INTRODUCTION

Intestinal fibrosis is a common complication of inflammatory bowel disease (IBD), especially in Crohn’s disease (CD) patients due to transmural inflammation and pathology usually affecting the entire thickness of the bowel wall. Although many cell types (fibroblasts, smooth muscle cells, endothelial or epithelial cells) and soluble factors including cytokines, growth factors, and proteases have been identified to be involved in intestinal fibrosis, the initiating mechanisms and the complex interplay between these factors during disease development remain elusive.

Proteases in the intestine are produced by various cell types and fulfill a number of important functions in health and disease, including tissue remodeling, nutrient digestion, modulating intestinal barrier function, activating signaling cascades, and modulating host defense to infections. Proteases can be classified as metallo-, aspartic, cysteine, serine, and threonine proteases, depending on the nature of the functional group at their active sites. The family of

Supported by: This work was funded by DFG Collaborative Research Center SFB 900 TP8 to G.A.G. Work in the lab was also supported by the DFG Priority Program SPP1656/2 and Infect-ERA Consortium Grant 031L0093B (G.A.G.) and the Canadian Academy of Health Sciences Foundation Grant 148408 (C.M.O.).

Address correspondence to: Guntram A. Grassl, PhD, Institute of Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany (grassl.guntram@mh-hannover.de).

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doi: 10.1093/ibd/izz070
Published online 8 May 2019
matrix metalloproteinases (MMPs) comprises 23 members in humans and mice and is divided into subfamilies according to their domain structure: collagenases, gelatinases, stromelysins, matrilysins, elastases, membrane-type MMPs, and others.\(^5\) MMPs act like “molecular scissors,” with their primary substrates being extracellular matrix (ECM) components such as collagens, laminins, fibronectins, and elastins; however, many more substrates including cytokines, chemokines, membrane receptors, and antimicrobial peptides have recently been identified.\(^8\) To prevent excessive proteolytic activity and tissue damage, protease synthesis, storage, and activity are tightly controlled. For example, MMPs are activated by proteolytic cleavage, and their activity is controlled by specific tissue inhibitors of metalloproteinases (TIMPs) and by nonspecific inhibitors (eg, \(\alpha_2\)-macroglobulin).\(^7\) Aberrant expression and/or activity of MMPs have been reported in several pathologies including cardiovascular diseases, obesity, type II diabetes,\(^8\) cancer, IBD, and fibrosis.\(^9, 10\) With regard to intestinal fibrosis, dysregulation of MMPs is thought to play a crucial role in the massive remodeling and excessive collagen accumulation that occurs within the intestinal wall.\(^11\) Dysregulation of MMPs and its inhibitors is in part due to the aberrant production of transforming growth factor–β (TGF-β) during fibrosis.\(^12\) Recently, it was shown that inhibition of MMP9 ameliorates intestinal fibrosis in a heterotopic intestinal transplant model.\(^13\)

Little is known about the early developmental stages of intestinal fibrosis. Various animal models are available for IBD; however, very few can be used to study fibrogenic processes in the intestine.\(^14\) Previously, we described characteristics of chronic colitis and intestinal fibrosis in \(Salmonella\) enterica serovar Typhimurium (\(S\). Typhimurium)–infected mice, which resembled many features frequently seen in fibrotic lesions of Crohn’s disease patients.\(^15, 16\) In the course of persistent infection, animals develop severe transmural inflammation, accompanied by extensive type I collagen deposition and increased levels of transforming growth factor–β1, connective tissue growth factor, and insulin-like growth factor 1. Our studies and others have demonstrated that \(S\). Typhimurium–induced colitis is a useful model to study early and progressive events in the development of intestinal fibrosis.\(^17–19\) Here, we identify proteases and their inhibitors associated with the development of intestinal fibrosis by using the \(S\). Typhimurium–induced colitis mouse model.

### METHODS

#### Mice and Bacterial Infection

129Sv/J mice were bred in the Animal Unit at the University of British Columbia (UBC). C57Bl/6j mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA) and housed at the Research Center in Borstel, Germany.

