Effect of polarisation and chronic inflammation on macrophage expression of heparan sulfate proteoglycans and biosynthesis enzymes.

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Summary

Heparan sulfate proteoglycans on immune cells have the ability to bind to and regulate the bioactivity more than 400 bioactive protein ligands, including many chemokines, cytokines and growth factors. This makes them important regulators of the phenotype and behaviour of immune cells. Here we review how heparan sulfate biosynthesis in macrophages is regulated during polarization and in chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, asthma, chronic obstructive pulmonary disease and obesity, by analyzing published micro-array data and mechanistic studies in this area. We describe that macrophage expression of many HS biosynthesis and core proteins is strongly regulated by macrophage polarization, and that these expression patterns are recapitulated in chronic inflammation. Such changes in HS biosynthetic enzyme expression are likely to have a significant impact on the phenotype of macrophages in chronic inflammatory diseases by altering their interactions with chemokines, cytokines and growth factors.

Keywords: Heparan sulfate, proteoglycans, macrophages, inflammation, rheumatoid arthritis

Macrophages perpetuate chronic inflammation

Inflammatory responses are essential for combatting infection and promoting tissue repair, and are normally quickly resolved. During chronic inflammation, unchecked inflammation persists for months to years and can contribute to the development of various diseases, such as rheumatoid arthritis (RA), cardiovascular disease and diabetes. Incidence of many chronic inflammatory diseases is on the rise, increasing the need to understand the molecular events that initiate and drive disease pathogenesis, in order to identify novel targets for intervention. For example, rheumatoid arthritis (RA) is a chronic inflammatory disease in which joint inflammation causes pain and loss of joint function. RA affects approximately 1% of the population, and while its treatment was revolutionised in the 1990s by development of anti-tumor necrosis factor (TNF)-targeted biologic therapies[1], 30-40% of patients exhibit only partial or temporary responses to treatment[2]. Novel approaches are thus required to improve treatment of RA and other chronic inflammatory conditions.

Chronic inflammatory conditions such as RA are characterised by elevated levels of pro-inflammatory cytokines (e. g. TNF, IL-1) and influx of many immune cell types into affected tissues[3]. Macrophages are a critical component of this elevated inflammatory response, and in RA, macrophage abundance in tissue correlates with joint erosion[4]. Macrophages respond to stimuli (such as danger signals) in their environment by adopting a range of activation signatures that define and regulate their biological responses. *In vitro*, two extreme activation signatures (also known as

polarisation states) are defined: namely, classically activated (pro-inflammatory; previously known as “M1”) and alternatively activated (reparative; previously known as “M2”) macrophages (Fig. 1A). Interferon-γ (IFNγ) and lipopolysaccharide (LPS) promote M1 polarisation and the expression of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF), whereas IL-4 and IL-13 result in M2 polarisation and the secretion of anti-inflammatory cytokines like IL-10. *In vivo*, macrophages are thought to adopt a range of activation states between these two extremes, fine-tuned by their local environment[5,6].

In active RA, as in many other chronic inflammatory diseases, macrophages are thought to adopt a persistently activated state, resembling an M1 signature, associated with secretion of pro-inflammatory cytokines (e.g. TNF, IL-6, IL-12 and IL-23)[7]. In several other chronic inflammatory conditions, a similar M1 signature has been demonstrated in macrophages isolated from affected tissues[8]. For example, macrophages from patients with atherosclerosis[8] and obesity[8] also exhibit an M1-like activation signature. Certain other chronic inflammatory conditions, such as asthma[9] and chronic obstructive pulmonary disease (COPD)[10], are associated with a more M2-like profile.

Macrophage phenotype is determined in response to molecules that the cells interact with in their environment. These include, for example, bacterial components (that serve as pathogen-associated molecular patterns or PAMPs), tissue components (such as extracellular matrix breakdown products that act as damage-associated molecular patterns or DAMPs), as well as cytokines, chemokines and growth factors (e.g. GM-CSF, IFNβ, IFNγ, IL-4, IL-10, IL-13) released by other cells[11–14]. The biological activity of cytokines, chemokines and growth factors in particular is highly regulated by their interaction with heparan sulfate (HS) proteoglycans, which

are structurally diverse molecules ubiquitously expressed on cell surfaces and in the extracellular matrix. HS proteoglycans consist of a core protein that carries one or more linear HS polysaccharide chains consisting of alternating N-acetylglucosamine (GlcNAc) and hexuronic acid monomers, decorated with variable patterns and levels of sulfation[15].

Heparan sulfate proteoglycans modulate the activity of inflammatory mediators

As comprehensively reviewed by Ori *et al.*[16], HS has been shown to bind to more than 400 proteins, including a multitude of chemokines, cytokines and growth factors. Several of these are known to affect macrophage activation and behaviour (Fig. 1B) and in turn their activity is modulated by binding to HS. Binding of proteins to HS can establish gradients (e. g. concentrate them on the cell surface), protect them from proteolysis, modulate their bioactivity (e. g. oligomerize chemokines) and affect receptor binding[17,18]. For example, IFNγ, which is critical for polarisation of macrophages towards a pro-inflammatory phenotype, binds to HS and this interaction inhibits IFNγ binding to its receptor[19]. Paradoxically, HS binding has been shown to increase the activity of IFNγ by promoting its local accumulation and inhibiting its rapid proteolytic degradation[20]. Many other HS binding proteins are also likely to or may affect macrophage function, e.g. Indian hedgehog (IHH) promotes alternative activation of macrophages[21].

Proteins bind to HS via positively charged amino acid residues (Arg, Lys) that interact with the negatively charged sulfate groups on HS[22]. Changes in HS structure change its binding to protein ligands, and consequently affect tissue homeostasis[23]. HS structure is dynamically regulated and has been shown to change with age[24–27], during development[28] and in many pathological contexts[29]. The structure of HS is

thought to be determined by the relative abundance of the 25 different HS biosynthetic enzymes (Fig. 2[30,31]), which collectively create a complex sulfation pattern that determines affinity for protein ligands[32]. However, little is known about the transcriptional and translational regulation of these HS biosynthetic enzymes. To date a systematic examination of how HS biosynthesis is regulated in chronic inflammation is lacking, and it is not known whether HS biosynthesis has an impact on the development of diseases such as RA.

Macrophage HS is regulated upon polarisation

Using published microarray data, we analysed expression of HS biosynthetic enzymes and core proteins in macrophages upon *in vitro* polarisation towards an M1 or an M2 phenotype[33–38]. This meta-analysis showed that HS sulfotransferase expression was largely downregulated in M1 compared to M2 macrophages, for both human and murine macrophages (Fig. 3). In line with this change in expression of HS biosynthetic enzymes, human M1 macrophages have been shown to contain half the amount of HS and have less 2-O and potentially less 3-O sulfation than M2 macrophages[39]. However, 3-O sulfation cannot be directly quantified due to the lack of commercial standards.

Macrophage HS is regulated in chronic inflammation

Similarly, we analysed published microarrays examining expression of HS-associated genes in macrophages isolated from patients with RA[40–42] (Fig. 4). This revealed that RA macrophages broadly resemble M1 macrophages in terms of HS biosynthesis gene expression, in line with studies indicating that RA macrophages exhibit an elevated chronic inflammatory state, with increased release of TNF[7]. In

contrast, macrophages from human chronic lung diseases (COPD and asthma) exhibited a more M2-like phenotype in terms of the HS biosynthesis gene expression profile, in line with reports that M2 macrophages predominate in these diseases[43–45].

