



# Proteolytic Cleavage and Loss of Function of Biologic Agents That Neutralize Tumor Necrosis Factor in the Mucosa of Patients With Inflammatory Bowel Disease

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**BACKGROUND & AIMS:** Many patients with inflammatory bowel disease (IBD) fail to respond to anti-tumor necrosis factor (TNF) agents such as infliximab and adalimumab, and etanercept is not effective for treatment of Crohn's disease. Activated matrix metalloproteinase 3 (MMP3) and MMP12, which are increased in inflamed mucosa of patients with IBD, have a wide range of substrates, including IgG<sub>1</sub>. TNF-neutralizing agents act in inflamed tissues; we investigated the effects of MMP3, MMP12, and mucosal proteins from IBD patients on these drugs. **METHODS:** Biopsy specimens from inflamed colon of 8 patients with Crohn's disease and 8 patients with ulcerative colitis, and from normal colon of 8 healthy individuals (controls), were analyzed histologically, or homogenized and proteins were extracted. We also analyzed sera from 29 patients with active Crohn's disease and 33 patients with active ulcerative colitis who were candidates to receive infliximab treatment. Infliximab, adalimumab, and etanercept were incubated with mucosal homogenates from patients with IBD or activated recombinant human MMP3 or MMP12 and analyzed on immunoblots or in luciferase reporter assays designed to measure TNF activity. IgG cleaved by MMP3 or MMP12 and antihinge autoantibodies against neo-epitopes on cleaved IgG were measured in sera from IBD patients who subsequently responded (clinical remission and complete mucosal healing) or did not respond to infliximab. **RESULTS:** MMP3 and MMP12 cleaved infliximab, adalimumab, and etanercept, releasing a 32-kilodalton Fc monomer. After MMP degradation, infliximab and adalimumab functioned as F(ab')<sub>2</sub> fragments, whereas cleaved etanercept lost its ability to neutralize TNF. Proteins from the mucosa of patients with IBD reduced the integrity and function of infliximab, adalimumab, and etanercept. TNF-neutralizing function was restored after incubation of the drugs with MMP inhibitors. Serum levels of endogenous IgG cleaved by MMP3 and MMP12, and antihinge autoantibodies against neo-epitopes of cleaved IgG, were higher in patients who did not respond to treatment vs responders. **CONCLUSIONS:** Proteolytic degradation may contribute to the nonresponsiveness of patients with IBD to anti-TNF agents.

Biologic therapies with agents that neutralize tumor necrosis factor (TNF) have revolutionized the treatment of human chronic inflammatory diseases.<sup>1</sup> Responsiveness, however, is difficult to predict, and there may be differences in efficacy and mode of action between different agents.<sup>2–4</sup> In particular, infliximab and adalimumab are monoclonal IgG<sub>1</sub> antibodies, and etanercept is a dimeric p75 TNF receptor-IgG Fc fusion protein.<sup>5,6</sup> The fact that etanercept is not helpful in Crohn's disease,<sup>7</sup> but is effective in rheumatoid arthritis and psoriasis,<sup>8,9</sup> remains unexplained. Moreover, information on the bioavailability of drugs in inflamed tissues, where TNF-neutralizing agents are expected to exert their action, is completely lacking.

Increased tissue levels of proteases, including matrix metalloproteinases (MMPs), are seen in inflammatory bowel disease (IBD), rheumatoid arthritis, asthma, and chronic obstructive pulmonary disease.<sup>10–14</sup> In particular, MMP3, MMP12, and MMP9 are highly up-regulated in the inflamed gut of IBD patients.<sup>12–14</sup> Interestingly, IgG<sub>1</sub> has a Thr-His human neutrophil elastase cleavage site in the upper hinge, and there is a Pro-Glu scissile bond susceptible to cleavage by MMP3 and MMP12 in the lower hinge.<sup>15,16</sup> Trastuzumab, a monoclonal antibody used in the treatment of breast cancer, can be degraded by a number of proteolytic enzymes, with consequent reduction of its immune effector functions.<sup>17</sup> Moreover, cleaved IgG has been detected within squamous cell carcinoma with a predominant localization at the invasive front, a site particularly rich in proteases.<sup>18</sup>

We therefore have examined the effects of MMPs and mucosal homogenates from inflamed human bowel on the integrity and function of commonly used TNF-neutralizing agents.

**Abbreviations used in this paper:** ELISA, enzyme-linked immunosorbent assay; HC, healthy control; IBD, inflammatory bowel disease; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

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## Patients and Methods

### Patients

This study was approved by the London City and Hackney Ethics Committee, and informed consent was obtained from all subjects. To assess the effect of proteins from human gut mucosa on the integrity and function of TNF-neutralizing agents, perendoscopic biopsy specimens from the inflamed colon of 8 Crohn's disease and 8 ulcerative colitis patients (Table 1) and from the normal colon of 8 healthy control (HC) subjects were collected and processed for routine histology or protein extraction after homogenization. Sera from 29 active Crohn's disease and 33 active ulcerative colitis patients, who were candidates to receive infliximab treatment, were collected. Responsiveness to infliximab treatment was defined as the concomitant presence of clinical remission and complete mucosal healing<sup>19</sup> at the first endoscopic examination after starting treatment.

### Protein Extraction

Biopsy specimens were placed in phosphate-buffered saline (PBS) (Oxoid, Ltd, Basingstoke, UK) and homogenized by sonication. Endogenous IgG was removed from the mucosal proteins by the Protein G SpinTrap (GE Healthcare, Little Chalfont,

UK), and the total protein concentration was normalized to 2 mg/mL by dilution in PBS.

### MMP Activity Assay

MMP3/MMP12 activity in IBD and HC homogenates was measured using the fluorescence resonance energy transfer-based Sensolyte 520 MMP12 assay kit from AnaSpec, Inc (Fremont, CA).

### Cleavage Reactions

Upon activation, recombinant human MMP3, MMP12, and MMP9 (R&D Systems, Abingdon, UK) were co-incubated for 24 hours with infliximab (Remicade; Schering-Plough, Welwyn Garden City, UK), adalimumab (Humira; Abbott Laboratories, Chicago, IL), or etanercept (Enbrel; Wyeth, Maidenhead, UK) to evaluate their effect on drug integrity and function. Moreover, infliximab, adalimumab, or etanercept was co-incubated for 24 hours with either PBS only or with IBD or HC mucosal homogenates. In parallel, cleavage reactions were performed with the addition of the MMP inhibitors marimastat (Sigma-Aldrich, Poole, UK) or UK370106 (Santa Cruz Biotechnology, Dallas, TX). Details of cleavage reactions are reported in the [Supplementary Materials and Methods](#) section. Cleavage reaction products then were stored at -70°C until further analysis.

### Immunoblotting

The effect of proteases on the integrity of TNF-neutralizing agents was assessed by immunoblotting under denaturing conditions. Rabbit anti-human immunoglobulin Fc antibody (Fisher Scientific UK, Loughborough, UK) or rabbit anti-human immunoglobulin  $\kappa$  light-chain antibody (Abcam, Cambridge, UK) were used as primary antibodies. Bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

### Protein-G Binding Assay

The effect of proteases on the Fc region of TNF-neutralizing agents was assessed by a protein-G binding assay. Untreated or MMP3-/MMP12-treated TNF-neutralizing agents were added to the wells of a protein-G-coated plate (Fisher Scientific UK). Because chicken antibodies are unable to bind to protein-G, chicken anti-human p75 TNF-receptor antibody (Abcam), previously conjugated with horseradish peroxidase using a commercial kit (Abcam), or horseradish peroxidase-conjugated chicken anti-human F(ab')<sub>2</sub> (Fisher Scientific UK), were added to it as a method to detect, respectively, the amount of etanercept or infliximab and adalimumab bound to the plate. Tetramethylbenzidine then was added and after stopping the reaction the optical density was measured on a plate reader.