### Table 1. Primers Used in This Study

| Primer Name | Sequence |
|-------------|----------|
| Cathepsin C F | CAAGGCTTCCAGATTGTGT |
| Cathepsin C R | CCACCCATCTAGGTTCCTCAT |
| Cathepsin D F | AGCATATTGTTCTCCCGT |
| Cathepsin D R | CCGGTCTTTGAAACCTGAT |
| Cathepsin K F | CGGCTATAGACCATGCCT |
| Cathepsin K R | TGCCTGGCGTTATACATAC |
| GAPDH F | ATGTGCAGAAATGGCATCCT |
| GAPDH R | ATGGACTGTTAAGCAGCC |
| Granzyme K F | CACAGGAGACCATAGCATG |
| Granzyme K R | TGGTATGCTGCACTCTTT |
| HPRT F | ATGTGGGATACAGGCCAGAC |
| HPRT R | GTGGACTGTGGTAAAGCC |
| Meprin beta F | TTAGCCGTGGCCATCTCTT |
| Meprin beta R | GTACCTGGTCTTACAGCC |
| klk1b5 F | GAAGGGTACCACAGCCCAT |
| klk1b5 R | CTAGCAGGTGGTACCTAGG |
| klk1b8 F | CAGGGTGGAATTTGTCCTT |
| klk1b22 F | CCTGGAGGGATTTCCATAC |
| klk1b22 R | CAGAGGGGATTTCCATAC |
| MMP10 F | AGCCACAAGTTGATGCTGC |
| MMP10 R | GTATGCTGTCAGCCAGCC |
| MMP13 F | TAGTGAAGACTGTGACAGC |
| MMP13 R | GCTTGGAGTATGCAGCC |
| MMP2 F | CAGCCAGGAGATGTCGGTC |
| MMP2 R | GGGCTCATTGTTCTTTT |
| MMP3 F | CATGATGAGACAGGATG |
| MMP3 R | AGCCCTGGATGATGTCGG |
| MMP7 F | AGTTCAGACAGCAGGATG |
| MMP7 R | CATGATGAGAGACAGGATG |
| MMP8 F | CCCGTTAGACACTTTTAT |
| MMP8 R | CGAGAATGAGTGGTTCG |
| PAR-1 F | GGGGGAGCCAGATTTAAGT |
| PAR-1 R | GTCCCATGAGAGCAAGC |
| PAR-2 F | GTCTACGGGAACCCCTTTC |
| PAR-2 R | TGAGAATGACTCCACG |
| PAR-4 F | CACTGGCTTGGAGCAAGAT |
| PAR-4 R | AGAGATACCGGGAGGAA |
| Serpin3m F | GGAGATACCGGAGCAG |
| Serpin3m R | TCTTGAAGAAGAGAGCAG |
| Stef A1 F | AACCTTGCCACAGCAAAAT |
| Stef A1 R | CTTACACGGCTTCGAAT |
| Timp-1 F | ATCTGGCATCTTTTCTGG |
| Timp-1 R | TGGGAACACCAAGGATTTAG |
| Trypsin g F | GTGTGCTGACTCTGTTG |
| Trypsin g R | GAGTTTGGAAACCTGGGATG |
FIGURE 1. Chronic Salmonella infection in mice induces expression of proteases and their inhibitors. 129SvJ mice were treated with streptomycin and 24 hours later infected with Salmonella Typhimurium. Twenty-one days post-infection, the mice were killed and their tissues collected. A, Histopathological changes in chronically inflamed ceca (hematoxylin and eosin staining). ECM deposition is apparent in the submucosa and mucosa of infected mice, as visualized by Masson’s Trichrome staining. Abbreviations: L, lumen; M, mucosa; SM, submucosa. B, Significance analysis for microarrays with hierarchical cluster analysis using a Pearson correlation average linkage method revealed 56 upregulated and (C) 40 downregulated proteases and inhibitor gene transcripts in the fibrotic cecum in comparison with the control cecum on murine CLIP-CHIP microarray.
S. Typhimurium SL1344\textsuperscript{20} and S. Typhimurium SL1344 \( \Delta \text{aroA} \)\textsuperscript{15} were grown overnight in Luria-Bertani broth at 37°C with shaking. At an age of 8–12 weeks, mice were given 20 mg of streptomycin by oral gavage 24 hours before infection by oral gavage with 3 \times 10^6 S. Typhimurium suspended in 100 \( \mu \)L of HEPES buffer (100 mmol/L, pH 8.0).

**Ethics Statement**

All experiments were conducted in accordance with the ethical requirements and approval of the Animal Care Committee at UBC and of the Animal Care Committee of the Ministry of Energy, Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Germany, and in direct accordance with the German Animal Protection Law. The protocols were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Germany (Protocol#: V244-7224.121.3). Approval for the use of human tissue samples was obtained by the Institutional Review Board of the Cleveland Clinic, Ohio. Tissue samples were collected from CD and UC patients or from non-inflamed tissue samples from non-IBD patients.