Here, we examine the expression of individual HS biosynthesis enzymes and core proteins in macrophages upon polarisation, in RA, as well as in other chronic inflammatory conditions. This gives insight as to the contribution of these genes to chronic inflammation.

Polymerases

EXT1 and EXT2

Exostosin glycosyltransferase 1 (EXT1) and EXT2 act as a heterodimer to synthesise the HS backbone by alternating addition of glucuronic acid (GlcA) and GlcNAc to the growing HS chain[46]. They are thus critical for HS biosynthesis, and mutation of either enzyme causes hereditary multiple exostoses (HME), characterized by the formation of multiple tumors on the lomg bones due to abnormal HS-dependent growth factor signalling[47]. EXT1 has a strong polymerising activity, whereas EXT2 is thought to act as a chaperone bringing biosynthetic enzymes to the correct position in the Golgi[46]. It has been hypothesized that EXT1 is the rate-limiting enzyme and that EXT2 is normally synthesized in excess[48]. This is supported by the predominance of EXT1 mutations in HME and the observation that EXT1 mutations correlate with more severe disease than EXT2[49]. A study by Bao *et al.*[50] indicates that EXT expression is important in immune responses, as *Ext1* deficiency in endothelial cells in mice has been shown to impair lymphocyte recruitment in a contact hypersensitivity model due to impaired chemokine presentation, but little is known about its role in macrophages.

Expression of EXT1 was increased by treatment of human macrophages with IFNγ and LPS[34], and expression of the enzyme increased 2-fold in *in vitro*-polarised M1 macrophages (Fig. 3)[33,34]. Increased expression of EXT1 in pro-inflammatory environments may imply that HS chain elongation is stimulated by pro-inflammatory signals. In support of this, HS chain length is also increased by pro-inflammatory cytokines such TNF and decreased by M2-like stimuli such as TGFβ[51] in other cell types, such as human umbilical endothelial cells (HUVEC). EXT1 expression was increased 3-fold in RA macrophages[40,41] and also in a murine atherosclerosis model[52], in line with the more M1-like phenotype of macrophages in these diseases. In contrast, EXT1 was largely not regulated in M2-like macrophages from patients with chronic lung diseases such as asthma and COPD[43,45], and was down-regulated in macrophages in murine obesity models[53,54].

On the other hand, EXT2 appeared to be unregulated or decreased by M1 polarisation[33–36] and in RA[40]. Changes in EXT2 expression may have more limited effects on HS structure as EXT2 is normally produced in excess[48].

EXTL1-3

The EXT like (EXTL) family of genes share sequence homology with the EXTs, but their role in HS biosynthesis is poorly understood[55]. EXTL1 is not expressed by human macrophages *in vitro,* while EXTL3 is expressed but not regulated by polarisation.

Among the family, EXTL2 appears to be most regulated in macrophages and its expression is dependent on the polarisation protocol. A study in which macrophages were polarised *in vitro* with IFNγ and LPS[34] found an increase in EXTL2 expression, while studies that used only IFNγ[33,36] found downregulation of the gene. This

suggests that EXTL2 expression is increased by LPS and decreased by IFNγ. This IFNγ polarisation profile mirrors the decrease of EXTL2 in RA[40]. However, Extl2 expression was increased in murine models of other M1 chronic inflammatory diseases[45,52,53,56,57], implying that the regulation of EXTL2 expression is complex.

Busse *et al.* hypothesized that EXTL2 initiated HS side chain formation by catalysing the addition of the first GlcNAc in the Golgi[48], but subsequent studies indicated that decreased expression of EXTL2 resulted in longer HS side chains[58]. Nadanaka *et al.* proposed that the addition of the first GlcNAc to a phosphorylated tetrasacharide linker (see 'Tetrasacharide linker region' below for more detail) by EXTL2 is involved in chain termination and quality control of HS production by avoiding over- and abnormal HS production[59]. It has been demonstrated that EXTL2 deficiency results in HS overproduction and structural changes, which affected cell signalling and liver and blood vessel remodelling upon injury[60,61]. Further studies are required to understand how inflammation changes expression of the EXTL family and how this affects HS chain length, amount, quality and ultimately macrophage signaling.

N-deacetylase/N-sulfotransferase

The N-deacetylase/N-sulfotransferase (NDST) family consists of 4 isoforms that perform the first HS modification step[32]. They deacetylate GlcNAc and subsequently N-sulfate glucosamine residues. NDST1 and 2 are ubiquitously expressed, while NDST3 and 4 are mainly expressed in the brain and fetal tissues[62,63], but not in macrophages.

NDST1 and NDST2 are expressed by macrophages and upregulated in human IFNγ-polarised M1 macrophages *in vitro*[33,36], indicating a pro-inflammatory role for the

NDST enzymes. Other studies reported that inflammatory mediators such as LPS or IFNγ+TNF also increase NDST1 and/or NDST2 expression in microvascular endothelial cells[64,65]. NDST1 and NDST2 levels were also increased 1.6-fold in RA macrophages[40]. In contrast, NDST expression is overall not regulated in macrophages from patients with more M2-driven diseases, such as asthma[43,45].

NDST expression seems to be differentially regulated in human and murine macrophages. For example, Ndst1 levels decreased in mouse M1 macrophages *in vitro*[37] and Ndst1 and Ndst2 were also decreased in murine M1-driven diseases, such as cardiovascular disease (CVD)[52,57]. In line, macrophage-specific inactivation of Ndst1 (LysMCre^{+}Ndst1$^{f/f}$) in mice diminished sulfation and accelerated atherosclerosis development and diet-induced obesity. The authors proposed that HS proteoglycan sulfation is required for the suppression of basal macrophage activation through interferon-β (IFN-β) sequestration[66,67], but many other factors that bind HS might be involved in the observed phenotype of these mice. In contrast to the detrimental effect of Ndst1 deficiency in M1-diseases, deficiency of Ndst1 in the endothelium and leukocytes (TekCre^{+}Ndst1$^{f/f}$) was found to be protective against allergen-induced airway remodelling (an M2-driven disease), by reducing the recruitment of inflammatory cells and the expression of IL-13, VEGF, TGFβ and FGF-2[68]. However, from this study it cannot be concluded whether the protective effect depends on Ndst1 deficiency in the endothelium or leukocytes. Overall, this indicates that the role of NDST enzymes is highly complex and that differences in regulation of their expression between mouse and human should be taken into account when performing murine studies.

D-Glucuronyl C5-epimerase

D-Glucuronyl C5-epimerase (GLCE) converts GlcA to iduronic acid (IdoA) in an irreversible reaction that increases the flexibility of the HS chain and so modulates ligand binding[69,70]. GLCE expression was found to be downregulated in human[33–36] and mouse[37] M1 macrophages, indicating an anti-inflammatory role for this enzyme. In support, GLCE expression was also decreased in RA macrophages[40]. Currently, the functional effects of GLCE in macrophages are unknown. Glce$^{-/-}$ mice displayed neonatal lethality with defects in kidney, lung, skeletal development[71], lymphoid organ development[72] and B-cell survival and maturation[73]. It is highly probable that GLCE also affects binding and signalling in macrophages, as all the characterised protein binding HS-sites contain at least one IdoA[32].