### Luciferase Assay

The effect of proteases on the TNF-neutralizing function of anti-TNF agents was assessed by means of a HeLa cell line, which had been stably transfected with the luciferase reporter gene under the control of nuclear factor- $\kappa$ B-enhancer elements.<sup>20</sup> HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF (R&D Systems),

**Table 1.** Clinical Features of 16 IBD Patients

	CD (n = 8)	UC (n = 8)
Median age, y (range)	28 (20–55)	30.5 (22–63)
Male/female (%)	5/3 (62.5/37.5)	4/4 (50/50)
Median duration of disease, y (range)	4 (0.5–11)	3 (0–15)
Extent of disease		
Ileocolon-CD (%)	4 (50)	NA
Colon-CD (%)	4 (50)	NA
Left-sided colitis-UC (%)	NA	5 (62.5)
Pancolitis-UC (%)	NA	3 (37.5)
Disease behavior		
Nonstricturing nonpenetrating-CD (%)	8 (100)	NA
Stricturing-CD (%)	0 (0)	NA
Penetrating-CD (%)	0 (0)	NA
Clinical disease activity		
Mild (%)	0 (0)	0 (0)
Moderate (%)	3 (37.5)	2 (25)
Severe (%)	5 (62.5)	6 (75)
Grading of histologic inflammation		
Mild (%)	0 (0)	0 (0)
Moderate (%)	0 (0)	1 (12.5)
Severe (%)	8 (100)	7 (87.5)
Treatment		
None	1 (12.5)	2 (25)
Mesalamine (%)	2 (25)	4 (50)
Corticosteroids (%)	3 (37.5)	4 (50)
Immunosuppressants		
Azathioprine/6-mercaptopurine (%)	2 (25)	3 (37.5)
Methotrexate (%)	1 (12.5)	0 (0)
Corticosteroids + immunosuppressants (%)	3 (37.5)	3 (37.5)

CD, Crohn's disease; UC, ulcerative colitis.

with or without increasing concentrations of infliximab, adalimumab, or etanercept, all pre-incubated for 24 hours with MMP activation buffer or active MMP3, MMP12, PBS, or mucosal homogenates prepared in PBS. In parallel, the same experiments were performed with the addition of the MMP inhibitors marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). The detailed description of experimental conditions is reported in the [Supplementary Materials and Methods](#) section. After culture, HeLa cells were lysated and assayed for luciferase activity with a commercial kit (Promega UK, Southampton, UK), following the manufacturer's instructions, using a luminometer.

### Clipped Endogenous IgG Detection

The presence of clipped endogenous IgG in IBD and HC sera was assessed by a new enzyme-linked immunosorbent assay (ELISA), by coating the plates with streptavidin (Sigma-Aldrich), followed by incubation with biotinylated rabbit anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody (kindly provided by R. J. Brezski), then serial dilutions of IBD and HC sera, followed by a horseradish peroxidase-conjugated donkey anti-human IgG heavy- and light-chain antibody (1:10,000; Jackson ImmunoResearch, West Grove, PA). The detailed description of the ELISA is reported in the [Supplementary Materials and Methods](#) section.

### Antihinge Autoantibody Detection

Antihinge autoantibodies against the immunogenic neopeptides generated by the proteolytic cleavage of endogenous IgG were assessed in IBD and HC sera by a new ELISA, by coating the plates with streptavidin (Sigma-Aldrich), followed by incubation with 1  $\mu\text{mol/L}$  biotinylated 14mer peptide analogue of the human IgG<sub>1</sub> hinge with a C-terminal amino acid correspondent to the MMP3/MMP12 cleavage site (kindly provided by R. J. Brezski), then serial dilutions of IBD and HC sera, followed by a horseradish peroxidase-conjugated goat anti-human IgG Fc antibody (1:10,000; Jackson ImmunoResearch). The detailed description of the ELISA is reported in the [Supplementary Materials and Methods](#) section.

### Statistics

Data were analyzed using the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) by means of comparisons between groups using the Mann-Whitney *U* test or the Student *t* test. A level of  $P < .05$  was considered statistically significant.

## Results

### Effect of MMP3, MMP12, and MMP9 on the Integrity and Function of TNF-Neutralizing Agents

We co-incubated increasing concentrations (0.001–1  $\mu\text{g/mL}$ ) of MMP3 or MMP12, or 1  $\mu\text{g/mL}$  MMP9 for 24 hours with 1  $\mu\text{g/mL}$  infliximab, adalimumab, and etanercept, and analyzed cleavage patterns in reducing gels by immunoblotting using an anti-human IgG Fc antibody. Infliximab, adalimumab, and etanercept were clipped in a concentration-dependent manner by both MMP3 and

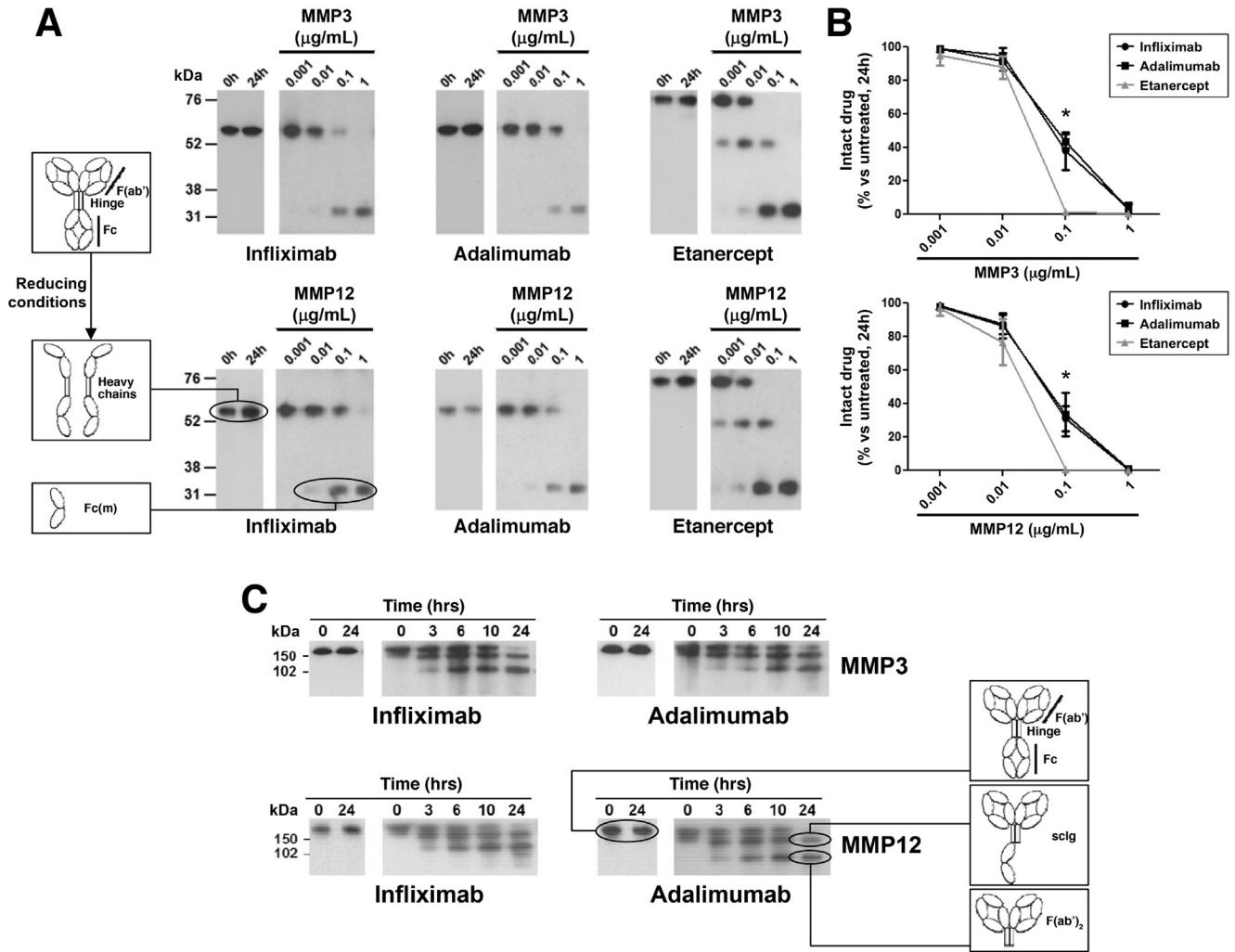
MMP12, with the formation of a 32-kilodalton fragment ([Figure 1A](#)), which likely corresponds to the Fc monomer observed after MMP digestion of native human IgG<sub>1</sub>.<sup>15</sup> An additional approximately 50-kilodalton etanercept fragment appeared at low MMP concentrations. Densitometry of the percentage of intact drug remaining at each concentration showed that etanercept was markedly more susceptible to clipping than infliximab and adalimumab ([Figure 1B](#)). Treatment of each TNF-neutralizing agent with a fixed concentration (1  $\mu\text{g/mL}$ ) of MMP3 or MMP12 and blotting in nonreducing gels with anti-human immunoglobulin  $\kappa$  light chain showed that degradation of infliximab and adalimumab occurs in a time-dependent sequential manner, with the formation of single-cleaved, upon removal of 1 Fc monomer, and, subsequently, double-cleaved, F(ab')<sub>2</sub>, intermediate products ([Figure 1C](#)). Etanercept could not be visualized using anti-human immunoglobulin  $\kappa$  primary antibody. Infliximab, adalimumab, and etanercept were not cleaved by MMP9 ([Supplementary Figure 1](#)).