**Cell Culture and In Vitro Infections**

NIH 3T3 mouse fibroblasts were grown in DMEM containing 10% newborn calf serum. Mode-K mouse intestinal epithelial cells were grown in DMEM containing 10% fetal calf serum (FCS) and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). To obtain primary bone marrow–derived macrophages (BMDM), the bone marrow from mouse femurs and tibia was flushed out with phosphate buffered saline (PBS), and the cells were differentiated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FCS, penicillin (100 U/mL), streptomycin (100 \( \mu \)g/mL), 1% HEPES, and 50 ng/mL of macrophage colony-stimulating factor (M-CSF). After 1 day, macrophage progenitors were separated from the adherent fibroblasts. BMDM were cultured for another 6 days before infection. All cells were cultured at 37°C in a humidified atmosphere with 5% CO\(_2\). One day before infection, cells were seeded into 24-well plates in their respective medium without antibiotics. Bacteria were grown overnight in Luria-Bertani broth at 37°C with shaking, diluted 1:30, and grown to mid-logarithmic phase. Fibroblasts and BMDM were infected with S. Typhimurium at a multiplicity of infection (MOI) of 10, and Mode-K cells were infected with an MOI of 50. Gentamicin protection assays were performed as previously described.\textsuperscript{21}

**Murine CLIP-CHIP Microarray Analysis**

The murine CLIP-CHIP is a custom DNA microarray that covers all murine proteases, inactive homologues, and their inhibitors.\textsuperscript{22} The murine CLIP-CHIP contains 70-mer oligonucleotides for 27 aspartic, 158 cysteine, 204 metallo-,

221 serine, and 26 threonine proteases, along with 187 protease inhibitor gene transcripts. Each glass slide contains 2 copies of the CLIP-CHIP microarray that can be used as a technical replicate for statistical analysis. Apart from protease and protease inhibitor oligonucleotides, the microarray also contains positive and negative control oligonucleotides.

Total RNA was isolated from cecal tissues using the RNeasy-Mini Kit with an on-column DNaseI treatment (Qiagen, Mississauga, ON, Canada). The CLIP-CHIP microarray sample preparation was conducted as described in Kappelhoff et al.\textsuperscript{22} and Kappelhoff and Overall.\textsuperscript{23} In brief, using the Message Amp II kit (Ambion, Austin, TX, USA), 1 \( \mu \)g of total RNA was reversed-transcribed into cDNA. Second-strand synthesis was performed using DNA polymerase. Purified dsDNA was used for linear amplification of amplified RNA (aRNA) by the T7 RNA polymerase.

Using the universal linking system (ULS) aRNA labeling kit (Kreatech, Amsterdam, the Netherlands), 2 \( \mu \)g of aRNA (from control or fibrotic tissue) was labeled with Cy5-ULS, and a universal reference RNA was labeled with Cy3-ULS. Experimental and reference RNAs were then pooled and hybridized to a murine CLIP-CHIP microarray.

Microarray scanning and image and data analyses were done as described in Kappelhoff et al.\textsuperscript{24} In brief, after stringent washes, the CLIP-CHIP microarrays were scanned using the 428 Array Scanner (MWG), and images were analyzed using CarmaWEB software was used to normalize the data, and MeV from the TM4 Microarray Suite (www.tigr.org) was used for statistical analysis. Significance analysis for microarrays (SAM) was performed according to Tusher et al.\textsuperscript{25} using 924 unique permutations in a 2-class unpaired analysis with a delta value of 2.254 for a false discovery rate of 0%. SAM was used to find significant changes in expression of proteases and protease inhibitors. The hierarchical cluster analysis of significant genes was done using Pearson correlation with a complete linkage method in MeV.

