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Neutrophilic HGF-MET signaling exacerbates intestinal inflammation

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Abstract

Background and Aims: Ulcerative colitis (UC) is associated with excessive neutrophil infiltration and collateral tissue damage, but the link is not yet completely understood. Since c-MET receptor tyrosine kinase (MET) is required for neutrophil chemoattraction and cytotoxicity in response to its ligand hepatocyte growth factor (HGF), we aimed to identify the function of HGF-MET signalling in neutrophils in UC patients and in mice during intestinal inflammation.

Methods: Serum and colonic biopsies from healthy controls and UC patients with active (Mayo endoscopic sub score 2-3) and inactive (Mayo endoscopic sub score 0-1) disease were collected to assess the level of serum and colonic HGF. Disease progression and immune cell infiltration was assessed during DSS colitis in wild type and MRP8-Cre MET-LoxP mice.

Results: Increased mucosal *HGF* expression was detected in patients with active UC, and in mice during the inflammatory phase of DSS colitis. Similarly, serum HGF was significantly increased in active UC patients and positively correlated with C-reactive protein and blood neutrophil counts. Flow cytometric analysis also demonstrated an upregulation of colonic MET^+ neutrophils during DSS colitis. Genetic ablation of MET in neutrophils reduced the severity of DSS-induced colitis. Concomitantly, there was a decreased number of T_H17 cells, which could be due to a decreased production of IL-1β by MET deficient neutrophils.

Conclusion: These data highlight the central role of neutrophilic HGF-MET signalling in exacerbating damage during intestinal inflammation. Hence, selective blockade of this pathway in neutrophils could be considered as a novel therapeutic approach in UC.

Key words: Neutrophils, Ulcerative Colitis (UC), receptor tyrosine kinase (MET)-hepatocyte growth factor (HGF) signalling.

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Introduction

Neutrophils are short-lived polymorphonuclear effector cells of the innate immune system, which are typically the first leucocytes recruited to an inflammatory area following tissue damage. They are crucial to protect the host from invading pathogens by performing a wide variety of antimicrobial functions such as phagocytosis of pathogens, production of reactive oxygen species, release of lytic enzymes by degranulation and liberation of neutrophil extracellular traps (NETs)[1-3]. Yet, their highly destructive capacity also leads to extensive damage in healthy tissues occurring in many chronic inflammatory conditions, such as inflammatory bowel disease (IBD). Neutrophil contribution in the pathogenesis of IBD remains controversial, and likely differs between the two main subtypes of IBD, i.e. Crohn's disease (CD) and ulcerative colitis (UC)[2-4]. While neutrophil infiltration in the lamina propria is often included in scoring systems of UC severity[5,6], their contribution in CD is less apparent[7]. Also experimental animal models of IBD show discrepancies in their results. Models based on direct toxicity on epithelial cells and disruption of the intestinal barrier integrity (dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS)) show an amelioration of colitis upon neutrophil depletion[8,9], while T cell transfer colitis shows a beneficial role of neutrophils with exacerbation of inflammation after their depletion[10,11]. However, these depletion models are based on the outdated dogma that neutrophils are a homogenous population of short-lived effector cells. In recent years, it has become apparent that both under homeostatic and pathological conditions multiple neutrophil subpopulations with distinct functions are present[12-14]. A complete understanding of neutrophil diversity and plasticity is currently lacking and markers need to be identified to discriminate between different neutrophil subpopulations.

Recently, a novel pathway in neutrophils has been identified that stimulates a pro-inflammatory phenotype, i.e. c-MET receptor tyrosine kinase (MET) - hepatocyte growth factor (HGF) signaling[15]. Finisguerra *et al*. showed that MET is upregulated in blood neutrophils by inflammatory stimuli[15].

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Furthermore, MET deletion in mouse neutrophils promoted tumor growth and metastasis in several cancer models including azoxymethane (AOM)/DSS-induced colon adenocarcinomas, as NO released by HGF-activated neutrophils promoted cancer cell killing[15]. However, the role of HGF-MET signaling in neutrophils during intestinal inflammation remains to be determined. Recently, Czarnewski *et al.* showed that colonic *HGF* is upregulated in a subgroup of UC patients which are refractory to anti-TNF therapy and present with a neutrophil gene signature[16]. MET, also named hepatocyte growth factor receptor (HGFR), is a 190 kDa tyrosine kinase receptor that is expressed primarily by epithelial cells and binds to its cognate ligand, HGF[17,18]. Originally identified as a potent mitogen for hepatocytes[19,20], HGF is currently described as a pleotropic factor of mesenchymal origin that controls cell proliferation, motility, differentiation and survival of different cell types in various organs[21]. For instance, in the gastro-intestinal tract, constitutive HGF secretion by submucosal stromal cells stimulates mucosal epithelial cell proliferation to maintain intestinal epithelial barrier integrity and homeostasis[22-25]. As neutrophilic MET signaling is activated in response to inflammatory stimuli and executes pro-inflammatory functions, MET-targeted therapies might ameliorate the symptoms of chronic inflammatory disorders such as IBD and should be evaluated.

In this study, we investigated the role of HGF-MET signalling in neutrophils during the pathophysiology of UC and during murine intestinal inflammation. Our findings using patient-derived samples and mouse studies indicate that *HGF* is strongly associated with disease severity during intestinal inflammation. In turn, we observed an increased percentage of blood and colonic MET^+ neutrophils upon murine intestinal inflammation. Genetic ablation of MET in neutrophils reduced the severity of both acute and chronic DSS-induced colitis. Concomitantly, there was a decreased number of pathogenic T_H17 cells in the lamina propria of mice lacking MET^+ neutrophils. The latter maybe explained by a decreased production of IL-1β by MET deficient neutrophils. Together, these data highlight the central role of neutrophilic HGF-MET signalling in exacerbating intestinal

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inflammation. Hence, selective blockade of this pathway in neutrophils may provide a novel strategy to reduce damage during inflammation

Material & Methods

Human

Study patients

This study was conducted at the University Hospitals Leuven (Leuven, Belgium). All included patients had given written consent to participate in the Institutional Review Board approved IBD Biobank (B322201213950/S53684). Non-IBD controls were included, who presented at the endoscopy department for screening colonoscopies and had a normal endoscopy. Inflamed colonic biopsies of UC patients with active disease (Mayo endoscopic sub score >2) were taken at the edge an ulcer, as well non-inflamed biopsies in healthy individuals and UC patients with inactive disease (Mayo endoscopic sub score ≤1) (**Supplementary Table 1**). Collected biopsies were put in RNAlater (Ambion) and stored at -80°C. Similarly, serum was collected in healthy individuals and in active UC patients (Mayo endoscopic sub score >2) initiating anti-TNF therapy (**Supplementary Table 2**). In anti-TNF treated patients, serum was also collected at the time of endoscopic re-assessment (week 8 adalimumab; week 14 infliximab according to local reimbursement criteria). Endoscopic response to anti-TNF was defined as a Mayo endoscopic sub score ≤ 1.