We next performed an ELISA using protein-G-coated plates as another way to look at loss of Fc. Untreated TNF-neutralizing agents bound strongly to protein G, but after 24 hours of incubation with MMPs, binding decreased in a dose-dependent fashion ([Figure 2A](#)). Etanercept binding was minimal after MMP treatment.

We then investigated the effect of MMP3 and MMP12 on the ability of biologic agents to neutralize soluble TNF. In the absence of MMPs, infliximab, adalimumab, and etanercept effectively inhibited TNF-induced luciferase activity ([Figure 2B](#)). Neutralization of TNF by infliximab and adalimumab did not change after overnight incubation with MMP3/MMP12, whereas etanercept showed a significant reduction in its ability to neutralize TNF.

### Proteolytic Cleavage of TNF-Neutralizing Agents by IBD Mucosal Proteins

We also prepared protein homogenates from normal HC and inflamed IBD colonic mucosa, and we observed that IBD protein extracts have significantly higher MMP3 and MMP12 activity compared with HC homogenates ([Figure 3A](#)). No significant correlation was found between the degree of histologic inflammation in IBD biopsy specimens and the level of MMP3 and MMP12 activity in paired IBD homogenates. We subsequently co-incubated IBD and HC homogenates with TNF-neutralizing agents. No cleavage by HC homogenates was detected, whereas infliximab, adalimumab, and etanercept were cleaved by IBD proteins ([Figure 3B](#)), suggesting that the increased proteolytic activity present in chronically inflamed tissues may affect drug stability. Although infliximab and adalimumab were partially cleaved, intact etanercept was completely lost after co-incubation with IBD homogenates. In some cases we could observe the 32-kilodalton fragment, indicative of lower hinge cleavage by MMP3/MMP12 ([Figure 3B](#), patient 2); nevertheless, the heterogeneous clipping profile between different patients suggests that the tissue proteolytic profile varies among individuals and other proteases also may contribute to IgG cleavage. At low concentrations (0.1  $\mu\text{g/mL}$ ),

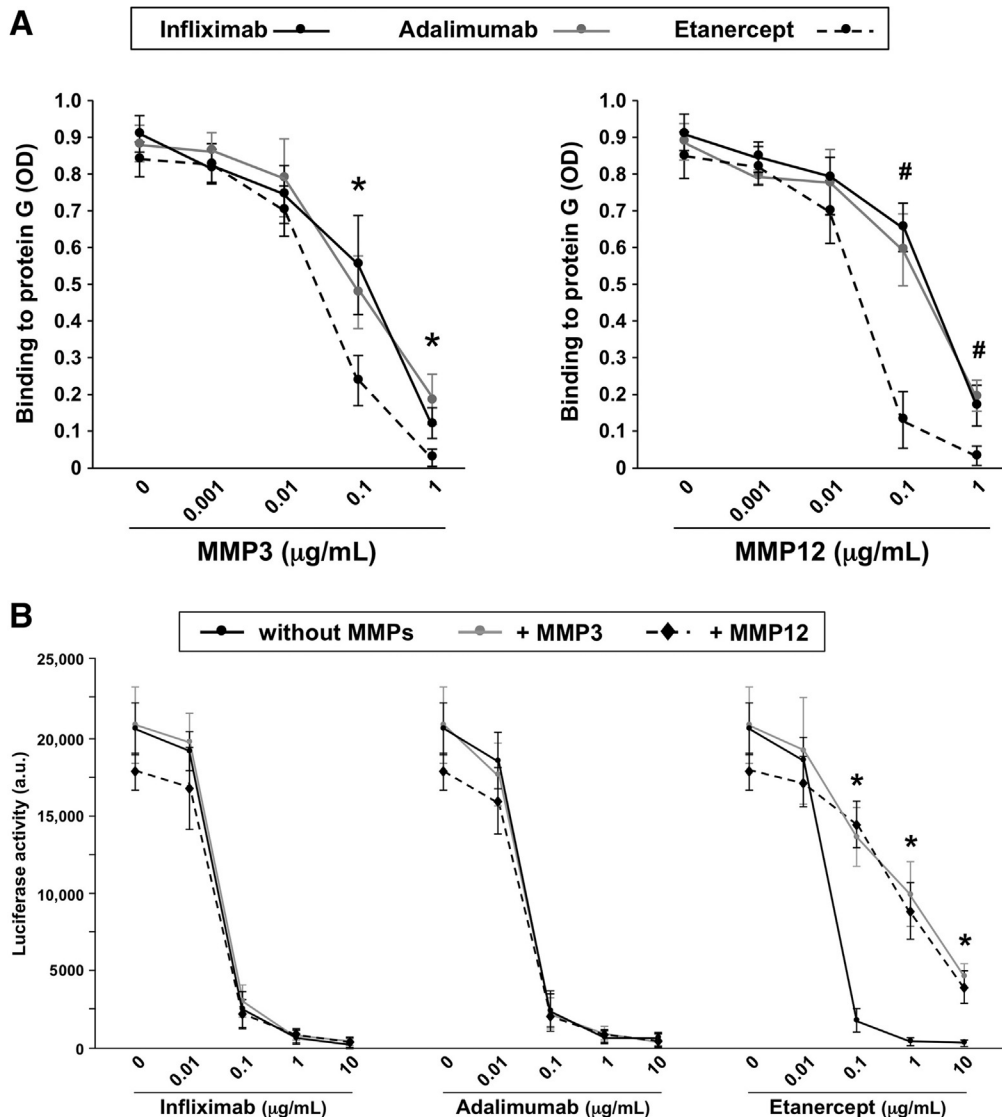


**Figure 1.** Degradation of TNF-neutralizing agents by MMP3 and MMP12. (A) Infiximab, adalimumab, and etanercept (1 μg/mL) were co-incubated at 37°C for 24 hours with increasing concentrations (0.001–1 μg/mL) of activated recombinant human MMP3 or MMP12, and were visualized by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. All TNF-neutralizing agents were cleaved by MMP3 or MMP12 with the formation of a 32-kilodalton band, likely to correspond to the Fc monomer [Fc(m)] observed after MMP digestion of native human IgG<sub>1</sub>. Cleavage occurred in a concentration-dependent manner, as shown by the increasing intensity of the 32-kilodalton band at higher MMP concentrations and the decreasing intensity of the bands correspondent to the intact heavy chain of infiximab and adalimumab and to the intact etanercept monomer. *Blots* are representative of 6 separate experiments. (B) Densitometry of the intact infiximab and adalimumab heavy chain, and of the intact etanercept monomer, after co-incubation with MMP3 or MMP12. Results are the mean percentage of the intact drug after MMP treatment compared with the correspondent untreated TNF-neutralizing agent ± SD (n = 6). \*P < .005 vs MMP3-/MMP12-treated etanercept at the correspondent MMP concentration. (C) Infiximab and adalimumab, each at a concentration of 1 μg/mL, were co-incubated at 37°C with 1 μg/mL activated recombinant human MMP3 or MMP12. The incubation was interrupted at fixed time points (3, 6, 10, and 24 hours), and the TNF-neutralizing agents were visualized by immunoblotting in nonreducing conditions using an anti-human immunoglobulin κ light-chain antibody. Infiximab and adalimumab were cleaved in a time-dependent manner by either MMP3 or MMP12 with the sequential formation of 2 additional approximately 130- and approximately 100-kilodalton bands, which likely correspond to the single-cleaved immunoglobulin intermediate (scIg) and F(ab)<sub>2</sub> fragments, respectively, and which increased in intensity, in parallel with the fading of the bands correspondent to the intact drugs, over time. *Blots* are representative of 3 separate experiments.

infiximab and adalimumab showed a reduction in their ability to neutralize soluble TNF after co-incubation with IBD proteins (Figure 3C). Etanercept largely lost its ability at all doses to neutralize TNF after co-incubation with IBD proteins. As expected, TNF concentration in both Crohn's disease and ulcerative colitis homogenates was significantly

higher compared with HC homogenates, however, the mean concentration of TNF in IBD homogenates was approximately 200 times lower than the concentration of exogenous TNF used for HeLa cell stimulation (Supplementary Figure 2A). Culture with IBD homogenates induced a small, not significant, increase in luciferase production by HeLa cells, both in