**Quantitative Real-time Polymerase Chain Reaction**

RNA was extracted from mouse cecal tissue using the High Pure RNA Tissue Kit (Roche). RNA was reverse-transcribed into cDNA using the eDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed with SYBR-Green Mastermix (Roche) and gene-specific primers (Table 1). Data were normalized to house-keeping genes Gapdh and Hprt1, and fold regulation was calculated using the \( \Delta \text{AC} \) method.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Samples included cecum tissue from uninfected (n = 5) and infected (n = 5) mice and human
| Merops ID | Description | Gene | RefSeq_1     | Fold Change |
|-----------|-------------|------|--------------|-------------|
| M10.006   | stromelysin 2 | Mmp10 | NM_019471 | 21.99       |
| M10.013   | collagenase 3 | Mmp13 | NM_008607 | 12.85       |
| C01.070   | cathepsin C  | Ctsc | NM_009982 | 11.43       |
| M10.002   | collagenase 2 | Mmp8  | NM_008611 | 10.62       |
| I04.xxx   | a1-antitrypsin member 3g | Serpina3g | XM_484175 | 10.17       |
| S01.196   | complement factor B | Bf | NM_008198 | 8.03        |
| S01.192   | complement component C1ra | C1ra | NM_023143 | 7.65        |
| I39.950ni | complement component 3 | C3 | NM_009778 | 7.15        |
| I35.001   | tissue inhibitor of metalloprotease-1 | Timp1 | NM_011593 | 6.73        |
| T01.013   | proteasome catalytic subunit 1i | Psmb9 | NM_013585 | 6.28        |
| T01.015   | proteasome catalytic subunit 3i | Psmb8 | NM_010724 | 6.25        |
| I04.024   | C1 inhibitor | Serping1 | NM_009776 | 6.09        |
| C01.040   | cathepsin H  | Ctsb | NM_007801 | 5.28        |
| M14.015np | carboxypeptidase X1 | Cpx1 | NM_019696 | 5.15        |
| I25.xxx   | stefin-2 like | Cstat1b | NM_173869 | 5.02        |
| S01.972np | haptoglobin-1 | Hp | NM_017370 | 4.79        |
| A02.059   | DDI-related protease | Ddi-rp | NM_026414 | 4.77        |
| I04.xxx   | a1-antitrypsin member 3m | Serpina3m | NM_009253 | 4.42        |
| S60.001   | lactotransferrin | Ltf | NM_008522 | 4.40        |
| C01.013   | cathepsin Z  | Ctsz | NM_022325 | 4.22        |
| C46.002   | sonic hedgehog protein | Shh | NM_009170 | 4.02        |
| I25.xxx   | stefin A3    | Stfa3 | NM_025288 | 3.79        |
| S01.155   | pancreatic elastase II (IIA) | Ela2a | NM_015779 | 3.79        |
| C01.037   | cathepsin W  | Ctsw | NM_009985 | 3.73        |
| M10.005   | stromelysin 1 | Mmp3 | NM_010809 | 3.72        |
| S01.246   | kallikrein hK10 | Mkklk10 | NM_133712 | 3.69        |
| I04.xxx   | a1-antitrypsin member 3n | Serpina3n | NM_009252 | 3.68        |
| C19.030   | USP18        | Usp18 | NM_011909 | 3.64        |
| M10.014   | MT1-MMP      | Mmp14 | NM_008608 | 3.49        |
| I04.005   | protein Z-dependent PI | Serpina10 | NM_144834 | 3.38        |
| C01.034   | cathepsin S  | CtsS | NM_021281 | 3.37        |
| I01.xxx   | follistatin-like 1 | Fstl1 | NM_008047 | 3.37        |
| C48.xxx   | sentrin/SUMO protease 14 | Senp14 | BN000389 | 3.32        |
| S01.146   | granzyme K   | Gzmk | NM_008196 | 3.19        |
| I04.xxx   | a1-antitrypsin member 3f | Serpina3f | BC049975 | 3.12        |
| M10.008   | matrilysin   | Mmp7  | NM_010810 | 3.02        |
| I39.001   | a-2-macroglobulin | A2m | NM_175628 | 3.00        |
| A01.009   | cathepsin D  | CtsD | NM_009983 | 2.99        |
| S01.131   | neutrophil elastase | Ela2 | NM_015779 | 2.96        |
| I01.xxx   | follistatin-like 2/IGFBP7 | Igfbp7 | NM_008048 | 2.92        |
| I04.014   | protease inhibitor 9/CAP3 | Serpinb9 | NM_009256 | 2.80        |
| I01.xxx   | osteonectin  | Sparc | NM_009242 | 2.76        |
| T01.014   | proteasome catalytic subunit 2i | Psmb10 | NM_013640 | 2.67        |
| C14.013   | caspase-12   | Casp12 | NM_009808 | 2.53        |
| T03.022   | gamma-glutamyltransferase 6 | Ggt6 | NM_027819 | 2.49        |
| I32.003   | cIAP2        | Birc2 | NM_007464 | 2.32        |
| M17.001   | leucyl aminopeptidase | Lap3 | NM_024434 | 2.31        |
colon samples from non-IBD (n = 5), UC (n = 5), or CD (n = 5) patients. Antigen unmasking was achieved by heat treatment in 10 mM of sodium citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) for 30 minutes. Specimens were incubated with blocking reagent containing 1% bovine serum albumin, 0.1% Triton X-100, 0.05% Tween 20, and 2% normal goat serum. Antigen retrieval, primary antibodies, and dilutions are listed in Supplementary Table 1. Horseradish peroxidase-labeled secondary antibodies were used and were followed by incubation with 3,3’Diaminobenzidine (DAB) substrate. Negative controls were done by omission of the primary antibody. The pattern (focal, patchy, or diffuse) and intensity (0–3) of antibody staining were analyzed in the epithelium, lamina propria, submucosa, and muscularis propria. Representative images are shown and were obtained using an Olympus BX41 microscope.