Gene expression studies in colonic biopsies

Total RNA from colonic biopsies was extracted using the AllPrep DNA/RNA Mini kit (Qiagen) and processed for RNA sequencing as described earlier[26]. In brief, next-generation single-end sequencing was performed using the Illumina HiSeq 4000NGS, after library preparation using the TruSeq Stranded mRNA protocol (Illumina). Raw RNA-sequencing data were aligned to the reference

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genome (GRCh37) using Hisat2 version 2.1.0 and absolute counts were generated using HTSeq, after which counts were normalized and differential gene expression assessed using the DESeq2 package, with false discovery rate (FDR) corrected p-values. Neutrophilic enrichment scores from bulk RNA sequencing were generated by cellular deconvolution using xCell[27].

Quantification of serum HGF

Serum HGF was measured using the MesoScale Discovery electrochemiluminescence technology according to manufacturer's instructions (MSD).

Mice

Experimental animals and ethics statements

Animal studies were conducted in a gender- and age-matched manner. 10-12 week old male wild-type (WT; C57BL/6J), c-MET$^{fl/fl}$ and MRP8$^{Cre/+}$ c-MET$^{fl/fl}$ mice were kept at the KU Leuven animal facility on a 12:12hour light–dark cycle and provided with commercially available chow (ssniff R/M-H; ssniff Spezialdiäten) and had *ad libitum* access to tap water. All experimental animal procedures were approved by the Animal Care and Animal Experiments Committee of KU Leuven (Leuven, Belgium).

Dextran sodium sulfate (DSS)-induced colitis

For the induction of acute DSS-induced colitis, mice received 2.5% DSS (reagent grade; MW 35,000–55,000 kDa; TdB Consultancy) in drinking water for 5 days. For the induction of chronic DSS-induced colitis, mice received three cycles of 2.5% of DSS for 5 days followed by 2 weeks of normal drinking water. Disease progression was assessed daily via a standardized disease activity index (DAI) including body weight loss, stool consistency and blood in the faeces as described previously[28,29]. The scores from these three parameters were summed and divided by three as the DAI, ranging from 0 (healthy) to 4 (maximal severity of colitis). Mice that lost more than 20% of their initial body weight

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were immediately euthanized with CO_2 overdose. Extra attention was paid to visual clues like long-lasting piloerection and isolation from the group. After assessment of the DAI, mice were killed by CO_2 overdose and cervical dislocation, and the colon was resected for further investigation.

Lamina propria cell isolation

After removing the residual fat, the colon was opened longitudinally, cut into 2 cm pieces and washed in Hank's balanced salt solution (HBSS; Sigma-Aldrich) with 1% FCS (wash medium). Next, the tissue was shaken vigorously and the supernatant was removed. To remove the epithelial cell layer, tissue was placed into pre-warmed HBSS containing 1% FCS, 100 µg/ml of Pen-Strep (Gibco) 1 mM EDTA (Invitrogen) and 1 mM DTT (Sigma-Aldrich) and incubated for 12 minutes at 37°C. After removing the supernatant, a second incubation step was performed for 10 minutes at 37°C in the same pre-warmed medium without DTT. After washing in wash medium, the remaining tissue was cut into small pieces and digested for 35 min at 37°C in pre-warmed alpha MEM (Lonza) containing 5% FCS, 100 µg/ml of Pen-Strep (Gibco), 5 U/ml DNase (Roche), 1 mg/ml dispase (Gibco), 1.25 mg/ml Collagenase D (Roche), 0.85 mg/ml Collagenase V (Sigma-Aldrich). After blocking the enzymes with cold wash medium and filtration through a 70 µM Nitex cell strainer (VWR), single cells were counted using the Countess II automated cell counter (ThermoFisher).

Flow cytometry

Before staining, single-cell suspensions were pre-incubated with fixable viability dye e450 (Thermo Fisher Scientific) for 30 min followed by blocking Fc receptors with an antibody for CD16/CD32 (BD Bioscience) for 10 min. After incubation, cells were stained for 45 min at 4°C with labelled antibodies as listed in **Supplementary Table 3.** To assess the mean fluorescent intensity (MFI) of MET in neutrophils, we subtracted the MFI of the fluorescence minus one (FMO) from the MFI of the samples to eliminate the effect of background fluorescence between different tissues. The intracellular expression of IL-17A and IFN-γ was analyzed using a Cytofix/Cytoperm Kit (BD Bioscience) according to the manufacturer's instructions. In brief, the single cell suspension was

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incubated for 1 hour at 37°C with 50 ng/mL phorbol 12-myristate 13- acetate (PMA; Sigma-Aldrich) and 1 µg/mL ionomycin (Sigma-Aldrich) in RPMI-1640 (Lonza) supplemented with 100 µg/ml of Pen-Strep (Gibco), β-mercaptoethanol (Gibco), L-glutamine (Gibco) and 10% FCS. Next, GolgiPlug (BD Bioscience) was added to the medium and the cells were incubated for another 3 hours at 37°C. After surface staining, cells were fixed and permeabilized and intracellular cytokine staining was performed using anti-IFN-γ mAb (XMG1.2; BD Biosciences) and anti-IL-17A mAb (eBio17B7, eBioscience, San Diego, USA). Finally, samples were acquired using a FACSCantoII (BD Biosciences) and analysed with FlowJo software (version 4.6.2, Treestar).