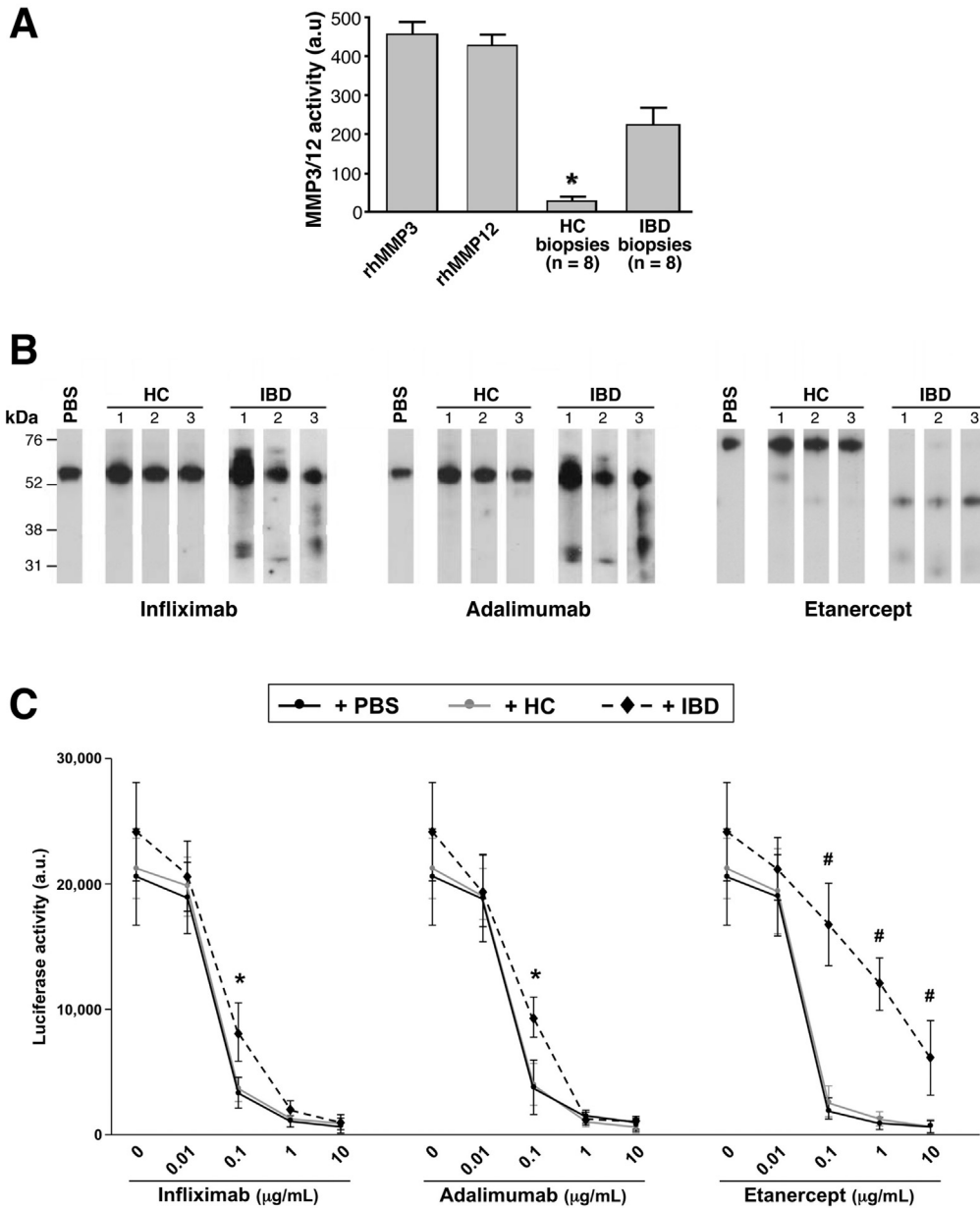
**Figure 2.** Effect of MMP3 and MMP12 on protein-G binding capacity and TNF-neutralization function of anti-TNF agents. (A) Untreated or MMP3- and MMP12-treated TNF-neutralizing agents were added to the wells of protein-G-coated plates. Horseradish peroxidase-conjugated chicken anti-human F(ab')<sub>2</sub> or anti-human TNF-receptor II antibodies, which do not bind to protein-G, were used to detect and quantify protein-G binding to TNF-neutralizing agents. Values, expressed as optical density (OD), are mean  $\pm$  SD of 6 separate experiments. \* $P < .01$  vs etanercept at the corresponding MMP concentration. # $P < .005$  vs etanercept at the corresponding MMP concentration. (B) Nuclear factor- $\kappa$ B reporter HeLa cells were cultured for 6 hours with TNF, with increasing concentrations (0.01–10  $\mu$ g/mL) of infliximab, adalimumab, or etanercept, pre-incubated for 24 hours with TCNB buffer (without MMPs), or 1  $\mu$ g/mL active MMP3 or MMP12. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means  $\pm$  SD of 6 separate experiments. \* $P < .005$  vs TNF plus TCNB-treated etanercept at the correspondent concentration.

the absence and presence of exogenous TNF (Supplementary Figure 2B).

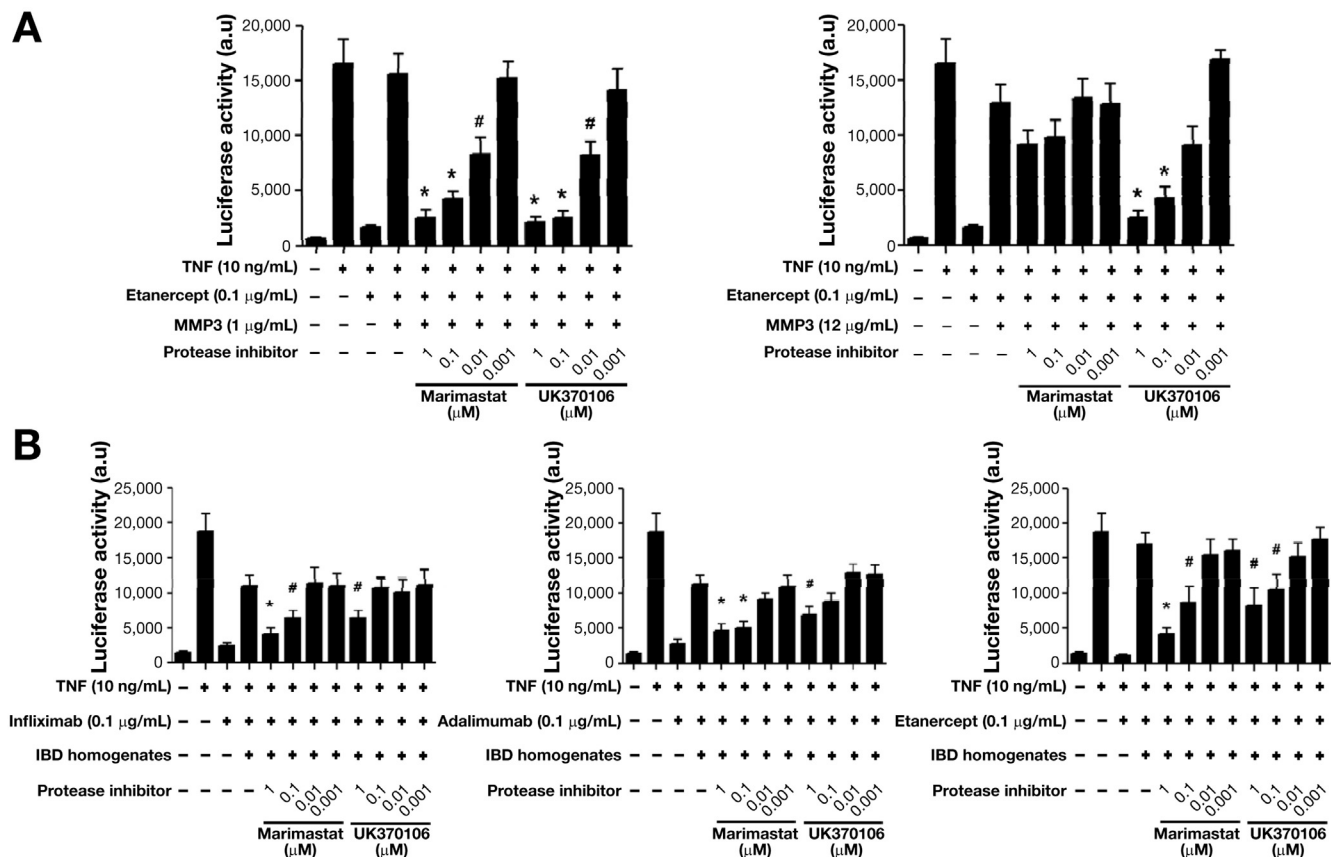
### Effect of MMP Inhibitors on Proteolytic Cleavage of TNF-Neutralizing Agents