Statistics

Statistical analysis of qPCR and bacterial colonization data was performed using the GraphPad Prism 5 software package (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests were used to determine significance between multiple data sets. The Student t test was used to compare two groups. A P value <0.05 was considered statistically significant.

RESULTS

Salmonella-Induced Intestinal Fibrosis in Mice Is Linked to the Differential Regulation of Multiple Proteases

We have previously shown that long-term infection with S. Typhimurium leads to chronic inflammation and the development of fibrosis in the cecum and colon of mice.15 129Sv/J mice infected with wild-type S. Typhimurium developed persistent fibrosis in the cecum, peaking 21 days postinfection. At this time point, ceca were inflamed and fibrotic. Crypt architecture was destroyed, and the mucosa, submucosa, and muscularis were infiltrated with large amounts of inflammatory cells and fibroblasts. In addition, collagen was deposited in the mucosa and submucosa, as visualized by Masson’s Trichrome staining (Fig. 1A). Microarray analysis on cecal tissue was performed to identify proteases and protease inhibitors regulated during chronic inflammation and fibrosis. We used the murine CLIP-CHIP degradome microarray, which is a dedicated and focused array that allows analysis of all 636 proteases and 187 protease inhibitor gene transcripts in the murine genome at the mRNA transcript level.22

Significance analysis for microarrays of the microarray data revealed 96 significantly regulated proteases and inhibitors. Fifty-six genes were upregulated and 40 genes were downregulated in fibrotic cecal tissue when compared with normal control tissue from mock-infected mice (Fig. 1B, C, Table 2; see Supplementary Data Table S2 for a complete list of CLIP-CHIP results).

The differential expression of proteases and protease inhibitors was further confirmed by qPCR analysis. Some of the most strongly upregulated proteases were from the MMP family. Expression of Mmp3, Mmp7, Mmp8, Mmp10, and Mmp13 was strongly increased in fibrotic cecal tissue 21 days post-infection (Fig. 2A). In contrast, expression of Mmp2 did not change during infection. Of note, the epithelial barrier-promoting metalloprotease meprin-β (Mep1b) was significantly downregulated in fibrotic tissue. As depicted in Figure 2B, expression of glandular kallikreins Klk1b5, Klk1b8, and Klk1b22 and tryptase-γ (Tpsg1) was significantly decreased, whereas expression of granzyme K (Gzmk) increased upon fibrosis development. The induction of several cathepsins (Ctsc, Ctsd, Ctsk) was confirmed by qPCR results (Fig. 2C). Various protease inhibitors including SerpinA3m and stefinA1 (Stfa1), along with the tissue inhibitor of metalloproteases-1 (Timp1), were also

| Merops ID | Description | Gene | RefSeq_1 | Fold Change |
|-----------|-------------|------|----------|-------------|
| A01.046   | napsin A    | Kdap | NM_008437| 2.28        |
| A01.010   | cathepsin E | Cte  | NM_007799| 2.20        |
| Cx1.xxxnp | HetF-like   | Hetf1| NM_024477| 2.04        |
| S01.099   | testis serine protease 4 | Tessp4 | NM_199471| 1.94        |
| A22.003   | presenilin homolog 3/SSP | Psh3 | NM_010376| 1.91        |
| C02.002   | calpain 2   | Capn2| NM_009794| 1.90        |
| I04.021   | proteinase nexin 1/GDN | Serpine2 | NM_009255| 1.90        |
| I04.xxx   | protease inhibitor 6b | Serpinb6b | NM_011454| 1.86        |
| S01.216   | coagulation factor Xa | F10  | NM_007972| 1.86        |

The letter in front of a number refers to the protease class (A, aspartic protease; C, cysteine protease; M, metalloprotease; S, serine protease; T, threonine protease; I, protease inhibitor).