Quantification of murine serum HGF

Blood was collected by cardiac puncture using a 23–gauge needle (Terumo), was allowed to clot for 30 minutes at room temperature and spun down at 2000 g for 20 minutes. Supernatant was removed and stored at -80°C until further processing. A murine HGF ELISA kit (MHG00; R&D Systems) was used according to manufacturer's protocols.

Bone marrow neutrophil isolation

Mouse neutrophils were isolated from the bone marrow with EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell Technologies) according to manufacturer's instructions. Briefly, the tibia and femur of mice were dissected and all tissue and muscle were removed from the bone. Bone marrow cells were flushed using a 24-gauge needle (Terumo) with DMEM high glucose (Lonza) supplemented with 10% FBS. After cells were collected and counted, neutrophils were isolated with the kit as mentioned above. Next, 10^5 neutrophils were stimulated with or without 15 ng/ml TNF-α (Peprotech) for 4 hours at 37°C followed by an incubation step with or without 100 ng/ml HGF (Peprotech) for 4 hours at 37°C.

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RNA isolation and gene expression

Cells were directly lysed in RLT buffer (Qiagen) containing 1% β-mercaptoethanol (Sigma-Aldrich) and stored at -80°C until further use. Colonic tissue was preserved in RNAlater (Qiagen) for 24 hours after sacrificing the mice and stored at -80°C until further use. Next, the tissues/cells were homogenized using the TissueLyser II homogenizer (Qiagen) and total RNA was extracted with the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Total RNA was transcribed into cDNA with qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. Gene expression levels were determined by quantitative reverse transcription PCR (RT-PCR) with LightCycler 480 SYBR Green I Master (Roche) using the LightCycler 96 (Roche) using primers for Rpl32 (FW: AAGCGAAACTGGCGGAAAC; RV: TAACCGATGTTGGGCATCAG), TNF-α (FW: TCTTCTCATTCCTGCTTGTGG; RV: CACTTGGTGGTTTGCTACGA), IL-1β (FW: GACCTTCCAGGATGAGGACA; RV: TCCATTGAGGTGGAGAGCTT). Results were quantified using the 2-ΔΔCT method[30] and expression levels of the genes of interest were normalized to the expression levels of the reference gene *Rpl32*. qPCR experiments were performed in duplicate and results are expressed as mean values.

Statistical analysis

Significance between two groups was determined by unpaired two-tailed Student's t-test or non-parametric Mann-Whitney U test, while one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was performed to compare multiple mean groups. Significance for the body weight and DAI curves was determined by two-way repeated ANOVA followed by Bonferroni post hoc analysis. GraphPad Prism 8.2.1 software (GraphPad) was used to generate graphs and perform statistical analysis, as well as R version 3.5.2.

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Results

Upregulation of colonic HGF and MET in patients with ulcerative colitis

To address the role of the HGF-MET pathway during chronic intestinal inflammation, we first evaluated the mRNA expression of both *HGF* and *MET* in colonic biopsies from patients suffering from UC compared to healthy non-IBD controls. Interestingly, both bulk *HGF* and *MET* mRNA expression were significantly increased in UC patients with active endoscopic disease compared to healthy controls **(Figure 1A-B)**. Moreover, *HGF* and *MET* mRNA levels were not increased in patients with quiescent UC disease **(Figure 1A-B)**, suggesting a possible correlation between the HGF-MET pathway and disease severity. In line, colonic *HGF* and *MET* expression levels positively correlated with C-reactive protein (CRP) (HGF ρ=0.23, p=0.04; MET ρ=0.22, p=0.04; data not shown). We also observed a positive correlation between colonic *HGF* and *MET* mRNA expression (**Supplementary Figure 1**). Finally, in order to correlate the expression of a specific gene signature involved in UC with immune cell types in inflamed colonic tissues, we performed a cellular deconvolution analysis using xCell to enable us to computationally estimate the proportions of neutrophils in colonic biopsies of patients with active UC[27]. We observed a positive correlation between the neutrophilic gene enrichment signature and the expression of *HGF* and *MET* **(Figure 1C-D)**, indicating a role for neutrophilic HGF-MET signaling in the colonic mucosa of UC patients.

Serum HGF protein levels correlate with disease activity in ulcerative colitis

Taking into account that colonic *HGF* mRNA levels were upregulated in active UC, we asked whether serum HGF may represent a potential biomarker of disease activity in UC. First, we measured serum HGF in 110 UC patients with active endoscopic disease and 28 healthy non-IBD controls. We observed a significant upregulation of serum HGF in active UC patients as compared to healthy

controls (**Figure 1E**) with significantly higher serum HGF in patients with increasing Mayo endoscopic sub scores (**Figure 1F**) and with a more extensive disease (**Supplementary Figure 2**). Although CRP is not a specific marker for intestinal inflammation and is often falsely low despite active mucosal inflammation in UC patients[31,32], it might have some correlation with clinical and endoscopic disease activity. Similarly, serum HGF levels positively correlated with CRP and with blood neutrophil count (**Figure 1G-H**). Of note, serum HGF more accurately predicted endoscopic remission (i.e. Mayo endoscopic sub score 0) as compared to CRP [area under the curve (AUC) 0.69, 95% CI 0.58 – 0.68 versus AUC 0.58, 95% CI 0.49 – 0.67; p=0.02, data not shown], suggesting serum HGF as a potential novel serological biomarker to monitor active mucosal disease in patients with UC.

Serum HGF levels do not predict response to anti-TNF therapy in ulcerative colitis

As serum HGF was upregulated in UC patients with active endoscopic disease[15], we decided to address if serum HGF could be used as a predictive biomarker of response to anti-TNF therapy in UC patients. Anti-TNF administration significantly reduced serum HGF levels ($p=2.0 \times 10^{-7}$) (**Figure 2A**) to comparable values as seen in healthy controls only in patients with positive response to anti-TNF therapy (Mayo endoscopic sub score of 0-1), but not in patients with ongoing inflammation (**Figure 2B**). However, baseline serum HGF was not predictive for future response to anti-TNF therapy 8-14 weeks after treatment initiation (**Figure 2B**). These findings indicate that serum HGF could be used as a potential biomarker for inflammation in UC, but cannot be used as a predictive biomarker for anti-TNF induced endoscopic remission.