We then evaluated the effect of the broad-spectrum MMP inhibitor marimastat or the selective MMP3/MMP12 inhibitor UK370106 on the function of TNF-neutralizing agents exposed to MMPs or IBD mucosal proteins. Although the addition of UK370106 to both MMP3 and

MMP12 effectively restored TNF-neutralizing function of etanercept in a dose-dependent manner, marimastat was able to prevent only MMP3- but not MMP12-induced loss of TNF-neutralizing ability of etanercept (Figure 4A). Conversely, both marimastat and, to a lesser extent, UK370106 were able to restore the ability of infliximab, adalimumab, and etanercept to neutralize TNF on exposure to IBD homogenates (Figure 4B). Neither marimastat nor UK370106 had any effect on luciferase production by HeLa cells, both in the absence and presence of exogenous TNF (Supplementary Figure 2B).



**Figure 3.** Effect of mucosal homogenates on the integrity and function of TNF-neutralizing agents. (A) MMP3 and MMP12 activity in the homogenates of normal colonic mucosa of 8 HC subjects and inflamed colonic mucosa of 8 IBD patients. Bars are mean  $\pm$  SD. Active recombinant human (rh)MMP3 and rhMMP12 have been used as positive controls. \* $P < .01$  vs IBD mucosa, rhMMP3, and rhMMP12. (B) Infliximab, adalimumab, and etanercept, all at a concentration of 1  $\mu$ g/mL, were co-incubated for 24 hours with PBS only or with mucosal proteins from the colon of HC subjects or from the colon of IBD patients, and subsequently were visualized by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. The heavy chains of infliximab and adalimumab appeared as a single band of approximately 55 kilodaltons, remained intact after 24 hours co-incubation with PBS or HC homogenates, and were partially cleaved by IBD mucosal homogenates with the appearance of 32-, approximately 35-, and approximately 40-kilodalton fragments. Etanercept, which was minimally cleaved by HC homogenates, was cleaved completely by inflamed IBD homogenates with the formation of 32- and approximately 40-kilodalton fragments. The 32-kilodalton band is consistent with lower-hinge cleavage. Blots are representative of experiments performed with colonic mucosal proteins from 6 HC subjects and 6 IBD patients. (C) Nuclear factor- $\kappa$ B reporter HeLa cells were cultured with TNF, with or without increasing concentrations of infliximab, adalimumab, or etanercept, all pretreated for 24 hours with either PBS or mucosal proteins from 8 HC subjects or 8 IBD patients. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means  $\pm$  SD of 8 separate experiments. \* $P < .001$  vs TNF plus PBS/HC-treated infliximab 0.1  $\mu$ g/mL, vs TNF plus PBS/HC-treated adalimumab 0.1  $\mu$ g/mL. # $P < .0005$  vs TNF plus PBS/HC-treated etanercept at the corresponding concentration.



**Figure 4.** Nuclear factor- $\kappa$ B reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF, with (A) 0.1  $\mu$ g/mL etanercept, pre-incubated for 24 hours with TCNB buffer or 1  $\mu$ g/mL active MMP3 or MMP12 in the absence or presence of increasing concentrations (0.001–1  $\mu$ mol/L) of marimastat or UK370106, or (B) 0.1  $\mu$ g/mL infliximab, adalimumab, or etanercept, all pretreated for 24 hours with either PBS or mucosal homogenates from 8 IBD patients in the absence or presence of increasing concentrations (0.001–1  $\mu$ mol/L) of marimastat or UK370106. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means  $\pm$  SD of (A) 6 and (B) 8 separate experiments. \* $P$  < .005 vs TNF plus MMP-treated etanercept and vs TNF plus the corresponding IBD-treated TNF-neutralizing agent. # $P$  < .05 vs TNF plus MMP-treated etanercept and vs TNF plus IBD-treated TNF-neutralizing agent.

### Levels of MMP3-/MMP12-Cleaved Endogenous IgG and Antihinge Autoantibodies in IBD and Control Sera

We next tried to detect clipped endogenous IgG in IBD sera using an antibody specific for the MMP3 and MMP12 cleavage site on IgG. Sera from patients with active IBD contained higher levels of cleaved endogenous IgG compared with HC subjects (Figure 5A). This result was confirmed by performing the assay using a range of different serum dilutions (Supplementary Figure 3). Stratification of IBD patients according to their response to biologic therapy showed that nonresponders had significantly higher serum levels of MMP3-/MMP12-cleaved IgG than responders (Figure 5A). Clipped infliximab in IBD patient sera collected immediately after the first infusion or before the second infusion was undetectable.

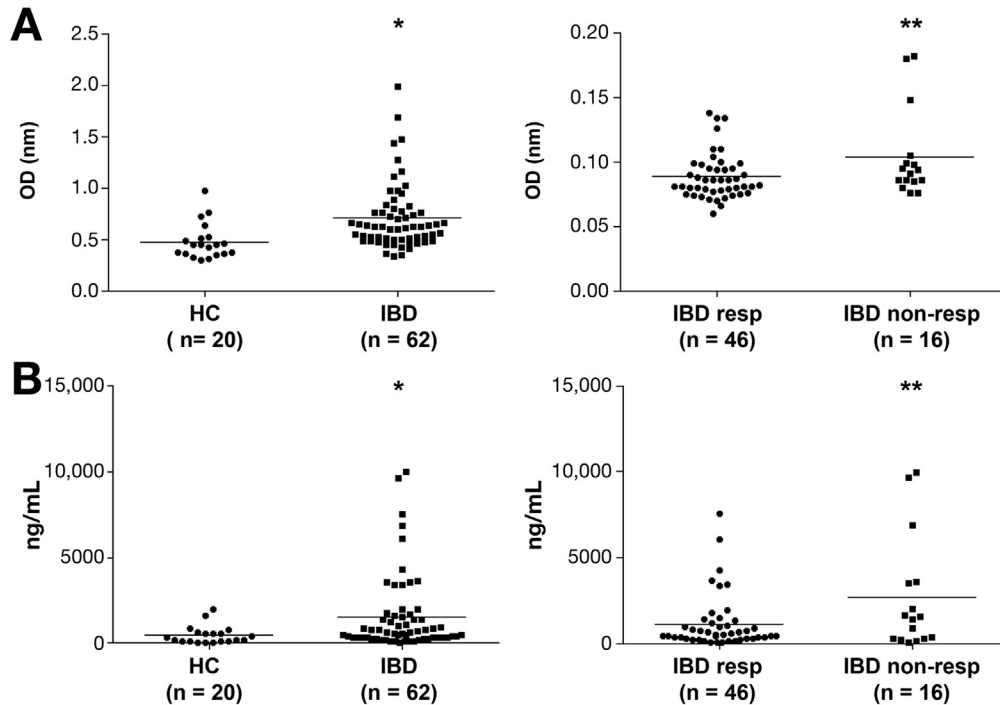
Cleavage of endogenous IgG creates immunogenic neoepitopes and autoantibody production.<sup>21,22</sup> Therefore, we performed an ELISA on the same IBD and HC sera by coating plates with the IgG hinge region peptide sequence generated by MMP3/MMP12 cleavage. Sera from active IBD patients contained higher concentrations of antihinge autoantibodies

compared with HC subjects (Figure 5B). Moreover, higher levels of antihinge autoantibodies were found in the sera of nonresponders (Figure 5B).

## Discussion

We show here that the proteases MMP3 and MMP12, but not MMP9, cleave infliximab, adalimumab, and etanercept in vitro in a dose-dependent manner. Mucosal homogenates from inflamed IBD biopsy specimens impair the integrity of infliximab, adalimumab, and etanercept. As a result of proteolytic degradation, only etanercept loses its TNF-neutralization ability. Sera from IBD patients who subsequently did not respond to biologic therapy contain higher levels of MMP3-/MMP12-cleaved endogenous IgG and antihinge autoantibodies.