Heparan sulfate sulfotransferases

Following epimerisation of GlcA into IdoA, one heparan sulfate 2-O sulfotransferase (HS2ST1) enzyme can O-sulfate IdoA at the carbon position 2 (Fig. 2). Finally, 3-O sulfotransferases (7 isoforms) and 6-O sulfotransferases (3 isoforms) can O-sulfate at carbons 3 and 6 of glucosamine[30,31]. Martinez *et al.* reported that M1 macrophages have decreased 2-O- and 3-O-, but not 6-O-sulfation, as determined by reverse phase high-performance liquid chromatography (RP-HPLC) and glycosaminoglycan specific antibodies[39]. The expression of HS2ST1, HS3ST1, HS3ST2 and HS6ST1 is dramatically downregulated up to 12.5-fold in human and murine M1 macrophages *in vitro*[33–35,37,38], strongly suggesting an anti-inflammatory role of O-sulfation. In line with these studies, HS2ST1, HS3ST1, HS3ST2 and HS6ST1 are also decreased up to 100-fold in macrophages from M1-driven diseases such as RA[40,41], while their expression is increased in macrophages from more M2-associated diseases, such as asthma and COPD[43,45].

In contrast to the other sulfotransferases, HS3ST3B1 is increased in M1 polarised macrophages up to 15-fold *in vitro*[34,35], implying an inflammatory role of this enzyme. In agreement, HS3ST3B1 is also upregulated 5-fold in RA macrophages[40], while it is decreased up to 3-fold in macrophages in more M2-driven diseases such as COPD[43].

Sulfotransferases are also downregulated in murine M1 macrophages *in vitro*[37], but the effects in murine disease models are more heterogenous. Hs3st1 and Hs3st2 are indeed downregulated in CVD and obesity[54,74], while Hs2st1 is slightly increased in obesity[54] and Hs6st1 is increased in CVD and obesity[52–54]. This illustrates that sulfotransferase expression is also affected by factors other than those (IFNγ, LPS, IL-4 and IL-13) which were used for the *in vitro* studies. This is supported by studies showing that TNF (but not IL-4, IL-6 and IFNγ) as well as TLR2 (peptidoglycan) and TLR4 (LPS) ligands increase HS3ST3B1 expression in human monocytes[75]. HS3ST1 is also upregulated by IL-4+IL-13 in the intestinal epithelium[76], whereas HS3ST2 is regulated by the circadium rhythm and increased by light[77,78] and HS3ST3A1 is increased by IL-6 in the canine urothelium[79]. HS6T1 expression is decreased by TGFβ lung fibroblasts[80], but not affected by TNF, IFNγ or TGFβ in renal epithelial cells[81]. Conversely, 6-O sulfation in HUVECs is reported to be decreased by stimulation with TNF and TGFβ and slightly increased upon IL-1α stimulation[51]. These studies indicate that sulfotransferases are highly regulated by inflammatory signals, but a systematic study of their regulation in macrophages and other immune cells is lacking.

Modifyers

HS can be post-synthetically modified by the extracellular 6-O endosulfatases (SULF1 and SULF2) and the endoglycosidase heparanase (HPSE)[82].

Sulfatases

Sulfatases act extracellularly to remove 6-O sulfate groups and so modulate ligand binding to HS. Moreover, it has been suggested that SULFs can also translocate to the nucleus where they might be involved in cell-cycle regulation[83]. SULF1 is not expressed by macrophages *in vitro*, while SULF2 is expressed and downregulated upon M1 polarisation *in vitro* of human and murine macrophages[33,34,37,38]. It is overall not regulated in M2-driven diseases such as asthma[45]. SULF2 appears to be variably regulated in M1-associated diseases, as its expression is decreased in obesity[54] and in one CVD[52] study, but increased in RA[40] and another CVD[57] study. This suggests that factors other than IFNγ, LPS, IL-4 and IL-13 affect SULF2 expression *in vivo*. This is supported by studies in other cell types. For example, SULF2 expression is increased by TGFβ in renal epithelial cells[81], by TNF in nucleus pulposis cells[84] and by IL-1α in HeLa cells[85]. Dhoot *et al.* demonstrated that SULF2 can be expressed as an alternatively spliced catalytically inactive variant and found that various tumors express this at high levels[86–88]. Mechanistically, the inactive variant titrates out the active variant and results in an increase in 6-O sulfation and receptor tyrosine kinase activation. It would thus also be important to check which splice variants of SULF2 are expressed in macrophages and other immune cell types during chronic inflammation.

Heparanase

The heparanase (HPSE) family consists of two isoforms: HPSE1 and HPSE2. HPSE1 degrades HS chains by specifically cleaving the bond between GlcA and GlcNAc in HS[89]. Although HPSE2 shares 40% similarity with HSPE1, it is unable to cleave HS chains[89]. HPSE1 expression is increased in various inflammatory conditions, such as inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis and also in the synovial fluid of patients with RA[90,91]. Inflammatory cytokines such as TNF, IFNγ and IL-1β increase HPSE secretion from U937 macrophages and endothelial cells[92–94]. HPSE1 is thought to increase inflammation through three different mechanisms. First, inflammation is increased in a positive feedback loop since heparanase stimulates pro-inflammatory cytokine secretion from macrophages by generating HS oligomers that signal via TLR4[95]. Secondly, increased HPSE1 expression might also indirectly enhance inflammation by accelerating HS turnover and promoting reshaping of the HS structure. In a tumor model, HPSE1 has been shown to increase levels of sulfation and consequently augment growth factor signalling[96]. Finally, HPSE1 might change the free concentration of inflammatory mediators in the microenvironment by promoting their release from HS[89].

Despite the evidence for an important inflammatory role of HPSE1 in chronic diseases, one microarray study showed a decrease in HPSE1 expression in RA macrophages[40]. It is unclear whether macrophage polarisation *in vitro* affects HPSE1 expression, as one study on human macrophages reported increased expression[33] upon polarisation with IFNγ, while murine studies reported decreased expression[37,38]. We cannot rule out differences in HPSE1 regulation in mouse and human. In addition, HPSE1 expression in other diseases does not correlate with the macrophage polarisation state. For example, its expression was decreased in

smoking and COPD[43,45] and upregulated by oxLDL[97] in human studies. In murine studies it was decreased in CVD[52], but increased in obesity[54]. It is tempting to speculate that feedback mechanisms might be in place to limit the inflammatory effects of HSPE1.

Expression of HPSE2 was also increased in RA[40], although its expression is undetectable in *in vitro*-polarised macrophages. Specific environmental cues may upregulate HPSE2 in RA. Nevertheless, the functional significance of HPSE2 is unknown.

Core proteins

HS proteoglycan core proteins carry the HS chains, and are also known to have biological functions themselves in some cases. They can be grouped into families based on their structure and location. The transmembrane syndecans (SDC; 4 members), the glycosyl phosphoinositol (GPI)-anchored glypicans (GPC; 6 members) and TGFβ receptor 3 (TGFβR3) are expressed on the cell surface, while agrin (AGRN) and perlecan (HSPG2) are present in the extracellular matrix.