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A similar profile for HGF expression is observed in rodents during acute DSS colitis

To further elucidate the role of HGF-MET signaling in neutrophils during intestinal inflammation, acute DSS colitis was induced in mice. Colonic *HGF* and *MET* expression were assessed at baseline, during acute inflammation and during the recovery phase (**Figure 3A-B**). While *HGF* transcripts remained significantly upregulated both during the acute phase and the recovery phase of acute DSS colitis, *MET* was unaltered during both the acute and recovery phase of the disease. Furthermore, there was an upregulation of HGF in mouse serum samples at day 7 following the induction of DSS colitis with a return to baseline at day 14 after the induction of DSS colitis (**Figure 3C**). However, no correlation was observed between colonic *HGF* and colonic *MET* or serum HGF during DSS-induced colitis (**Supplementary Figure 3A-B).** Taken together, the pattern of murine HGF expression in the blood and colon is therefore similar to the pattern observed in UC. However, MET is upregulated during active UC while it remains unaltered during murine intestinal inflammation.

Acute DSS colitis alters MET expression on neutrophils in the circulation and in the colon

To assess the contribution of MET^+ neutrophils during DSS colitis, we determined the amount of circulating and infiltrating MET^+ neutrophils during intestinal inflammation (**Figure 4**). Flow cytometric analysis revealed that intestinal inflammation did not alter the percentage of circulating blood neutrophils (**Figure 4A-B**). However, both the percentage and mean fluorescent intensity of MET^+ blood neutrophils was significantly increased during the acute inflammatory phase of the disease (**Figure 4C-D**). The percentage of MET^+ blood neutrophils remained elevated during the course of intestinal inflammation until the recovery phase (**Figure 4C)**. In comparison, the percentage of neutrophils was significantly increased in the colon during the acute phase of DSS colitis and remained elevated during the recovery phase (**Figure 4E-F**). Similar to the blood, an upregulation in the percentage and MFI of MET^+ neutrophils was noticed in the colon during acute

colitis, which decreased towards the recovery phase of the disease (**Figure 4G-H**). A similar increase was observed for both neutrophils and MET^{+} neutrophils when the cell number was assessed during the acute and recovery phase of DSS colitis as compared to control mice (**Supplementary Figure 4A-B)**. These data indicate that neutrophilic MET signaling is involved during the course of DSS-induced colitis.

Neutrophilic MET deficiency leads to reduced severity of DSS-induced colitis

To determine the exact contribution of the MET-HGF axis in neutrophils during intestinal inflammation, we subjected $MRP8^{Cre/+}$ $MET^{fl/fl}$ (cMET KO) mice and its littermate $MET^{fl/fl}$ (WT) controls to acute and chronic DSS colitis. Interestingly, cMET KO mice showed a reduced body weight loss and an improved disease activity index (DAI) in comparison with its littermate controls during both acute and chronic DSS-induced colitis (**Figure 5A-B and Supplementary Figure 5A-B)**. For the stool consistency and blood in the faeces, we only observed a significant reduction for the blood at day 5 and a trend towards a reduced stool consistency over time in cMET KO mice compared to its littermate controls during acute DSS-induced colitis (**Figure 5C-D).** In addition, flow cytometric analysis (**Figure 5E-J**) showed a reduced amount of $CD45^{+}$ immune cells in the colonic lamina propria of cMET KO mice during the recovery phase of acute colitis compared to its littermate controls (**Figure 5E**). More specifically, we observed a significant reduction in the number of $Ly6G^{+}$ neutrophils (**Figure 5F**) and $Ly6C^{hi}$ monocytes (**Figure 5G**) in the cMET KO mice during acute DSS colitis. When we assessed the same cellular subsets during early and late recovery in cMET KO mice during chronic DSS colitis, we could not observe any differences between cMET KO mice and its controls in cell numbers during the early recovery (**Supplementary Figure 5C-E**). However, during the late recovery of chronic DSS colitis, we observed a similar pattern as seen in acute DSS colitis with a reduced number of $CD45^{+}$ immune cells (**Supplementary Figure 5C**), $Ly6G^{+}$ neutrophils

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(**Supplementary Figure 5D**) and Ly6C^{hi} monocytes (**Supplementary Figure 5E**) in cMET KO mice compared to its littermate controls. Of note, the cMET KO mice seemed to resolve inflammation faster as compared to the littermate controls, where cell numbers remained elevated during the late recovery, implying a protective effect of MET deletion in MRP8^{+} neutrophils during colonic inflammation.

Neutrophilic MET signaling increases T_H17 expansion in an IL-1β dependent fashion

To further elucidate the observed phenotype in the cMET KO mice during colonic inflammation, we evaluated the amount of CD4^{+} T cells, more specifically T_H1 and T_H17 cells during acute and chronic DSS colitis (**Figure 5H-K and Supplementary Figure 5F-H**). Strikingly, CD4^{+} T cells were reduced in both acute (**Figure 5I**) and chronic (**Supplementary Figure 5F**) DSS colitis. Furthermore, there was a predominant decrease in the number of T_H17 cells in cMET KO mice compared to WT mice in both models (**Figure 5K and Supplementary Figure 5H**), while no differences were observed in the number of T_H1 cells (**Figure 5J and Supplementary Figure 5G**). These results showed that neutrophilic MET drives the expansion of T_H17 cells during colonic intestinal inflammation.

As IL-1β is known to be an important factor regulating the differentiation of naïve T cells into T_H17 cells[33,34], we assessed the production of *IL-1β* by WT and cMET KO bone marrow derived neutrophils upon stimulation with TNF-α and HGF (**Figure 6A)**. At baseline, cMET KO neutrophils displayed a similar expression of *IL-1β* and *TNF-α* compared to WT neutrophils (**Figure 6B-C**). Moreover, while TNF-α activation and HGF stimulation could increase *IL-1β* in WT neutrophils, cMET KO neutrophils had a blunted response and had an overall decreased expression of *IL-1β* as compared to WT

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neutrophils (**Figure 6B).** In comparison, cMET KO neutrophils also remained unresponsive to TNF-α and HGF stimulation for *TNF-α* expression compared to WT neutrophils (**Figure 6C**). Taken together, these data indicate that activation of neutrophilic MET stimulates the production of IL-1β.