TABLE 2. Continued
confirmed to be upregulated (Fig. 2D) during *S. Typhimurium*-induced intestinal fibrosis. Taken together, our analysis indicated an enhanced expression of MMPs and an impairment of serine protease expression.

Proteases Are Expressed by Epithelial Cells and Infiltrating Cells During Inflammation and Fibrosis

Next, we tested whether the gene expression changes we observed with the microarray and by qPCR can also be observed at...
the protein level. Antibody staining of tissues from mock-infected and chronically infected mice demonstrate that MMP3 is weakly and MMP8 is strongly expressed in the uninfected cecum epithelium and upregulated upon S. Typhimurium–induced fibrosis in the cecum epithelium and infiltrating cells (Fig. 3). Positive staining for MMP7, MMP10, MMP13, cathepsin D, and granzyme K is not observed in uninfected ceca; however, in infected fibrotic tissue, MMP13 and cathepsin D are found in the inflammatory infiltrate, MMP7 is upregulated in the epithelium, and MMP10 and granzyme K are seen in both the inflammatory infiltrate and the epithelium of fibrotic tissue. Meprin-β is strongly expressed in epithelial cells in uninfected mice. Upon experimental fibrosis, meprin-β is downregulated and is no longer detectable by immunohistochemical staining (Fig. 3), in agreement with the microarray and qPCR data.

We next tested whether some of the proteases we see regulated in our experimental model of intestinal fibrosis are also regulated in human IBD patients (see Supplementary Tables 3 and 4 for clinical data of human subjects). Tissue sections from noninflamed controls (C) were compared with inflamed fibrotic tissue from CD and UC patients (Fig. 4A). Similar to our mouse model, the epithelium of non-inflamed controls stained positive for MMP3 and MMP8, whereas in addition to the epithelium of CD and UC patients, there were MMP3- and MMP8-positive inflammatory cells in the lamina propria (Fig. 4B). MMP7, MMP10, and granzyme K were not detected in the noninflamed control intestines. However, in UC and CD patients, a subset of crypt epithelial cells stained strongly for MMP7. In addition, epithelial cells and inflammatory cells in IBD tissues were positive for MMP10 and granzyme K. In noninflamed control tissue, a few cells in the lamina propria were positive for cathepsin C, cathepsin D, and cystatin A. In tissues from CD patients, there was a slight increase in cathepsin C–positive cells, and there was a strong increase in cathepsin D– and cystatin A–positive cells in both CD and UC patients. Strong staining for meprin-β and kallikrein 5 was found in the epithelium and lamina propria of all noninflamed controls, but only mild expression was detected in CD or UC patients. In noninflamed colon, we observed strong TIMP1-positive cells, which are presumably enteroendocrine cells, whereas in IBD patients other epithelial cells and some cells in the lamina propria stained positive for TIMP1 as well.

In summary, the pattern of protease staining in intestinal tissues from UC patients was very similar to that of CD
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patients but drastically different from that of noninflamed controls. In addition, protease staining observed to be up- or downregulated in inflamed intestinal mouse tissues followed a comparable pattern in human IBD tissues.

**Salmonella Induces Proteases in 129SvJ and C57Bl6/J Mice to a Similar Level**

Chronic mouse infections with wild-type *S. Typhimurium* can only be performed in resistant mouse strains such as 129SvJ. However, the majority of knockout mice that would allow investigation into the contribution of specific proteases to fibrosis are generated in a *Salmonella*-sensitive C57Bl/6J background. Therefore, we infected C57Bl6J mice with the attenuated *S. Typhimurium ΔaroA* strain, which also induces chronic intestinal inflammation and fibrosis (Fig. 5A). We analyzed the expression kinetics of selected proteases at days 7, 14, and 21 postinfection. As shown in Figure 5B, the MMPs tested were induced to a similar degree compared with 129SvJ mice infected with wild-type *Salmonella*. Therefore, C57Bl/6J mice and their gene-deficient strains can be used to study the role of specific proteases during intestinal fibrosis.