Agrin and perlecan

Perlecan does not appear to be regulated by macrophage polarisation *in vitro* or by chronic inflammation, while AGRN expression is increased 2- to 2.5-fold in murine[37,38] and human[34] macrophages upon M1 polarisation *in vitro*. Inflammatory stimuli, such as IL-1β, have also been shown to increase agrin secretion from other cell types[51].

In contrast, AGRN is significantly downregulated in RA (by to 2 fold[41]) and also in other chronic inflammatory conditions (i. e. asthma[45], COPD[43] and obesity[54]), but

increased in CVD[57]. Other factors are likely to regulate AGRN expression in these contexts, or a negative feedback loop may be responsible for AGRN downregulation. Transgenic mice studies indicate that AGRN is vital for macrophage function. Agrn$^{-/-}$ mice demonstrate neonatal lethality due to neuromuscular synapse dysfunction[98]. This phenotype can be partially rescued by selective expression of Musk in skeletal muscle (*Musk-L;*Agrn$^{-/-}$)[99]. Such mice displayed reduced differentiation and viability of monocytes, as well as a reduction in subsequent differentiation into macrophages and impaired biological function, with reduced phagocytosis and cytoskeletal remodeling[99].

Glypicans

Among the glypicans, expression of GPC3 and GPC4 is regulated in macrophages upon chronic inflammation, whereas the other family members are overall not affected. For example, GPC3 is downregulated in RA and other chronic inflammatory M1- and M2-driven conditions[40,45,54,57], but is not differentially regulated by macrophage polarisation[33–38]. Therefore, mediators other than IFNγ, LPS and IL-4 might be important for GPC3 regulation. GPC3 is likely to be important for the generation of macrophages, as Gpc3$^{-/-}$ mice have impaired haematopoiesis of monocyte/macrophage progenitors[100], while peripheral cell numbers are unaffected. In addition, defective hematopoiesis has not been reported in patients with loss of function mutations in GPC3, which causes Simpson-Golabi-Behmel Syndrome. It could be hypothesized that compensatory mechanisms maintain peripheral cell numbers, but it would be interesting to address this during chronic inflammation.

In contrast, GPC4 is strongly upregulated by up to 33-fold in RA[40,41] and other M1-driven conditions such as obesity[54]. As GPC4 is decreased upon M1

macrophage polarisation *in vitro*[33], factors other than IFNγ are likely to be responsible for the upregulation of GPC4 during chronic inflammation. GPC4 expression has previously been shown to increase in the synovial lining layer and blood vessels in RA[101], but its function is currently unknown. We speculate that GPC4 contributes to inflammation in RA, since GPC4 has been shown to bind and enhance Wnt3a and Wnt5a signaling[102], which is increased in RA[103] and promotes pro-inflammatory cytokine release by macrophages (e.g. IL-6 and TNF)[104]. However, GPC4 is shed in obesity and levels of shed GPC4 correlate with BMI and insulin resistance[105]. This raises the question of whether GPC4 is shed in RA and why GPC4 is not upregulated in other chronic inflammatory conditions.

Syndecans

Sdc1 is expressed at high levels in M2 macrophages and not in M1 macrophages[106], and is thought to exert an anti-inflammatory effect by sequestering inflammatory mediators on its glycan chains[107]. Additionally, Angsana *et al*. showed that macrophage motility and consequently resolution of inflammation is impaired in Sdc1$^{-/-}$ mice[106].

Among the syndecan family, only SDC2 and SDC3 are differentially expressed in RA. SDC2 expression was found to increase slightly in RA[42], CVD[56,74] and obesity[54] models, but was also increased in M2-driven diseases, such as asthma and COPD[43–45]. Previous studies indicated that macrophages increase SDC2 expression upon IL-1α, TNF and LPS treatment[108] and IL-1β, IL-6 and TGFβ have also been shown to promote SDC2 expression in other cell types[109–111]. In contrast, SDC2 was downregulated in human macrophages upon IFNγ-dependent M1 polarisation *in vitro*[33,34,36].

SDC2 is also important for the response of macrophages to growth factors and cytokines through its core protein. For example, the core protein binds TGFβ, and SDC2 upregulates TGFβR expression and enhances its signalling[112]. Finally, SDC2 expression is increased in many cancer types, where it is important for cancer cell adhesion and migration[113]. SDC2 may also modulate immune cell adhesion and migration. Shedding of SDC2 is also increased by inflammatory signals, such as TNF[114], potentially via ADAM17 activation[115]. Such shedding may enable secondary downregulation of SDC2 biological activity on the cell surface. Moreover, levels of matrix metalloproteinases (MMP) that shed SDC2, such as MMP-7 and MMP-14, are elevated in various chronic inflammatory diseases[116,117]. Systemic study of SDC2 expression, shedding and function in chronic inflammation is required to better understand the contribution of this proteoglycan.

Little is currently know about the role and regulation of SDC3 in inflammation. In contrast to SDC2, SDC3 is downregulated in macrophages from RA[40,41] and models of M1-driven diseases such as CVD[52] and obesity[54]. SDC3 was upregulated in human macrophages by *in vitro* IFNγ polarisation, but downregulated by IFNγ and LPS, suggesting that regulation may be complex. Its biological activity is also likely to be complex, as one study demonstrated that SDC3 is pro-inflammatory in the joint, but protective in the skin[118].

Tetrasaccharide linker region

Synthesis of HS as well as chondroitin (CS) and closely related dermatan sulfate (DS) starts at the tetrasaccharide linker that attaches the sugar chain to the core protein (reviewed by Mikami *et al.*[119]), so HS biosynthesis in macrophages during inflammation might also be regulated at the level of this tetrasacharide linker.

Modifications of the linker region determine whether the linker will continue to be synthesized as either HS or CS/DS chains and can therefore act as a switch between HS or CS/DS synthesis[120].

The tetrasaccharide linker is formed by the action of 4 transferase enzymes, namely xylosyltransferase (XYLT1 and XYLT2), β1, 4-galactosyltransferase-I (GalT-I), β1,3-galactosyltransferase-II (GalT-II) and β1,3-glucoronyltransferase-I (GlcAT-I) that sequentially add a xylose, 2 galactose (Gal) and a GlcA residue[121]. Of these enzymes, XYLT1 has been found to be most drastically regulated by macrophage polarisation, with a decrease of up to a 20-fold in M1 macrophages [33–36,38] (Fig. 5) and a 3-fold decrease in expression in RA macrophages[40]. This suggests that a decrease in linker formation might be an important contributor to the decrease in HS content in M1 macrophages observed by Martinez *et al.*[39] However, fibroblasts from patients with mutations in XYLT1 produce less CS, but not less HS, suggesting that XYLT1 might be more associated with the formation of CS chains[122].

The expression of XYLT2, GalT-I and GalT-II is also downregulated in *in vitro* IFNγ–polarised macrophages[36] and macrophages from RA patients[40]. In contrast, expression of these enzymes is increased upon *in vitro* stimulation with IFNγ in combination with LPS [34,37].

