An antibody against triggering receptor expressed on myeloid cells 1 (TREM-1) dampens proinflammatory cytokine secretion by lamina propria cells from patients with inflammatory bowel disease

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Short title:
Blocking TREM-1 down-regulates inflammation in IBD

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Abstract

Background: Triggering receptor expressed on myeloid cells 1 (TREM-1) is a potent amplifier of inflammation. Recently the antimicrobial peptide PGLYRP-1 was shown to be the ligand of TREM-1. Here, the ability of an anti-TREM-1 antibody to dampen the release of proinflammatory cytokines by colon lamina propria cells from IBD patients was investigated and correlated with PGLYRP-1 levels.

Methods: Biopsies from patients with ulcerative colitis (UC, n=45) and Crohn's disease (CD, n=26) were compared to individuals undergoing colonoscopy for other reasons (n=17). TREM-1 expression was analyzed on myeloid cells by flow cytometry. Cell culture experiments with lamina propria cells were used to analyze PGLYRP-1 and inflammatory cytokine levels and assess the effect of anti-TREM-1 on cytokine secretion.

Results: The frequency of TREM-1-expressing neutrophils and recruited macrophages was higher in inflamed than non-inflamed biopsies. The PGLYRP-1 level in inflamed tissue was higher than in non-inflamed tissue, it was produced primarily by neutrophils and its level correlated with the secretion of proinflammatory cytokines. Secretion of myeloperoxidase, TNF-α, IL-1β and IL-8 by lamina propria cells stimulated with the potent TREM-1 agonist consisting of PGLYRP-1 complexed with peptidoglycan was reduced in the presence of anti-TREM-1. Moreover, a blocking effect of anti-TREM-1 was apparent when lamina propria cells from a subset of inflamed individuals with elevated PGLYRP-1 were stimulated with killed bacteria.

Conclusions: An anti-TREM-1 antibody can dampen secretion of proinflammatory cytokines in inflamed patients with elevated PGLYRP-1. Moreover, PGLYRP-1 +

myeloperoxidase is a potential biomarker for predicting the effect of anti-TREM-1 therapy.

Keywords: TREM-1, PGLYRP-1, inflammatory bowel disease, ulcerative colitis, Crohn's disease

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract, with the two most prevalent disease types being Crohn's disease (CD) and ulcerative colitis (UC). Although intestinal commensals are important in maintaining the homeostatic balance in the healthy intestine, aberrant reactivity to the microbiota contributes to breakdown of this balance and drives the inflammation that underlies these diseases[1-3]. Cells of myeloid origin, particularly macrophages and dendritic cells (DCs), are central to maintaining intestinal homeostasis in the steady-state by, for example, promoting the generation of regulatory T cells, as shown primarily in mouse studies[4-7]. However, these cells can instead promote inflammation upon exposure to inflammatory signals[6,8-10]. Similarly, macrophages and DCs from healthy human intestine have anti-inflammatory properties[5,11-13] while those from inflamed intestine are pro-inflammatory[6,14-18]. The alternate functions of intestinal myeloid cells in steady-state versus inflammation underscores their pivitol role in homeostasis.

Triggering receptor expressed on myeloid cells 1 (TREM-1) is constitutively expressed on neutrophils and most monocytes/macrophages and potently amplifies inflammation[19-22]. TREM-1 is up-regulated by microbial components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) as well as toll-like receptor (TLR) engagement and TNF-α[19,23]. It can synergize with other microbial recognition receptors, particularly LPS/TLR4, to further potentiate the expression of pro-inflammatory cytokines, thus creating a vicious cycle that amplifies inflammation[19-21,23]. Indeed, the interaction between TREM-1 and TLR4 appears to be crucial, where TREM-1 modulates the activity and availability of key proteins in the TLR4 signaling cascades[24]. TREM-1 plays a critical role in the innate immune response to microbial

pathogens, and blocking TREM-1 enhances survival to bacterial sepsis and reduces serum proinflammatory cytokines[19,25].