Discussion

Novel therapeutic strategies in IBD are mainly aimed at blocking and modulating the adaptive immune response. However, accumulating findings have underlined a central role of the innate immune system, more specifically neutrophils, in the pathogenesis of IBD[35]. In the current study, we unraveled the role of the neutrophilic HGF-MET axis in intestinal inflammation, and additionally investigated whether HGF could be used as a UC disease activity biomarker. We showed that both patients with active UC and mice during the acute inflammatory phase of DSS-induced colitis have increased mucosal and serum expression of HGF. Moreover, human serum HGF was positively correlated with CRP and blood neutrophil counts, but was not predictive for future response to anti-TNF therapy. Furthermore, we showed that the absence of MET in MRP8^{+} neutrophils reduced the severity of DSS-induced colitis. This phenotype was correlated with a decreased infiltration and differentiation of immune cells, more specifically T_H17 cells. In addition, we observed that MET deficient neutrophils that were activated by TNF-α and HGF, produced less IL-1β and TNF-α, which can be correlated possibly to a decreased differentiation of naïve T cells into T_H17 cells.

In UC, there is extensive neutrophil infiltration in the lamina propria, the surface epithelium and crypt epithelium, which is associated with the release of NETs, erosion and ulceration during progressively worsening disease[4,5]. Therefore, neutrophil infiltration has been used an important diagnostic tool for the histological assessment of UC and has been included in commonly used UC

scoring system, such as in the Geboes score[36,6]. Interestingly, UC patients in remission with only a few remaining mucosal neutrophils are at increased risk of adverse clinical outcomes, similar to UC patients with more prominent neutrophil infiltration[37]. In line, neutrophil-derived biomarkers including fecal calprotectin (FCP)[38-41], lactoferrin[38,39,42] and serum matrix metalloproteinase-9[43] have been extensively investigated and proved to be essential to monitor disease activity without invasive endoscopic procedures. With the increasing incidence of IBD in newly industrialized countries[44] and the high percentage of patients with IBD still unresponsive to therapy[45], there is an unmet need to prevent inflammatory flares and to sustain remission. In this regard, neutrophils might pose as a novel target to develop new therapeutic strategies in IBD.

In line with previous studies showing increased colonic *HGF* in the inflamed mucosa of adult UC patients[46] and elevated serum HGF in children with UC[47], we observed an increased expression of *HGF* and *MET* in colonic biopsies of active UC patients, which positively correlated with a neutrophil enrichment score and thus reflected a neutrophilic involvement during active UC. Moreover, we confirmed the increase of serum HGF in a large cohort of patients with active UC, which might be a surrogate marker of colonic inflammation.

To investigate whether serum HGF could compete with commonly used biomarkers for inflammation[48], we compared the levels of serum HGF with CRP. Upon an acute phase stimulus during inflammation such as IL-6, TNF-α or IL-1β, hepatocytes rapidly produce CRP[31]. However, CRP has only limited correlation with clinical and endoscopic disease activity in patients with UC, as many UC patients with active endoscopic disease present with normal CRP levels[31,32]. In this study, we showed that serum HGF was able to track endoscopic remission more precisely as compared to CRP. However, comparisons with other non-invasive markers including FCP are currently lacking, as there were no sufficient paired samples in our patient cohort.

To evaluate whether serum HGF could also be predictive for endoscopic response upon anti-TNF therapy, we subdivided serum HGF levels prior to and after anti-TNF treatment based on endoscopic remission. However, serum HGF was not predictive for future response to anti-TNF therapy. All in all, these results indicate that serum HGF could be employed in daily clinical practice as a surrogate biomarker for intestinal inflammation, as this non-invasive blood biomarker could be easily implemented on a broader scale compared to the more invasive tissue biomarkers from biopsies. Further validation is needed in larger independent cohorts from patients suffering from UC and CD and other intestinal inflammatory disorders diseases such as infections, irritable bowel syndrome or intestinal tumors to identify clear cut-off values for translation into daily clinical practice.

To further investigate the role of the HGF-MET pathway during intestinal inflammation, we employed a DSS colitis model which induces a UC-like pathology due to its toxicity to colonic epithelial cells associated with impaired mucosal barrier integrity, body weight loss, diarrhea and occult blood in stool. While similar expression patterns were observed for murine HGF in the colon and the serum as compared to UC, *MET* remained unaltered in the colon of mice undergoing DSS-induced colitis compared to naïve controls in contrast to a clear increase of *MET* in colonic biopsies of patients with active UC compared to healthy controls. As MET is expressed on epithelial cells in many organs during both embryogenesis and adulthood[49], we hypothesize that the severe epithelial erosions observed during DSS-induced colitis blocked the upregulation of the MET gene during the acute phase of the disease.

In agreement with previous studies[50,51], we employed the MPR8-Cre line to delete MET specifically from neutrophils, as limited cross-over has been detected in macrophages/monocytes, dendritic cells, natural killer cells, mast cells, basophils or eosinophils. MET signaling has been described in the regulation of the immune response in different immune cell types and is also responsible for migration of immune cells[15,52,53]. However, only a limited number of studies have been performed to elucidate the role of MET in neutrophils. Neutrophilic MET deletion has only been performed in

different cancer models as the MET proto-oncogene has been implicated in many solid tumors[49]. Thus, this is the first study exploring the role of MET in neutrophils in an inflammatory setting. In this study, we observed an amelioration of DSS-induced colitis upon neutrophilic MET deletion based on body weight and DAI. This phenotype was associated with a reduced infiltration of immune cells, more specifically neutrophils and monocytes and a reduced accumulation of T_H17 lymphocytes. In previous studies, neutrophils have already been implicated in the polarization of T_H17 cells. For instance, Tamassia *et al.* showed that TLR8 stimulated human neutrophils promote T_H17 differentiation in an IL-23 dependent fashion [54]. In addition, neutrophils have been found to produce IL-1β[55,56], which has been shown to be involved in T_H17 differentiation during inflammation[33,34]. Therefore, we explored alteration in cytokine production upon neutrophilic MET deletion. Of note, we found that MET deficient neutrophils produced decreased levels of the pro-inflammatory cytokines IL-1β and TNF-α, which can be linked to a reduced differentiation of naïve T cells into T_H17 cells in MET deficient mice. Similarly, Finisguerra *et al.* showed that MET deficient neutrophils in Lewis lung carcinoma produced reduced levels of *NOS2* compared to WT neutrophils, indicating that MET activation in neutrophils leads to a more pro-inflammatory phenotype[15]. In light of these findings, Czarnewski *et al.* identified two distinct subgroups of UC patients of which UC1 patients have active neutrophilic inflammation and are refractory to anti-TNF thereapy[16]. TNF-α and IL-1β are among the top signaling cytokines that discriminate between the two subgroups, suggesting that neutrophilic HGF-MET signaling could lie at the basis of this signature. Taken together, these results indicate that targeting neutrophils during intestinal inflammation can aid in limiting inflammation and preventing flares during UC.

