BrdU incorporation in colonic organoids.**
(**A**) Representative confocal images showing the presence of pSTAT3 immunofluorescent labelling (green) in the nuclei (red) of crypt organoids following 1 hour IL-22 (100 ng / ml) stimulation. Scale bar 50 μm. (**B**) Histogram showing the percentage of BrdU positive nuclei in small intestinal organoids following 24 hours stimulation with IL-22 (0.1- 1000 ng / ml) compared to control (n=3,$^{***}$P<0.001). (**C**) Histogram showing the average number buds per small intestinal organoid following IL-22 (10 ng / ml) treatment (n=3, $^{*}$P<0.05) compared to control. (**D**) Histogram showing the percentage of BrdU positive nuclei in colonic organoids following 24 hours stimulation with IL-6 (100 ng / ml) or IL-22 (100 ng / ml) compared to control (n=3,$^{**}$P<0.01; $^{***}$P<0.001).




**Supplemental Figure 4. IWP2 has no effect on cell death and survival of small intestinal organoids.**
Histogram showing no change in (**A**) the percentage of caspase 3 positive cells per crypt or (**B**) the percentage survival of organoids, following 17h culture with IWP2 (5μM) +/- IL-6 (100 ng / ml) compared to control (DMSO).