All four of the sugars in the linker region can be modified to regulate the HS-CS/DS switch. The xylose subunit can be transiently phosphorylated by FAM20B at C2. This phosphorylation substantially increases addition of the third and fourth sugar units by GalT-II and GlcAT-I, and is thus a prerequisite for the completion of the linker region[123,124]. Once the linker has been synthesised, this xylose residue must be dephosphorylated for further HS/CS polymerisation. If this does not occur, EXTL2 can add a fifth (GlcNac) unit, leading to chain termination[60]. This series of events is

poorly understood. For example, while FAM20B overexpression increases both HS and CS synthesis *in vitro*[125], expression of the enzyme was increased in RA[40] as well as M1[33,34,38] macrophages, which have lower HS content[39]. Additionally, the enzyme responsible for dephosphorylating the xylose residue is unknown.

The second and third residues of the linker region can be respectively 6-O, and 6-O and 4-O sulfated, with these modifications thought to switch synthesis towards CS/DS, but little is known about the enzymes involved[118].

Finally, the fourth sugar (GlcA) can be 3-O-sulfated by human natural killer 1 sulfotransferase (HNK1ST), promoting chain termination[119]. Expression of HNK1ST is not regulated by macrophage polarisation[33-38].

Several of the enzymes responsible for synthesis of the tetrasaccharide linker region are thus regulated by macrophage polarisation and in RA, suggesting they may contribute to changes in HS synthesis in acute and chronic inflammation. Further investigation is required to evaluate the role(s) of these enzymes and whether their expression and activity may be co-ordinately regulated.

Chondroitin and dermatan sulfate

HS only accounts for 37-45% of the total GAG content in primary human M1 and M2 macrophages, while CS/DS makes up to 60%-77% of the GAG content in human macrophages[39] and the murine macrophage cell line J774A.1[126]. CS/DS differ from HS in that their backbone consists of GlcA and GalNAc monomers instead of GlcA and GlcNAc, and DS is distinguished from CS by the presence of IdoA instead of GlcA[119]. CS/DS can also be sulfated by various sulfotransferases and while CS binding to bioactive proteins is less well studied than for HS, the available data suggest that CS can bind to and modulate the activity of mediators in a similar

fashion to HS. For example, CS is known to influence Wnt3a signalling[127,128]. Our microarray analysis indicates that all of the CS sulfotransferases are downregulated in IFNγ–polarised macrophages[33,36] (Fig. 5) and overall that their expression is also decreased in RA macrophages[40,41]. Therefore, it is likely that changes in CS also contribute to altered binding of mediators in acute and chronic inflammation. This under-studied area might prove to be important for understanding macrophage behaviour during inflammation.

Conclusions and future directions

Macrophage heparan sulfate biosynthesis and core protein expression is dramatically affected by polarisation *in vitro* and upon chronic inflammation, indicating that HS is likely to be important for the function of macrophages in inflammation. While there are many similarities between HS expression profile in chronic inflammatory diseases and following *in vitro* polarisation, these patterns do not fully mirror each other, suggesting that other factors in the microenvironment fine-tune expression of these genes. The importance of microenvironment is further supported by studies showing that HS expression is not significantly different in *in vitro* monocyte-derived macrophages from the blood of patients with atherosclerosis or systemic lupus erythematosus and healthy donors[97,129–131]. Therefore, to understand the biological function of HS in macrophages, it is necessary to study macrophages isolated directly from the diseased tissue. Unfortunately, there are currently no published datasets with the proper controls to analyse macrophage expression of HS-associated genes in human atherosclerosis, obesity or IBD. Additionally, differences in the macrophage isolation method (e.g. markers for FACS sorting, microdissection)

between studies might be partly responsible for the observed differences, since purification methods can affect the purity or subset of macrophages analyzed.

It is difficult to predict how changes in expression of HS biosynthesis enzymes will specifically affect HS structure. For example, although M1 macrophages express more EXT1 and NDST1/2 than M2 macrophages, they have lower amounts of HS and lower HS sulfation. This highlights the importance of validating changes in mRNA expression at the protein level, and of analysing effects on HS structure directly. Limited availability of good antibodies for HS biosynthetic enzymes can make this challenging. Furthermore, Esko and Selleck hypothesized that HS biosynthetic enzymes might function as a physical complex, which they termed the GAGosome, in which the relative abundance of the biosynthetic enzymes affects their function and ultimately HS structure and modification[32]. Various studies demonstrate that changes in the expression of one of the biosynthesis enzymes can affect the function of other HS biosynthesis enzymes, which supports the existence of the GAGosome[32]. For example, one study showed that the relative abundance of EXT1, EXT2 and NDST1 determine the activity of NDST1[132]. Overexpression of EXT2 increased expression and activity of NDST1, while EXT1 had the opposite effect. NDST1 might compete with EXT1 for binding to EXT2 and require EXT2 for transport to the Golgi to perform its activity. In another study, inactivation of Hs2st1 ($TieCre^{+}Hs2t1^{f/f}$ mice) has been shown to not only decrease 2-O sulfation, but also to increase N-sulfation and 6-O sulfation and, as a result to alter cytokine and chemokine binding[133]. Moreover, the catalytic activity of HS3ST enzymes has been reported to depend on 2-O sulfation[134]. The complexity of these inter-relationships makes it difficult to predict how the changes in expression of HS-associated genes shown in Fig. 3 and Fig. 4 impact HS structure. Fig. 6 depicts how we think the

changes in expression of HS-associated genes in RA macrophages might affect HS structure. Nevertheless, studies directly examining macrophage heparan sulfate structure in disease are likely to be challenging, but are clearly required.

Most of the studies that address functional changes in heparan sulfate gene expression in disease focus on the role of one heparan-binding protein, with FGF, HGF, PDGF, WNT, VEGF and TGFβ commonly studied. In chronic diseases, it is likely that changes in HS structure affect more than one signalling pathway due to the heterogeneity and complexity of HS. Immune cells, such as macrophages (Fig. 1B), frequently depend on multiple signalling molecules for their function. Therefore, it would be beneficial to study differences in the whole macrophage heparan-binding proteome in chronic inflammatory diseases in an unbiased way (e.g. by quantitative mass spectrometry[135]). This approach might help to identify potential therapeutic targets to treat chronic inflammatory diseases. HS binding of these targets can be inhibited using small peptides that resemble their HS-binding site[136]. This approach has been effective for IFNγ and chemokines[137,138].

Finally, we should be careful in interpreting the effect of macrophage heparan sulfate biosynthetic enzymes and core proteins in mouse models of chronic inflammatory diseases, since some of the genes (e.g. NDST1) appear to be differentially expressed in human and mouse macrophages.

In summary, changes in expression of HS core proteins and biosynthetic enzymes have the potential to drastically alter signals that macrophages receive and therefore to affect their function in chronic inflammation. Systematic investigation of how HS changes in chronic inflammation and which signalling pathways this affects will broaden our understanding of how macrophage behaviour is regulated in chronic inflammation and disease.

Author contributions

MS performed micro-array meta-analysis, drafted and revised the review and LT critically revised the review. All authors have read and approved the final manuscript.

Competing interests

The authors declare they have no competing interests.