In conclusion, we showed that both patients with active UC and mice during the acute inflammatory phase of DSS-induced colitis have increased mucosal and serum expression of HGF. Moreover, serum HGF was positively correlated with CRP and blood neutrophil counts, while tissue *HGF* significantly correlated with a neutrophil signature. Furthermore, we showed that the absence of MET in $MRP8^+$

neutrophils reduced the development of DSS-induced colitis, presumably because MET deficient neutrophils produced less IL-1β and TNF-α, which was associated with a decreased differentiation of naïve T cells into T_H17 cells. Although additional preclinical and clinical studies are required to further unravel the HGF-MET pathway, our study highlighted that serum HGF might be used as a non-invasive biomarker to monitor disease activity in UC. Furthermore, neutrophilic MET targeting holds great promise and should be further investigated as a novel mode-of-action in the treatment of UC.

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Author contributions

M.S. and B.V. designed and performed experiments, analyzed data and wrote the paper; E.M., G.G., V.D.S., M.D.M., P.C. and S.V. performed experiments and analyzed results; S.V., M.F., M.M., G.B. and E.J.V. gave technical support and conceptual advice; G.M. conceived and supervised the project. All authors edited and revised the manuscript.

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Disclosure

The authors declared no conflict of interest regarding the current study.

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Figure legends

Figure 1. HGF and MET expression during colonic inflammation in active ulcerative colitis. A-B Normalized *HGF* (A) and *MET* (B) mRNA counts (RNA-sequencing) in colonic biopsies from healthy controls and ulcerative colitis (UC) patients with active and quiescent disease. Statistical significance was determined using an unpaired t-test. **p<0.01; ***p<0.001; NS = not significant. C-D Correlation between neutrophilic gene signature obtained by xCell and normalized *HGF* (C) or *MET* (D) mRNA counts in colonic biopsies of UC patients with active disease. E Serum HGF protein levels in healthy volunteers and patients with active UC. F Results from UC patients with active disease in figure 1E are subdivided based on Mayo endoscopic sub score. Statistical significance was determined using an unpaired t-test. **p<0.01. G-H Correlation between serum HGF levels in patients with active UC and blood neutrophil counts (G) or C-reactive protein (CRP) (H). Statistical significance and correlation coefficient were determined using the spearman's rank-order correlation.

Figure 2. Serum HGF is not predictive for response upon anti-TNF therapy. A Serum HGF protein levels in patients with active ulcerative colitis (UC) prior to and after anti-TNF therapy. Statistical significance is determined by an unpaired t-test. ***p<0.001. B Serum HGF protein levels from figure 6A are subdivided based on endoscopic remission after anti-TNF therapy.

Figure 3. DSS-induced colitis shows similar expression patterns for HGF in the colon and serum. A-B Log_2 of the fold induction of *HGF* (A) and *MET* (B) mRNA levels during a time course of acute DSS-induced colitis relative to naïve mice. C Serum HGF protein levels in mice during the course of acute DSS-induced colitis. Data are shown as mean ± SEM and were compared via one-way ANOVA to the naïve control. *p<0.05; **p<0.01; ***p<0.001 (n = 5-10 per group).

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Figure 4. Acute DSS colitis alters MET expression in neutrophils in the blood and the colon. A Gating strategy for the quantification of MET in blood neutrophils. Representative expression of live/dead and CD45 (left column), CD11b and Ly6G (middle column) and Ly6G and MET (right column) from the blood of naïve mice and 5 and 10 days after the induction of acute DSS colitis. B Percentage of CD11b+ Ly6G+ blood neutrophils among live CD45+ immune cells from naïve mice and 5 and 10 days after the induction of acute DSS colitis. C Percentage of MET+ blood neutrophils among live CD45+ CD11b+ Ly6G+ neutrophils. D Mean fluorescent intensity (MFI) of MET in the population of live CD45+ CD11b+ Ly6G+ blood neutrophils. Data are expressed as mean ± SD. Statistical significance was determined by an unpaired t-test. *p<0.05; **p<0.01; ***p<0.001 (n=5 per group). E Gating strategy for the quantification of MET in colonic neutrophils. Representative expression of live/dead and CD45 (left column), CD11b and Ly6G (middle column) and Ly6G and MET (right column) from the colon of naïve mice and 5 and 10 days after the induction of acute DSS colitis. F Percentage of CD11b+ Ly6G+ colonic neutrophils among live CD45+ immune cells from naïve mice and 5 and 10 days after the induction of acute DSS colitis. G Percentage of MET+ colonic neutrophils among live CD45+ CD11b+ Ly6G+ neutrophils. H Mean fluorescent intensity (MFI) of MET in the population of live CD45+ CD11b+ Ly6G+ colonic neutrophils. Data are expressed as mean ± SD. Statistical significance was determined by an unpaired t-test. *p<0.05; **p<0.01; ***p<0.001 (n=5 per group).