Multiple factors contribute to nonresponsiveness to biologic agents that neutralize TNF in IBD. In particular, the formation of antidrug antibodies, especially against infliximab, exerts a profound influence on the persistence of functional TNF-neutralizing agents in the circulation and, more importantly, in the inflamed mucosa.<sup>23,24</sup> However,



**Figure 5.** Serum levels of MMP3-/MMP12-cleaved endogenous IgG and antihinge autoantibodies. ELISA plates were coated with streptavidin, followed by (A) a biotinylated rabbit anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody or (B) the biotinylated 14mer peptide analogue of the human IgG<sub>1</sub> hinge with a C-terminal amino acid corresponding to the MMP3/MMP12 cleavage site. Sera from 62 active IBD patients and from 20 HC subjects were serially diluted and added to the plate, then (A) a horseradish peroxidase-conjugated donkey anti-human IgG heavy- and light-chain antibody or (B) a horseradish peroxidase-conjugated goat anti-human IgG Fc antibody were used to detect bound cleaved IgG or antihinge autoantibodies. Levels of clipped endogenous IgG are expressed as optical density (OD). For antihinge autoantibodies, a concentration curve was constructed based on the OD recorded for the standard (a chimeric rabbit/human anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody), and the corresponding equation was used to convert the serum antihinge autoantibody OD into ng/mL. IBD patients were stratified as responders (IBD resp; n = 46) or nonresponders (IBD non-resp; n = 16; 6 Crohn's disease and 10 ulcerative colitis patients) according to their subsequent response to infliximab treatment. Data shown here were obtained at a (A, left panel) 1:50 or a (A, right panel); (B) 1:1350 dilution of the sera. (A) \**P* < .005 vs HC sera. \*\**P* < .05 vs IBD resp sera. (B) \**P* < .05 vs HC sera. \*\**P* < .05 vs IBD resp sera.

antidrug antibodies are important in the late induction phase and are unlikely to play a role in primary nonresponse. Our results suggest that proteolytic degradation occurring at the site of inflammation may be a novel mechanism affecting the bioavailability of TNF-neutralizing agents and contributing to primary nonresponsiveness in IBD. Human IgG<sub>2</sub> are resistant to proteolytic cleavage by MMPs, however, their Fc-dependent cell killing functions are weak.<sup>25</sup> Specific mutations can be incorporated into the Fc region of protease-resistant monoclonal IgG<sub>1</sub> antibodies, resulting in enhanced cell killing properties.<sup>18</sup> Therefore, it may be possible to apply the same strategy to generate TNF-neutralizing agents resistant to proteolytic cleavage without compromising their immunologic properties.

Similarly to what has been shown for the monoclonal antibodies trastuzumab and rituximab,<sup>26</sup> the presence of the Fc region is necessary for some important immunologic properties of TNF-neutralizing agents, such as antibody-dependent, cell-mediated cytotoxicity and complement activation.<sup>3</sup> A genetic polymorphism in IgG Fc receptor IIIa that enhances its affinity for IgG<sub>1</sub> is associated with an increased biological response to infliximab in Crohn's disease.<sup>27</sup> Moreover, Vos et al<sup>28,29</sup> showed that the Fc region is

required for the ability of TNF-neutralizing monoclonal antibodies to promote the development of regulatory M<sub>2</sub> macrophages, which are induced by infliximab in responder, but not in nonresponder, IBD patients. We have observed that proteolytic digestion by MMP3 and MMP12 leads to the complete removal of the Fc region of TNF-neutralizing agents. As a result, despite infliximab and adalimumab still being able to neutralize soluble TNF as F(ab')<sub>2</sub> fragments even at the lowest anti-TNF/MMP concentration ratio of 1:10, proteolytic degradation of TNF-neutralizing agents is likely to be clinically relevant in vivo in IBD patients because of the consequent impairment of Fc-mediated properties. Moreover, because binding of the IgG to the neonatal Fc receptor has been shown to protect them from catabolism,<sup>30</sup> the removal of the Fc region from anti-TNF agents operated by MMPs may result in decreased serum trough levels of these drugs.

In our in vitro experiments we only studied MMP3, MMP12, and MMP9, and we have not assessed the effects of a range of other proteases with known ability to cleave IgG, including MMP7, MMP13, and the serine proteases elastase and cathepsin G,<sup>15,16</sup> which are likely to play a relevant role in biologic agent clipping in vivo. Moreover, in our



experiments we used single doses of MMPs added at a single time and do not know if these reflect the *in vivo* situation, in which there is continuing production of MMPs by activated fibroblasts and inflammatory cells into the pericellular spaces.<sup>31</sup> The multiple bands detected after incubation of adalimumab and infliximab with IBD mucosal proteins did not correspond to the 32-kilodalton band seen with recombinant MMPs, suggesting that, in the complex situation of inflammation, clipping of biologic agents may be variable in different patients. Our observations on the effects of IBD mucosal proteins on anti-TNF agents in the absence or presence of MMP inhibitors suggest that proteolytic cleavage in the inflamed tissue may influence the efficacy of TNF-neutralizing agents, however, in the present study we have not examined our hypothesis by means of *in vivo* experiments. This instead could be the focus of a future preclinical interventional study on the treatment of experimental colitis with anti-TNF antibodies combined with the broad-spectrum MMP inhibitor marimastat. Although this approach would not provide clear evidence that MMP3 and MMP12 are the key enzymes involved in proteolytic degradation, it will be useful to evaluate whether MMP inhibition may enhance the efficacy of anti-TNF therapy.

Our findings provide an explanation for the failure of etanercept in Crohn's disease,<sup>7</sup> which is very susceptible to proteolytic cleavage and the only one drug to substantially lose its TNF-neutralizing ability after Fc removal by MMP3/MMP12 and IBD mucosal proteins. This may be related to a conformational change in the p75 TNF receptor when cleaved from the IgG tail. On the other hand, etanercept is clinically useful in rheumatoid arthritis,<sup>8</sup> and there are many reports showing that MMP3 is increased in the rheumatoid joint.<sup>10</sup> However, the concentration and activity of MMPs and their natural inhibitors in the affected tissues may be different in different diseases; therefore, local quantification of proteolytic activity would be important for interpretation of our results and translation into practical consequences. In more fulminant diseases, such as IBD, cleavage and loss of function of biologic agents may be quantitatively greater than in the rheumatoid joint. The high susceptibility of etanercept to proteolytic cleavage suggests that there may be an inherent problem with Fc-receptor fusion proteins at sites of inflammation rich in proteases. The recent failure of the cytotoxic T-lymphocyte-associated protein (CTLA)-4 fusion protein abatacept both in Crohn's disease and in ulcerative colitis<sup>32</sup> supports this idea.

We tried to detect clipped infliximab in IBD patient sera collected immediately after the first infusion or before the second infusion, however, these experiments uniformly were negative. This may be owing to the low ratio of clipped vs intact drug within the sera collected 1 hour after the infusion, or to the catabolism of degraded IgG in the samples collected before the following administration. An additional reason may be the fact that the F(ab')<sub>2</sub> fragments generated as a result of proteolytic cleavage become self-antigens that are recognized by autoantibodies,<sup>21</sup> which may prevent the possibility of detecting clipped infliximab with an immunoassay directed against the same epitope. Interestingly, patients with rheumatoid arthritis have a higher incidence

of serum antihinge autoantibodies compared with HC subjects.<sup>22</sup> We performed an ELISA on IBD and HC sera by coating the plate with the peptide sequence on the IgG hinge region generated by MMP3/MMP12 cleavage. Sera from active IBD patients contained significantly higher concentrations of antihinge autoantibodies compared with HC subjects. We then stratified IBD patients according to their response to biologic therapy, and we observed that patients who subsequently did not respond to treatment had significantly higher levels of antihinge autoantibodies than responders. Moreover, sera from active IBD patients and nonresponder IBD patients contained significantly higher serum levels of MMP3-/MMP12-cleaved endogenous IgG than HC subjects and responders.

Therapeutic drug monitoring by serial measurement of serum trough levels of anti-TNF agents is a valuable strategy for optimizing the management of nonresponsiveness to biologic therapy in IBD.<sup>33-35</sup> However, there is a strong need for biomarkers able to stratify IBD patients according to their subsequent possibility to respond to biologic therapy.<sup>36</sup> The evaluation of mucosal proteolytic potential through the quantification of serum levels of cleaved IgG and antihinge autoantibodies opens the possibility of developing a biomarker of responsiveness to biologic therapy in IBD. In addition, our results may contribute to clarifying the mechanisms underlying primary nonresponse to TNF-neutralizing agents, especially in severely active IBD patients who are likely to have high mucosal proteolytic activity and therefore may benefit from a higher anti-TNF loading dose.<sup>37</sup>

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at <http://dx.doi.org/10.1053/j.gastro.2015.07.002>.