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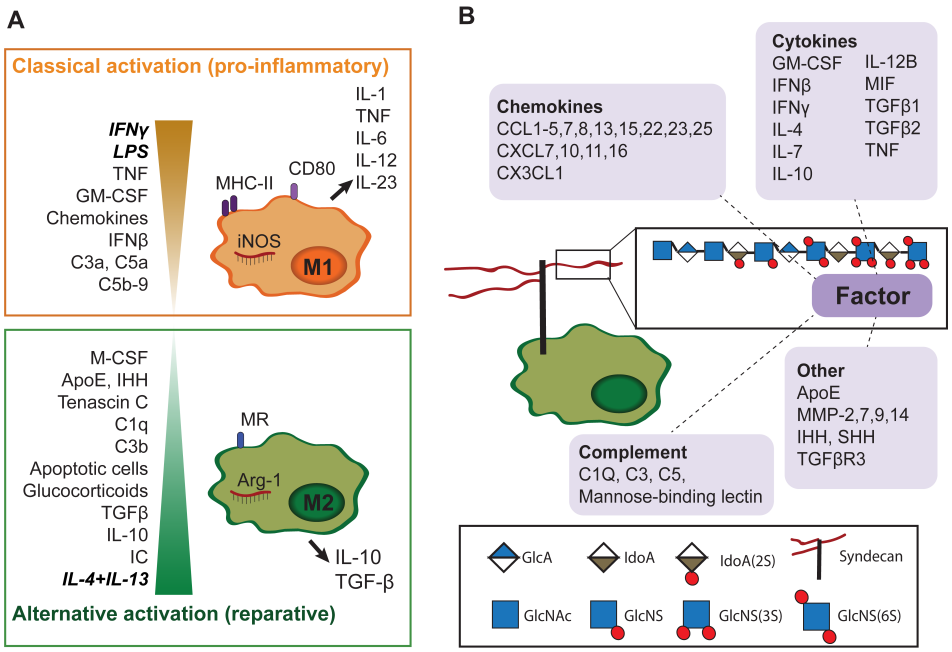

Figure 1. Several macrophage-modulating bioactive proteins bind to HS. **A**. *In vitro,* two extreme activation signatures are defined for macrophages. Interferon-γ (IFNγ) and lipopolysaccharide (LPS) or tumor necreosis factor (TNF) are responsible for classical activation (previously termed "M1"), which is characterised by nitric oxide synthase (iNOS), CD80 and MHC-II expression and secretion of pro-inflammatory cytokines. IL-4 and IL-13 result in alternative macrophage activation (previously termed "M2"), which is associated with arginase (Arg-1) and mannose receptor (MR, or CD206) expression and secretion of IL-10 and TGFβ. *In vivo*, heterogenous activation states are present, depending on stimuli in their local environment[5,6]. GM-CSF, various chemokines[139] and the complement factors C3a, C5a and C5b-9[140] also promote M1-like differentiation, while immune complexes (IC), IL-10, TGFβ, glucocorticoids[6], the complement factors C1q and C3b[140], tenascin C[14], Indian hedgehog signalling (IHH)[21] and ApoE[141] contribute to M2-like activation. Many additional factors are also likely to affect macrophage polarisation. **B**. Many HS-binding proteins (as reviewed by Ori *et al.*[16]) are known to modulate macrophage function. In addition to the well-known cytokines and chemokines[139], proteins such as complement factors can modulate macrophage activation[140]. MMPs are involved in macrophage infiltration[142–144] and MMP-7 suppress M1 polarisation, while SHH has been shown to act as a macrophage chemoattractant[145].

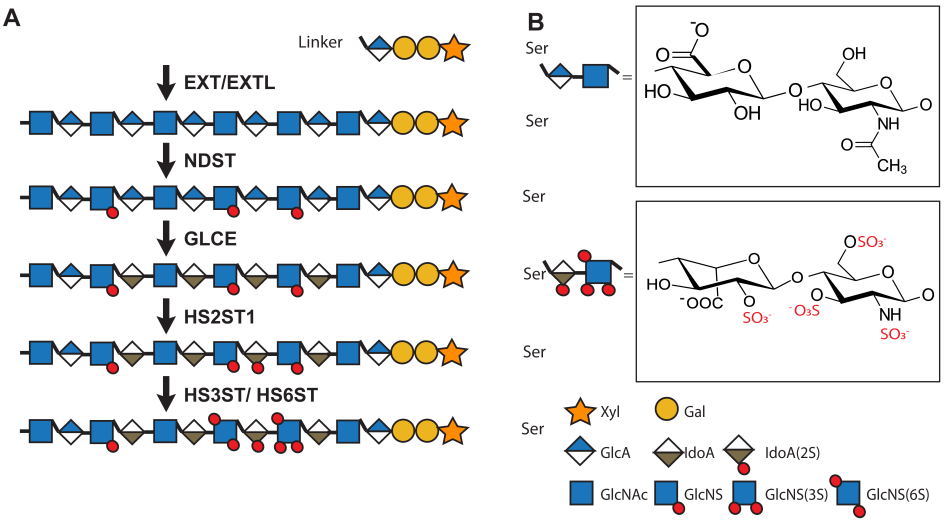

Figure 2. Enzymes responsible for heparan sulfate biosynthesis. HS biosynthesis is initiated in the Golgi by the formation of a tetrasaccharide linker region (on serine residues of the core protein) consisting of a xylose, 2 galactose and a glucuronic acid (GlcA) residue. Subsequently, HS chains are synthesized and modified in a template-independent manner by up to 25 different enzymes. First, the exostosis (EXT) and exostosin-like (EXTL) glycosyltransferase enzyme family elongate the HS chain by alternating the addition of N-acetyl glucosamine (GlcNAc) and GlcA. Next, the N-deacetylase/ N-sulfotransferase (NDST) family starts the modification of the HS backbone by deacetylating GlcNAc, followed by N-sulfation. Afterwards, glucuronic acid epimerase (GLCE) converts some GlcA residues into iduronic acid (IdoA) and a 2-O-sulfotransferase (HS2ST1) can then sulfate IdoA. Alternatively, glucosamine can be sulfated by 6-O-sulfotransferases (HS6ST1-3) and 3-O-sulfotransferases (HS3ST1-6). As these enzymes do not completely sulfate the HS chain, they generate complex sulfation patterns that form structurally diverse protein-binding sites. 6- and 3-O-sulfotransferases are thought to be particularly important, since these 10 enzymes have slightly different substrate specificities and are highly evolutionarily conserved. Even after HS biosynthesis in the Golgi system, HS can be

extracellularly modified by the endosulfatases SULF1 and SULF2, which can specifically remove sulfate groups from the 6-O position. Moreover, heparanase can degrade the HS chain into shorter oligosaccharides. Together, these biosynthetic enzymes account for the structural diversity of HS[30,31].