Figure 5. MET deletion in neutrophils reduces the severity of acute DSS-induced colitis and intestinal myeloid cell infiltration. A-D MRP8$^{Cre/+}$ MET$^{fl/fl}$ (cMET KO) and its littermate controls (WT) were subjected to acute DSS colitis using 2.5% of DSS in drinking water for 5 days. Body weight (A), disease activity index (B), stool consistency (C) and blood in stool (D) were assessed daily. Data are expressed as mean ± SEM as determined by the repeated-measures two-way ANOVA test. *p<0.05 (n= 11-13 mice per group). Representative image of two independent experiments. E-G Number of CD45+ immune cells (E), Ly6G+ neutrophils (F), Ly6Chi monocytes (G) in the colon of WT and cMET

KO mice during the recovery phase of acute DSS induced colitis. Data are expressed as mean ± SD and statistical significance is determined by an unpaired t-test. *p<0.05, **p<0.01. H Gating strategy for the quantification of T_H1 and T_H17 lymphocytes. Representative image of the expression of CD3 and CD4 (left column) among live $CD45^+$ cells and IFN-γ and IL-17A (right column) among live $CD45^+$ $CD3^+$ $CD4^+$ cells in the colon of WT and cMET KO mice during the recovery phase of acute DSS-induced colitis. I-K Number of CD3+ CD4+ T cells (H), T_H1 cells (I), T_H17 cells (J) in the colon of WT and cMET KO mice during the recovery phase of acute DSS colitis. Data are expressed as mean ± SD and statistical significance is determined by an unpaired t-test. *p<0.05; **p<0.01; NS=not significant.

Figure 6. Neutrophilic MET activation by TNF-α and HGF stimulates IL-1β and TNF-α production. A Bone marrow-derived neutrophils were stimulated with 25 ng/ml TNF-α for 4 hours at 37°C to induce the expression of the MET receptor. After incubation, cells were washed and stimulated with 100 ng/ml HGF for an additional 4 hours after which cells were lysed for RNA isolation. B-C Fold induction of *IL-1β* (B) and *TNF-α* (C) RNA expression in bone marrow-derived neutrophils from WT and cMET KO mice after stimulation with/without TNF-α for 4h followed by stimulation with/without HGF for 4h relative to WT unstimulated neutrophils. Data are expressed as mean ± SD and statistical significance is determined by an unpaired t-test (n=4-9/condition). *p<0.05; **p<0.01; ***p<0.001; NS=not significant.