**Salmonella Induces Protease Expression in Macrophages and Epithelial Cells**

We observed upregulation of specific proteases during *Salmonella*-induced chronic inflammation and fibrosis in mice. Although many cell types can express different proteases during inflammation, the staining pattern in fibrotic mouse

![Protease expression in intestinal tissue of mice chronically infected with *S. Typhimurium*. Cecum sections of mice were stained for proteases as indicated. Original magnification 400×; scale bar: 50 µm.](image-url)
and human tissue indicated specific expression patterns by epithelial cells, inflammatory cells, and fibroblasts present in the inflamed intestine. Therefore, we tested whether in vitro *Salmonella* infection of these cell types directly induces protease expression. Mouse primary BMDMs, fibroblasts (NIH-3T3), and epithelial cells (Mode-K) were infected with *S. Typhimurium* for up to 3 days. As shown in Figure 6, *Salmonella* invaded and persisted in all three cell types (Fig. 6A–C). Upon infection, BMDMs strongly upregulated expression of *Mmp3*, *Mmp8*, *Mmp10*, and *Mmp13* (Fig. 6D–G) but not *Mmp7* (not shown). *Salmonella* infection also induced expression of *Mmp3*, *Mmp10*, and *Mmp13* in epithelial cells (Fig. 6H–J), but not *Mmp7* or *Mmp8*. In contrast, infection of fibroblasts did not induce expression of any of the tested proteases (not shown). These data demonstrate that *S. Typhimurium* infection directly stimulates protease expression in macrophages and epithelial cells, but not in fibroblasts.

**DISCUSSION**

Proteases and their inhibitors are thought to be key mediators of the intestinal fibrotic process, but their specific roles remain poorly understood. Until recently, there was a paucity of animal models to study intestinal fibrosis. The *S. Typhimurium* model is now established in the field of intestinal fibrosis as it recapitulates the pathology of human disease including transmural tissue fibrosis, a Th1/Th17 immune response, and the induction of pro-fibrotic genes. Using this model, we discovered that a plethora of proteases and protease inhibitors are regulated during intestinal fibrosis. Site-specific upregulation of several MMPs was observed, and a similar protease expression pattern was validated in human intestinal fibrotic tissue. In addition, we demonstrate that *S. Typhimurium* is capable of directly inducing expression of several proteases in a cell type–specific manner.

During intestinal fibrosis development, it is thought that tissue destruction during chronic inflammation causes an excessive healing response, leading to an imbalance between ECM deposition and ECM turnover by MMPs. Association studies show that single nucleotide polymorphisms in the stromelysin genes *MMP3* and *MMP10* are associated with an increased risk for IBD and that the *MMP3* 5A/6A genotype is especially associated with fibrostenosing complications and fistula formation in CD patients. *MMP10* and *MMP13* are also described to be upregulated in the intestine of CD and UC patients. In mice chronically infected with *Salmonella*, we observed upregulation of *Mmp3*, *Mmp10*, and *Mmp13*. At first glance, this may appear counterintuitive as MMPs have been implicated in ECM degradation, but the function of MMPs goes beyond cleavage of matrix molecules. For example, MMP3 and macrophage MMP12 can act intracellularly as transcription factors to induce profibrotic connective tissue growth factor (CTGF) and IFN-α gene transcription, respectively. MMP2, where we did not detect altered expression upon *Salmonella* infection, cleaves and inactivates CTGF, thereby unmasking VEGF. Thus, fibrosis represents a complex inflammatory environment where the interplay of cytokines and growth factors can be orchestrated by proteases.

Although many MMPs are involved in epithelial barrier destruction, TIMP function is generally thought to be critical for epithelial barrier restitution. On the other hand, excessive TIMP expression can also result in fibrosis: *TIMP1* is upregulated in inflammatory and fibrotic lesions in patients with Crohn’s disease and in other mouse models of intestinal fibrosis. In agreement with these studies, we found a strong upregulation of *Timp1* during *Salmonella*-triggered fibrosis. Besides inhibiting the action of MMPs, TIMP1 also stimulates cell proliferation and activates neutrophils. Recently, it was shown that TIMP1-deficient mice have less fibrosis and
FIGURE 4. Protease expression in tissues from human IBD patients. Tissue sections were taken from Crohn’s disease (CD) or ulcerative colitis (UC) patients and from non-inflamed controls (C). A, Hematoxylin and eosin and Masson’s Trichrome (MT) staining showing pathological changes during disease and extracellular matrix deposition, respectively. Original magnification 100×; scale bar: 100 µm. B, Protease staining. Original magnification 200×; scale bar: 50 µm.
inflammation upon stimulation with DSS. Conversely, treatment of CD myofibroblasts with the anti-inflammatory drug infliximab increases TIMP1 production in a dose-dependent manner, leading to an antifibrotic effect by enhancing cell migration and decreasing collagen production.