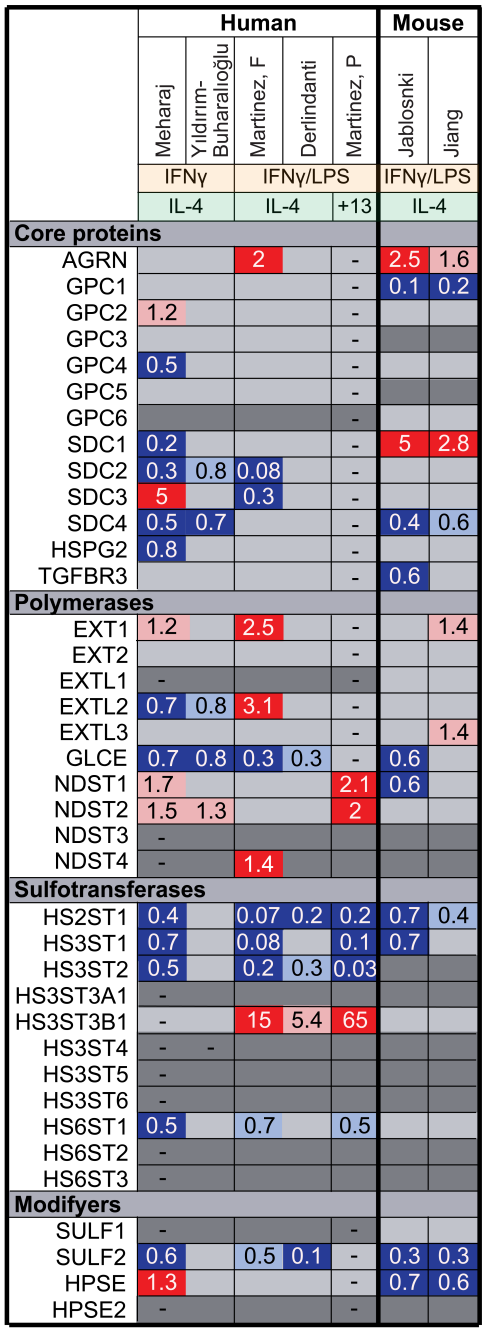

Figure 3. Fold change in the expression of HS biosynthetic enzymes and core proteins in in vitro polarised M1 compared to M2 macrophages. Using microarray data, we analysed the expression of HS core protein and biosynthetic enzymes upon macrophage polarisation *in vitro* with either IFNγ/LPS (M1) or IL-4/IL-13 (M2). Genes marked in dark gray show minimal expression in macrophages in our experience. Genes upregulated in M1 compared to M2 macrophages are marked in red (P<0.05) or pink (P>0.05), genes downregulated in M1 macrophages are marked in dark blue (P<0.05) or light blue (P>0.05) and genes that were not measured are marked with a

dash (-). Mehraj[33], Martinez F[34], Derlindanti[35], Yıldırım-Buharalıoğlu[36] compared human M1 and M2 macrophages by microarray. Jablonski[37] and Jiang[38] compared M1 and M2 macrophages by microarray from C57BL/6 or BALB/c mice respectively. Martinez P[39] performed individual qPCRs for NDST's and sulfotransferases in human M1 and M2 macrophages.

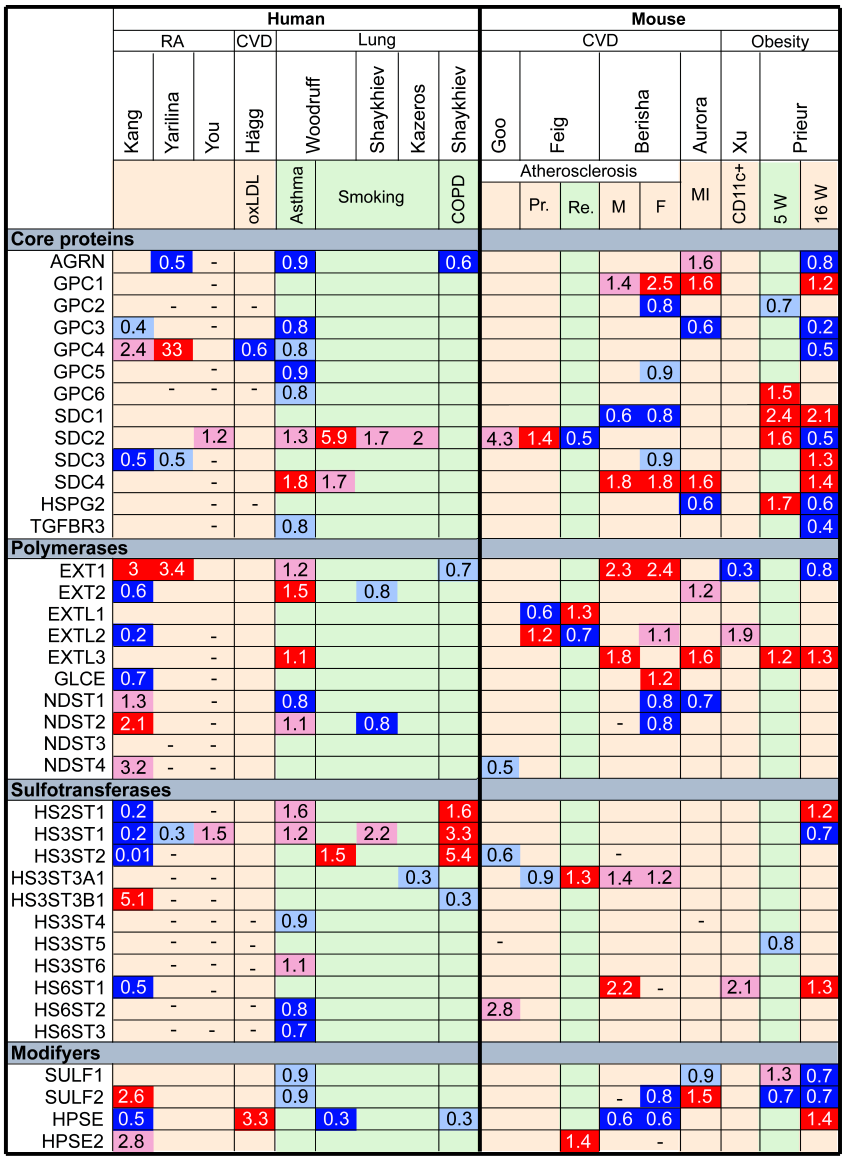

Figure 4. Fold change in the expression of HS biosynthetic enzymes and core proteins in chronic inflammatory diseases. We analysed microarray data on expression of HS core protein and biosynthetic enzymes in macrophages isolated from chronically inflamed tissues from more M1- (yellow) or M2-associated (green) diseases by FACS or laser-capture microdissection. Genes which were increased in disease compared to control are marked in red (P<0.05) or pink (P>0.05) and downregulated genes are marked in dark blue (P<0.05) or light blue (P>0.05). Kang[40], Yarilina[41] and You[42] compared freshly isolated CD14+ cells from human RA synovial fluid to CD14+ monocyte-derived macrophages (MDM) from healthy donors. Hägg[97] compared MDMs isolated from blood of patients with subclinical atherosclerosis and a family history of CVD after exposure to oxidised LDL (oxLDL) to mimic the atherosclerotic environment with those from healthy donors. Woodruff[45],

Shaykhiev[43] and Kazeros[44] compared macrophages isolated from broncheoalveolar lavage from smokers, patients with asthma or COPD to those of healthy donors. Goo[74] compared microdissected macrophages from the aorta of $ApoE^{-/-}$ mice on a high fat western-type diet (WTD) for 14 weeks to a normal diet. Feig[56] compared microdissected macrophages in a progression (Pr) or regression (Re) model of atherosclerosis, which promoted M1 or M2 polarisation respectively. Berisha[52] compared bone marrow-derived macrophages (BMDM) from male (M) or female (F) atherosclerosis-resistant $ApoE^{-/-}$ AKR mice to BMDM from atherosclerosis-prone $ApoE^{-/-}$ DBA/2 mice after loading with acetylated LDL (acLDL). Aurora[57] compared $CD11b^{+}Ly6G^{-}$ macrophages from the heart of mice 3 days after experimental myocardial infarction at postnatal day 14 (P14; non-regenerating) to P1 (regenerating). Xu[53] compared $CD11c^{+}$ and $CD11c^{-}$ (generally considered to represent M1 and M2 macrophages) $CD11b^{+}F480^{+}$ macrophages from the perigonodal cells of leptin-deficient obese mice to wild-type mice. Although HS-associated genes were differentially expressed upon obesity in $CD11c^{+}$ macrophages, no differences were observed in $CD11c^{-}$ macrophages. Prieur[54] compared $CD11b^{+}$ macrophages from gonadal white adipose tissue of leptin-deficient obese mice after 5 (5W) or 16 weeks (16W) of WTD to wild-type mice. They demonstrated that macrophages have a more M2-like phenotype after 5 weeks and more M1-like after 16 weeks of diet.