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**Conflicts of interest**

The authors disclose no conflicts.

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## Supplementary Materials and Methods

### Cleavage Reactions

Upon activation, recombinant human MMP3, MMP12, and MMP9 (R&D Systems) were co-incubated with TNF-neutralizing agents to subsequently evaluate their effect on the drug integrity and function. Recombinant human pro-MMP3 and pro-MMP9 (both from R&D Systems) were activated by 24-hour incubation at 37°C with p-aminophenylmercuric acetate (Sigma-Aldrich) 1 mmol/L in 50 mmol/L Tris, 10 mmol/L CaCl<sub>2</sub>, 150 mmol/L NaCl, 0.05% Brij 35 (Sigma-Aldrich) (TCNB) buffer, whereas recombinant human pro-MMP12 (R&D Systems) was auto-activated by 30-hour incubation at 37°C in TCNB buffer, following the manufacturer's instructions. Infliximab (Remicade; Schering-Plough), adalimumab (Humira; Abbott Laboratories), or etanercept (Enbrel; Wyeth), each one at a concentration of 1 µg/mL, were incubated for 24 hours at 37°C with active MMP3, active MMP12 (0.001–1 µg/mL), active MMP9 (1 µg/mL), or p-aminophenylmercuric acetate 0.02 mmol/L in TCNB buffer or TCNB buffer alone. In parallel, infliximab, adalimumab, or etanercept, each one at a concentration of 1 µg/mL, were incubated for 24 hours at 37°C with 1 µg/mL active MMP3, MMP12, or p-aminophenylmercuric acetate 0.2 mmol/L in TCNB buffer or TCNB buffer alone, and the reaction was stopped at fixed time points (3, 6, 10, and 24 h). In parallel, increasing concentrations (0.01–10 µg/mL) of infliximab, adalimumab, or etanercept were co-incubated for 24 hours with p-aminophenylmercuric acetate 0.02 mmol/L in TCNB buffer or 1 µg/mL active MMP3 or MMP12. In parallel, etanercept 0.1 µg/mL was incubated for 24 hours at 37°C with 1 µg/mL active MMP3 or MMP12 or p-aminophenylmercuric acetate 0.2 mmol/L in TCNB buffer or TCNB buffer alone, in the absence or presence of increasing concentrations (0.001–1 µmol/L) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). Moreover, to assess the effect of mucosal protein extracts on TNF-neutralizing agents, infliximab, adalimumab, and etanercept, all at a concentration of 1 µg/mL, were co-incubated for 24 hours with either PBS only or with mucosal homogenates. In parallel, increasing concentrations (0.01–10 µg/mL) of infliximab, adalimumab, or etanercept were co-incubated for 24 hours with either PBS only or with mucosal homogenates. In parallel, infliximab, adalimumab, and etanercept, all at a concentration of 0.1 µg/mL, were incubated for 24 hours at 37°C with either PBS only or with IBD mucosal homogenates, in the absence or presence of increasing concentrations (0.001–1 µmol/L) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). Cleavage reaction products then were stored at -70°C until further analysis.

### TNF Detection in Mucosal Homogenates

The TNF concentration in the homogenates from colonic mucosa of HC and IBD patients was measured using the Human TNF DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions.

### Luciferase Assay

The effect of proteases on the TNF-neutralizing function of anti-TNF agents was assessed by means of a HeLa cell line that had been stably transfected with the luciferase reporter gene under the control of nuclear factor-κB enhancer elements. HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF (R&D Systems), with or without 1 µg/mL active MMP3 or MMP12 or increasing concentrations (0.01–10 µg/mL) of infliximab, adalimumab, or etanercept, all pretreated for 24 hours with either p-aminophenylmercuric acetate 0.02 mmol/L in TCNB buffer or 1 µg/mL active MMP3 or MMP12. In parallel, HeLa cells were cultured in the absence or presence of 10 ng/mL recombinant human TNF, with 0.1 µg/mL etanercept, preincubated for 24 hours with TCNB or 1 µg/mL active MMP3 or MMP12, in the absence or presence of increasing concentrations (0.001–1 µmol/L) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). In parallel, HeLa cells were cultured with 10 ng/mL recombinant human TNF, with or without increasing concentrations (0.01–10 µg/mL) of infliximab, adalimumab, or etanercept, all pretreated for 24 hours with either PBS or mucosal homogenates prepared in PBS. In parallel, HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF, with or without 0.1 µg/mL of infliximab, adalimumab, or etanercept, all pretreated for 24 hours with either PBS or mucosal proteins from IBD patients, in the absence or presence of increasing concentrations (0.001–1 µmol/L) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). The direct effect of IBD homogenates and MMP inhibitors on the production of luciferase by HeLa cells also was assessed. HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF (R&D Systems), with or without IBD mucosal homogenates prepared in PBS, in the absence or presence of 1 µmol/L marimastat (Sigma-Aldrich) or 1 µmol/L UK370106 (Santa Cruz Biotechnology).

After culture, HeLa cells were lysated and assayed for luciferase activity with a commercial kit (Promega UK), following the manufacturer's instructions, using a luminometer.

### Clipped Infliximab Detection

We tried to detect clipped infliximab in human sera of IBD patients by a new ELISA. Recombinant human TNF (R&D Systems) 10 µg/mL was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After overnight incubation at +4°C, plates were blocked for 30 minutes at room temperature in 3% bovine serum albumin (Sigma-Aldrich) in PBS. Serial dilutions of IBD sera, and of MMP3- and MMP12-cleaved infliximab (kindly provided by R. J. Brezski, starting from 2 µg/mL), which we used as standard, were added to the plates. After 1 hour at room temperature, 2.5 µg/mL biotinylated rabbit anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody (kindly provided by R. J. Brezski) was added. After 1 hour at room temperature, horseradish peroxidase-conjugated streptavidin



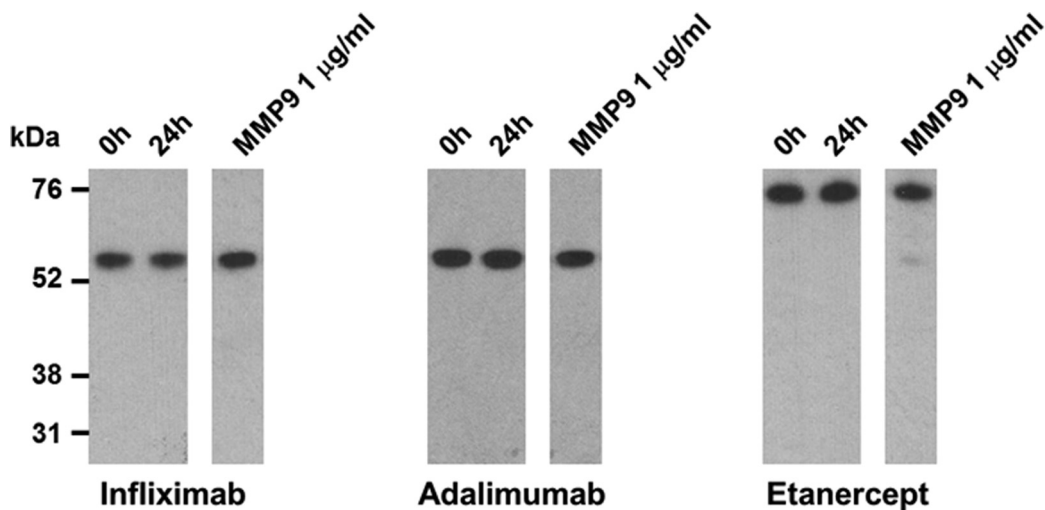
(R&D Systems) 1:10,000 was added to the plates. After 1 hour and thorough washing, tetramethylbenzidine was added, and after stopping the reaction the optical density was measured on a plate reader.