The ligand for TREM-1 has recently been identified as PGN receptor binding protein-1 (PGLYRP-1)[26]. PGLYRP-1 is an antimicrobial peptide stored in neutrophil granules with PGN binding activity[27]. Neutrophil degranulation releases PGLYRP-1 that, multimerized with itself or complexed with peptidoglycan, potently activates TREM-1 causing proinflammatory cytokine release[26]. Peptidoglycan does not bind TREM-1 directly and, although it enhances binding of PGLYRP-1 to TREM-1, it is not absolutely required[26]. Peptidoglycan rather provides a scaffold allowing PGLYRP-1 multimerization to optimize binding of PGLYRP-1 to TREM-1. As peptidoglycan is a cell wall component of all bacteria, activation of TREM-1^+ cells by PGLYRP-1/peptidoglycan complexes in bacteria-rich environments potently amplifies inflammation[19]. Blocking TREM-1 with antagonistic peptides reduces serum inflammatory cytokines and attenuates chemically-induced colitis in mice[28]. However, the role of TREM-1 in driving the chronic inflammation of human IBD is not known.

Here we demonstrate that the frequency of TREM-1-expressing neutrophils and macrophages is increased in inflamed colonic biopsies from UC and CD patients. Moreover, an anti-TREM-1 antibody down-modulates proinflammatory cytokine secretion by lamina propria cells from biopsies taken from the inflamed colon of patients. High PGLYRP-1 levels in biopsies correlate with increased secretion of pro-inflammatory cytokines and the effect of the anti-TREM-1 antibody. Overall, blocking TREM-1 dampens release of inflammatory mediators in a subgroup of IBD patients with elevated PGLYRP-1 and MPO, suggesting that TREM-1 blockade could be a treatment for IBD.

Material and Methods

Patients and specimens

Approval for the study was granted by the regional Ethical Review Board in Gothenburg (permit 040-08) and informed written consent was obtained from all study participants. Patients with UC or colitic CD undergoing colonoscopy were recruited at Sahlgrenska University Hospital, Gothenburg, Sweden (Table 1). Individuals without inflammation undergoing colonoscopy for other reasons (polyps, weight loss, pain, etc.) were used as non-inflamed controls and will hereafter be called controls. Six to eight biopsies per inflamed or non-inflamed colon region were collected using a single-use forceps (Olympus, Tokyo, Japan). Macroscopic assessment of inflammation was made by the attending gastroenterologist and biopsies were subsequently examined microscopically by a pathologist. Peripheral blood was collected in heparinized collection tubes (Greiner Bio-One, Kremsmünster, Austria) and mononuclear cells were isolated by Ficoll density gradient (GE Healthcare, Little Charlfont, UK) according to the manufacturer's protocol.

Isolation of lamina propria cells (LPCs)

LPCs were isolated as previously described[29] Briefly, to remove epithelial cells, biopsies were incubated in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (Invitrogen, Carlsbad, USA) containing 2 mM EDTA, 15 mM HEPES (Invitrogen, Carlsbad, USA), 2% FBS (PAA, Pasching, Austria) for 3 x 15 min at 37°C with gentle agitation. This was followed by one or two 45 min incubations at 37°C in RPMI GLUTAMAX (Invitrogen, Carlsbad, USA) with 10% FBS, 60 K/ml DNase I (Sigma-Aldrich, St. Louis, USA), 40 CDU/ml of Collagenase D (Roche, Basel, Switzerland) or 25 CDU/ml Collagenase VIII (Sigma-Aldrich, St. Louis, USA) and

2.5mM $CaCl_2$ with gentle agitation. Digested material was filtered through a 100-µm strainer (TP-filter, Upplands Väsby, Sweden).