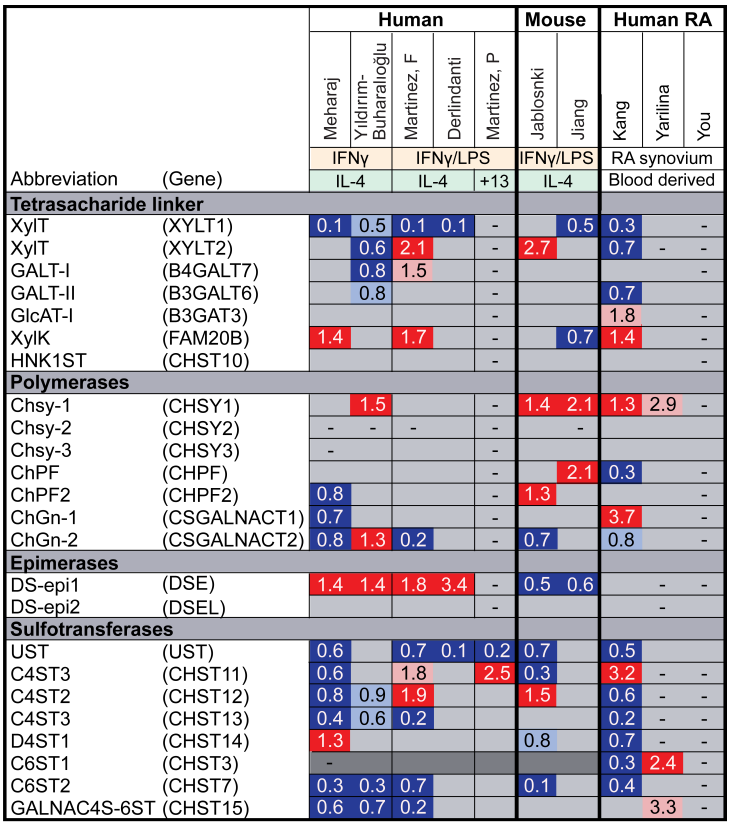

Figure 5. Fold change in the expression of enzymes involved in the formation of the tetrasaccharide linker and CS biosynthetic enzymes and core proteins upon macrophage polarisation and RA. Using microarray data, we analysed the expression of HS core protein and biosynthetic enzymes upon macrophage polarisation *in vitro* with either IFNγ/LPS (M1) or IL-4/IL-13 (M2) or in rheumatoid arthritis (RA). Genes marked in dark gray show minimal expression in macrophages in our experience. Genes upregulated in M1/ RA compared to M2/ healthy macrophages are marked in red ($P<0.05$) or pink ($P>0.05$), genes downregulated in M1/ RA macrophages are marked in dark blue ($P<0.05$) or light blue ($P>0.05$) and genes that were not measured are marked with a dash (-). Mehraj[33], Martinez F[34], Derlindanti[35], Yıldırım-Buharalıoğlu[36] compared human M1 and M2 macrophages by microarray. Jablonski[37] and Jiang[38] compared M1 and M2 macrophages by microarray from C57BL/6 or BALB/c mice respectively. Martinez P[39] performed individual qPCRs for NDST's and sulfotransferases in human M1 and M2 macrophages. Kang[40], Yarilina[41] and You[42] compared CD14+ cells freshly isolated by FACS from human RA synovial fluid to CD14+ monocyte-derived macrophages (MDM) from healthy donors.

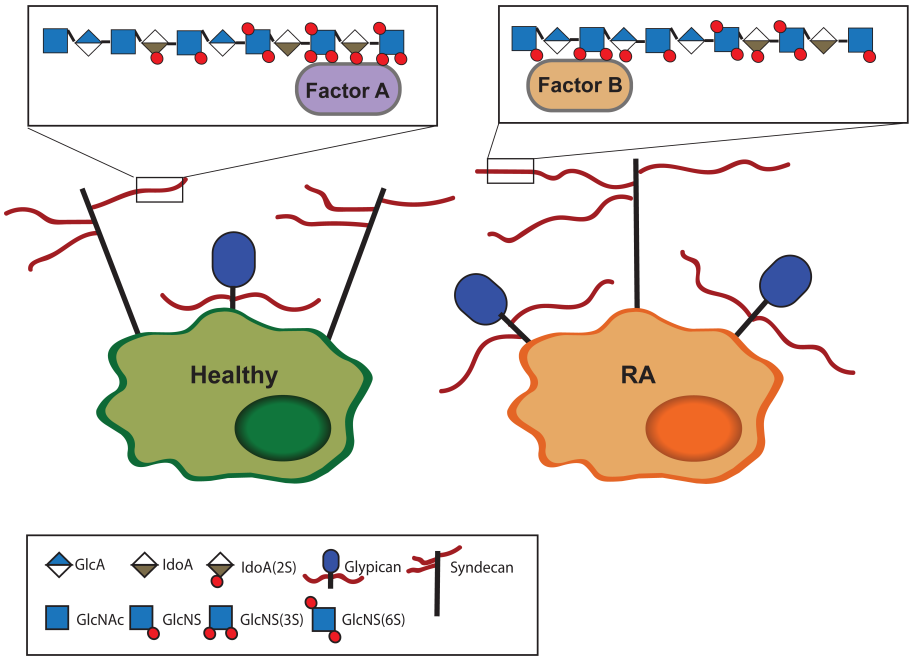

Figure 6. Schematic summary of changes in HS biosynthesis in macrophages from RA patients**.** Analysis of microarray studies demonstrated that macrophages from RA synovial fluid express more glypican-4. As EXT1 was also increased, we hypothesize that the HS chain length is increased in RA. NDST expression was also increased, suggesting increased N-sulfation, while decreased GLCE and 2-O sulfotransferase expression suggest decreased epimerisation and 2-O sulfation. Decreased 6-O sulfatase expression combined with an increase in SULF2 point to a net decrease in 6-O sulfation. The pattern of 3-O sulfation might also be altered, as the HS3ST1 and 2 isoforms are decreased, while HS3ST3B1 was increased. Nevertheless, expression data alone cannot be used to predict HS structure and studies directly studying HS structure are required. Abbreviations: GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc , N-acetyl glucosamine; GlcNS, N-sulfated glucuronic acid; NS, N-sulfation; 2S, 2-O sulfation; 3S, 3-O sulfation; 6S, 6-O sulfation.