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Figure 1

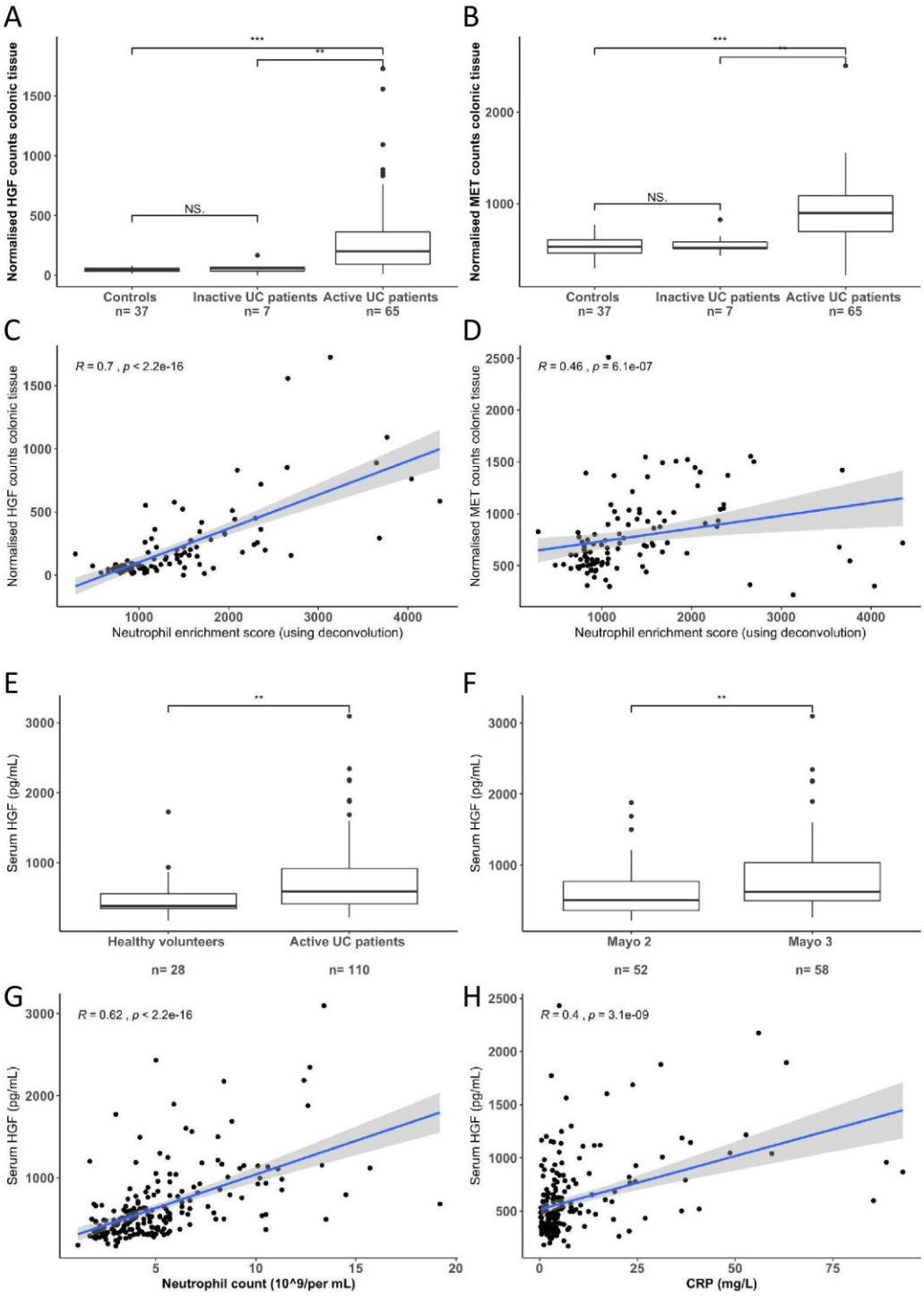

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Figure 2

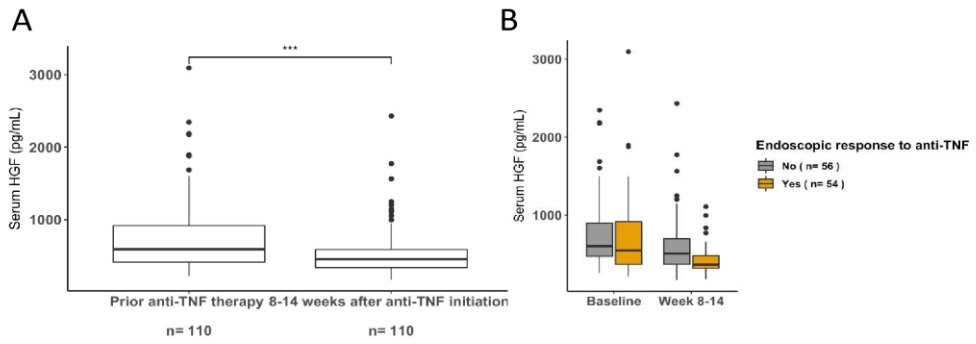

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Figure 3

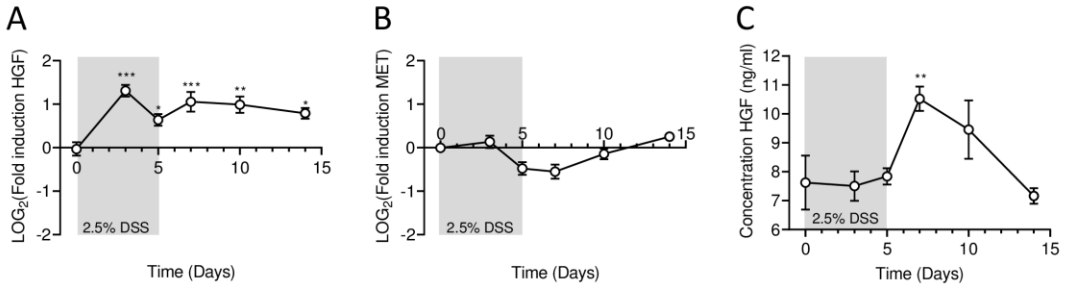

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Figure 4

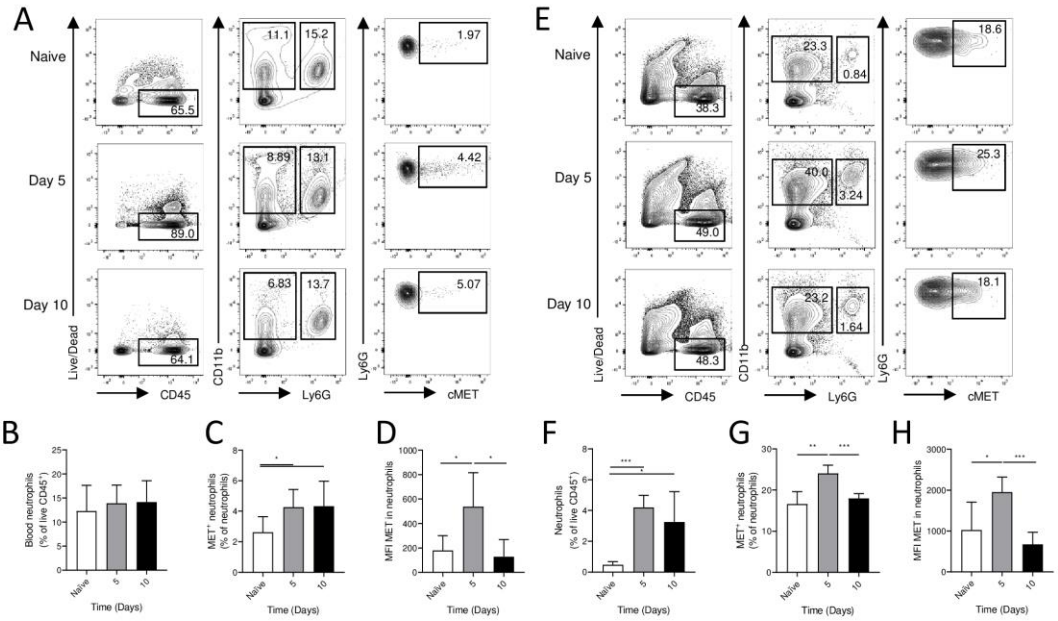

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Figure 5

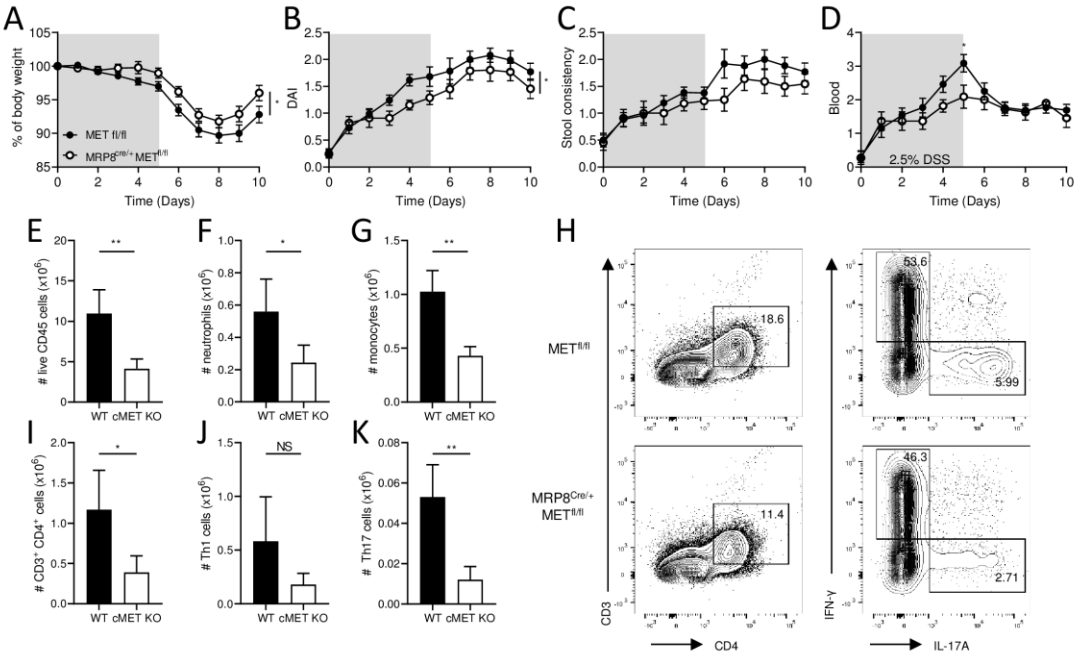

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Figure 6