Flow cytometry

Cells were stained as described previously[29] using a combination of the following antibodies: anti-CD66b FITC (Biolegend, San Diego, USA., 305104), anti-CD56 PE-CF594 (BD Biosciences, New Jersey, USA., 562289), anti-CD19 PE-CD594 (BD, 562294), anti-CD3 PE-CF594 (BD, 562280), anti-CD45 APC-H7 (BD, 560178), anti-CD16 FITC (e-Bioscience, USA, 11016842), anti-CD354 Biotin (Biolegend, 314904), anti-CD14 FITC (BD, 555397), anti-CD11c BV450 (BD, 560369), anti-CD14 PE-Cy7 (BD, 557742), anti-CD15 PB (e-Bioscience, 57015973), anti-CD103 APC (e-Bioscience, 17103842), anti-CD141 PE (Miltenyi, 130090514), anti-CD1c PerCP/Cy-5.5 (Biolegend, 331514), anti-HLA-DR Alexa Fluor 700 (Biolegend, 327014), SA-PE (BD, 554061), SA-PE/Cy7 (BD, 557598), Trustain FcX (Biolegend, 422302), anti-CD141 PE/Cy7 (Biolegend, 344110), anti-CD64 APC (Biolegend, 305014) and LIVE/DEAD Aqua (Life Technologies, L34957). Cells were analyzed using a LSRII flow cytometer (BD Biosciences, Franklin Lakes, USA) and data were analyzed using FlowJo software (Tree Star, Ashland, USA)

Cytokine and ligand quantitation

Cytometric bead array (CBA) was performed according to the manufacturer's instructions using the Th1/Th2 11-plex kit (e-Bioscience, BMS810FF) with added simplex kits for MCP-1, myeloperoxidase (MPO) and IL-17A (all from e-Bioscience). Data were analyzed using a LSRII flow cytometer (BD Biosciences). PGLYRP-1 in

LPC supernatants was measured by ELISA (PGRP-S, R&D systems, DY2590) according to the manufacturer's recommendation.

Cell culture

2-5 x10^5 LPCs/well were seeded in 96-well flat bottom plates (Nunc, Roskilde, Denmark) and stimulated with or without one of the following: heat-killed (hk) *E. coli* LF82 isolated from a Crohn's disease patient[30], hk-*Bacillus subtilis* (ATCC, 23857) or 10 μg/ml peptidoglycan (PGN) (Invivogen, tlrl-pgnb2) plus 1 μg/ml of PGLYRP-1 (produced in house, Novo Nordisk, Målov, Denmark). Anti-TREM-1 antibody (IgG4; produced in house, Novo Nordisk) or isotype control (hIgG4-TNP; in house, Novo Nordisk) was added and cells were incubated for 24 h at 37°C with 5% CO_2 before supernatants were harvested and frozen at -20°C until further analysis.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA). For Comparison of two independent groups, the Mann-Whitney-U test was applied with a Bonferroni correction for multiple comparisons when needed. Wilcoxon signed rank test was used to evaluate differences between two paired groups and a Spearman's ranked test was used to evaluate correlation. A P value below 0.05 was considered statistically significant.

Results

TREM-1$^+$ neutrophils and TREM-1$^+$ CD14$^+$HLA-DR^{int} macrophages are increased in the lamina propria of inflamed colon

TREM-1 expression on LPCs, particularly myeloid cells, from biopsies of UC and CD patients was compared to the same cell population from controls. TREM-1-expressing neutrophils were defined as CD66b$^+$CD15^{hi} (Fig. 1a) and were distinguished from eosinophils based on the level of CD15 and CCR3 expression (Fig. S1). TREM-1$^+$CD66b$^+$CD15^{hi} neutrophils were more frequent in inflamed lamina propria (LP) of patients compared to LP of non-inflamed patients and to LP of controls (Fig. 1b). Importantly, the frequency of neutrophils expressing TREM-1 in inflamed colon was higher than that in adjacent non-inflamed regions where biopsies were taken during the same colonoscopy (Fig. 1c). Essentially all peripheral blood neutrophils, identified as CD66b$^+$CD15^{hi} cells (Fig. S2a), expressed TREM-1 (Fig S2c) and there was no difference in the frequency of TREM-1$^+$ neutrophils in the blood between controls, non-inflamed and inflamed UC and between controls and inflamed Crohn's patients (Fig. S2b-c).