Meprin-β has various roles and can lead to cytokine induction and inactivation of certain cytokines by cleavage. Meprin-β-deficient mice have increased amounts of pro-inflammatory cytokines and increased inflammation in a DSS colitis model. Our data show that meprin-β is downregulated in the fibrotic intestinal tissue of Salmonella-infected mice and also in IBD patient tissue, consistent with previous reports in patients.

Various cells have been shown to be sources for proteases. In CD patients, MMP3 was seen to be produced by fibroblasts and mononuclear cells, and MMP7 was exclusively found in enterocytes adjacent to ulcers. In vitro, we also observed MMP3 upregulation in Salmonella-infected macrophages, and in chronically infected mice, MMP7 is upregulated in enterocytes. In vitro, human intestinal fibroblasts can be a major source of MMPs (MMP1, -2, -3, and -9) when stimulated with IL-21 or TNF-α. However, in our experiments using a mouse fibroblast cell line infected with S. Typhimurium, we did not detect upregulation of Mmp3, Mmp7, Mmp8, Mmp10, or Mmp13. We speculate that in an in vivo setting, bacterial infection would trigger IL-21 and TNF-α production in other cell
types, which would then indirectly stimulate fibroblasts to produce proteases. *S. Typhimurium* infection of BMDM directly triggered upregulation of *Mmp3*, *Mmp8*, *Mmp10*, and *Mmp13*. Also to consider, many proteases are produced as inactive zymogens and need to be activated (eg, by proteolytic cleavage). Our data showing upregulation of proteases thus do not directly imply that these proteases are present in their active form.

Several MMPs have been shown to play a role in *Salmonella* infection. *Mmp2* and *Mmp9* are highly upregulated during acute *Salmonella* infection in mice, and *Mmp2/Mmp9−/−* mice are resistant to *S. Typhimurium*-induced colitis. In contrast, in our chronic infection experiments, we did not detect significant changes in expression of these MMPs compared with uninfected controls, indicating that these MMPs play a role in acute but not chronic infections.

Although the etiology of IBD is still not completely understood, an aberrant response to normal microbial populations, intestinal dysbiosis, and infection with various enteric pathogens such as adherent invasive *Escherichia coli*, *Mycobacterium paratuberculosis*, *Campylobacter*, or *Salmonella* have been associated with IBD. However, whether particular

**FIGURE 6.** *Salmonella* infection induces protease expression in macrophages and epithelial cells. BMDMs, NIH-3T3 fibroblasts, and Mode-K epithelial cells were infected with *S. Typhimurium*. At indicated time points, cells were lysed, and intracellular bacterial counts were determined by gentamicin protection assay, demonstrating that all cell types were infected and that *Salmonella* persisted for at least 3 days inside macrophages (A), epithelial cells (B), and fibroblasts (C). Individual values and the mean +/- SD from 1 representative out of 2 independent experiments are shown (n = 4–5 per group). Statistical significance was analyzed using one-way ANOVA with Tukey’s post-test. ***P < 0.001. RNA was isolated from macrophages (D–G) and epithelial cells (H–K) at the indicated time points postinfection, and protease expression was analyzed by qPCR. Individual values and the mean +/- SD from 1 representative out of 2 independent experiments are shown (n = 4–5 per group). Statistical significance was analyzed using Student’s t-test. **P < 0.01; ***P < 0.001.
infections cause intestinal fibrosis or whether their presence simply reflects the competitive advantage of these pathogens to expand in the inflamed gut is still under debate.  

To our knowledge, this is the first protease screen performed on bacterial-induced fibrosis in mice and will serve as the basis for future studies into the role of specific proteases in disease development. The S. Typhimurium model is now poised to provide further insight into the molecular mechanisms of bacterial-induced intestinal fibrosis. Genes regulated in the mouse model can be validated in the fibrotic tissue removed from Cronh's disease patients. In the future, specific inhibition of proteases could be a promising target for treatment of chronic inflammation and fibrosis in IBD.  

SUPPLEMENTARY DATA
Supplementary data are available at Inflammatory Bowel Diseases online.

ACKNOWLEDGMENTS
We would like to thank Katrin Seeger and Janin Braun for expert technical help.

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