### Clipped Endogenous IgG Detection

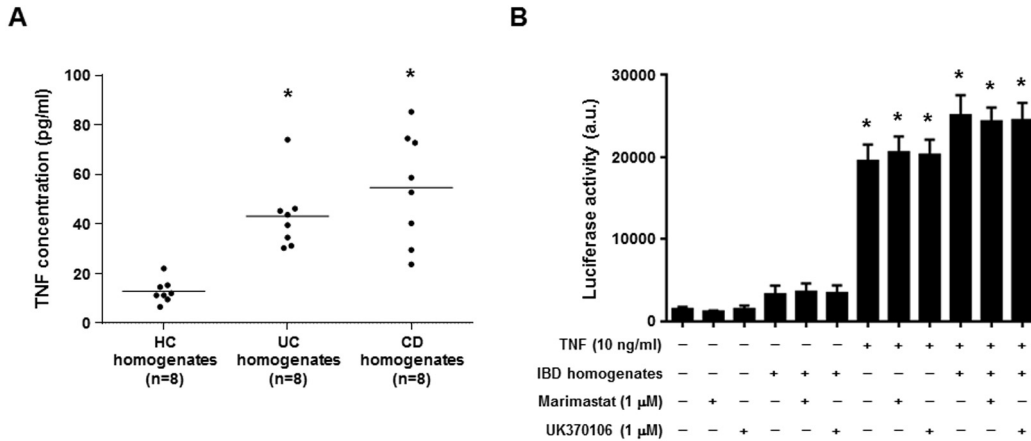
The presence of clipped endogenous IgG in human sera of IBD patients and HC subjects was assessed by a new ELISA. Streptavidin (Sigma-Aldrich) 10  $\mu\text{g}/\text{mL}$  was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After overnight incubation at  $+4^\circ\text{C}$ , plates were blocked for 30 minutes at room temperature in 3% bovine serum albumin (Sigma-Aldrich) in PBS, and then incubated for 1 hour at room temperature with 15  $\mu\text{g}/\text{mL}$  biotinylated rabbit anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody (kindly provided by R. J. Brezski). Plates then were incubated for 1 hour at room temperature with serial dilutions of IBD and HC sera, followed by a horseradish peroxidase-conjugated donkey anti-human IgG heavy- and light-chain antibody (1:10,000; Jackson ImmunoResearch), that has minimum cross-reactivity with rabbit IgG. After 1 hour and thorough washing, tetramethylbenzidine was added, and after stopping the reaction the optical density was measured on a plate reader.

### Antihinge Autoantibody Detection

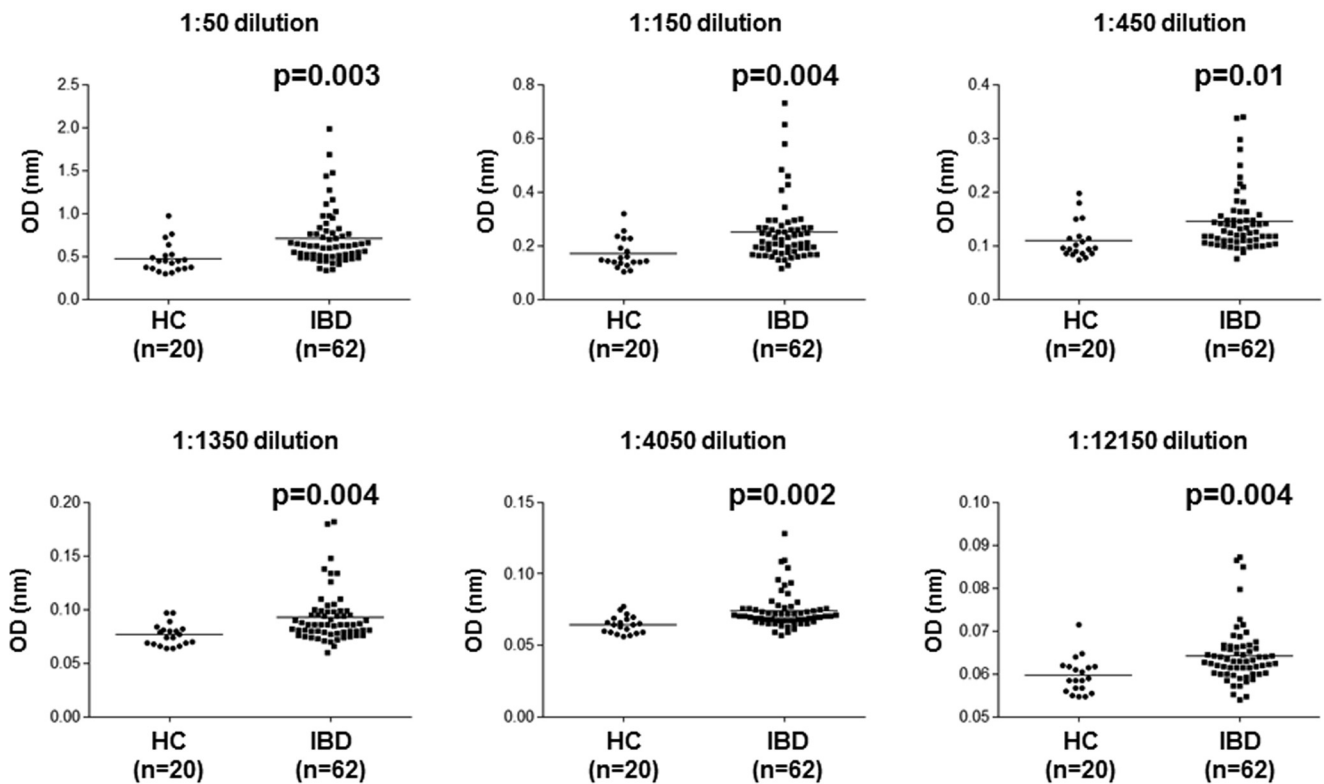
Antihinge autoantibodies against the immunogenic neopeptides generated by the proteolytic cleavage of endogenous IgG were assessed in IBD and HC sera by a new ELISA. Streptavidin (Sigma-Aldrich) 10  $\mu\text{g}/\text{mL}$  was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After overnight incubation at  $+4^\circ\text{C}$ , plates were blocked for 30 minutes at room temperature in 3% bovine serum albumin (Sigma-Aldrich) in PBS, and then incubated for 1 hour at room temperature with 1  $\mu\text{mol}/\text{L}$  biotinylated 14mer peptide analogue of the human IgG<sub>1</sub> hinge with a C-terminal amino acid corresponding to the MMP3/MMP12 cleavage site (kindly provided by R. J. Brezski). Plates then were incubated for 1 hour at room temperature with serial dilutions of a chimeric rabbit/human anti-human MMP3/MMP12 IgG hinge cleavage site monoclonal antibody, which we used as a standard, or IBD and HC sera, followed by a horseradish peroxidase-conjugated goat anti-human IgG Fc antibody (1:10,000; Jackson ImmunoResearch). After 1 hour and thorough washing, tetramethylbenzidine was added, and after stopping the reaction the optical density was measured on a plate reader. A concentration curve was constructed based on the optical densities recorded for the standard, and the correspondent equation allowed conversion of serum antihinge autoantibody optical densities into ng/mL.



**Supplementary Figure 1.** Effect of MMP9 on the integrity of TNF-neutralizing agents. Infliximab, adalimumab, and etanercept (1  $\mu\text{g}/\text{mL}$ ) were co-incubated at  $37^\circ\text{C}$  for 24 hours with 1  $\mu\text{g}/\text{mL}$  of activated recombinant human MMP9, and visualized by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. Infliximab, adalimumab, and etanercept were not cleaved by MMP9. Blots are representative of 6 separate experiments.



**Supplementary Figure 2.** (A) Concentration of TNF, expressed as pg/mL, in the homogenates of normal colonic mucosa of 8 HC subjects and from inflamed colonic mucosa of 16 IBD patients (ulcerative colitis [UC], n = 8; Crohn’s disease [CD], n = 8). Bars are mean values. \**P* < .0005 vs HC homogenates. (B) Nuclear factor-κB reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/mL recombinant human TNF, with or without mucosal homogenates from 8 IBD patients, in the absence or presence of 1 μmol/L marimastat or 1 μmol/L UK370106. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means ± SD of 8 separate experiments. No significant difference was observed between culture with or without MMP inhibitors. No significant difference was observed between culture with PBS or with IBD homogenates. \**P* < .0001 vs the corresponding condition without TNF.



**Supplementary Figure 3.** Levels of MMP3/MMP12-cleaved endogenous IgG in active IBD patients compared with HC subjects, determined using a range of different serum dilutions. Sera from 62 active IBD patients and from 20 HC subjects were serially diluted from 1:50 down to 1:12,150 and added to the plate. Levels of clipped endogenous IgG are expressed as optical density (OD). Horizontal bars indicate the mean OD.