Analysis of TREM-1-expression on resident CD14$^+$HLA-DR^{hi} and recruited CD14$^+$HLA-DR^{int} LP macrophages (Fig. 2a;[29]) revealed a higher number of TREM-1$^+$HLA-DR^{int} macrophages among CD45$^+$ LPCs in inflamed colon of UC and CD patients compared to non-inflamed regions and to controls (Fig. 2b). Similarly, the number of TREM-1$^+$HLA-DR^{int} macrophages in inflamed colon was significantly higher than non-inflamed regions from the same patient taken at the same endoscopy for both UC and CD (Fig. 2c). The frequency of TREM-1$^+$HLA-DR^{int} macrophages among HLA-DR^{int} macrophages in inflamed colon of UC patients was higher compared to non-inflamed biopsies from the same patient (Fig. 2d). For CD patients,

5 out of 6 patients showed increased frequencies of TREM-1 expressing HLA-DR^{int} macrophages (Fig. 2d). In contrast to TREM-1^{+}HLA-DR^{int} macrophages, the number of TREM-1^{+}HLA-DR^{hi} macrophages in inflamed tissue was similar to non-inflamed tissue (Fig. 2 e-f) with the exception of increased TREM-1^{+}HLA-DR^{hi} macrophages in inflamed colon of CD patients compared to controls (Fig. 2e). In peripheral blood, TREM-1 expression on monocytes was highest in the CD14^{++}CD16^{-} population followed by CD14^{+}CD16^{+} and CD14^{+}CD16^{++} cells (Fig. S2d).

The number of TREM-1-expressing dendritic cells, identified as lineage^{-}HLA-DR^{+}CD14^{-}CD11c^{+} LP cells and further divided into CD141^{+}CD103^{+} and CD1c^{+}CD103^{+} subsets (Fig. S3a-b;[29]), was not increased in inflamed versus non-inflamed colon (Fig. S3c-d). While the frequency of TREM-1^{+}CD141^{+}CD103^{+} was unaltered in inflamed and non-inflamed LP of UC and CD patients (Fig. S3c,d), TREM-1^{+}CD1c^{+}CD103^{+} DCs were decreased in inflamed tissue of UC but not CD patients when compared to controls (Fig.S3c,d).

Thus, neutrophils and HLA-DR^{int} macrophages expressing TREM-1, but not TREM-1-expressing HLA-DR^{hi} macrophages or dendritic cell subsets, are increased in inflamed colon of UC and CD patients.

Neutrophils are the predominant source of increased PGLYRP-1 in inflamed colon LP

PGLYRP-1 has recently been identified as a ligand for TREM-1[26] and whether PGLYRP-1 levels are altered in inflamed intestinal tissue of IBD patients is not known. We thus examined PGLYRP-1 levels in supernatants of LPCs from inflamed and non-inflamed colon of UC and CD patients and controls cultured *ex vivo,* without stimulation, for 24 hrs. PGLYRP-1 was higher in LPC supernatants from inflamed

biopsies compared to non-inflamed regions of patients, as well as to controls, for both UC and CD (Fig. 3a). Removal of $CD66b^+$ cells from LPCs isolated from inflamed biopsies dramatically reduced PLGYRP-1 levels (Fig. 3b). This suggests that neutrophils are the predominant source of the increased PGLYRP-1 in LPCs from inflamed colon of UC and CD patients.

PGLYRP-1 levels in intestinal tissue correlate with increased levels of several proinflammatory cytokines

To investigate the relationship between PGLYRP-1 and release of proinflammatory cytokines in colon LP, cytokines were quantitated from the same *ex vivo* LPC supernatants as PGLYRP-1 above (in Fig. 3a). Myeloperoxoidase (MPO) was also measured as a relative indicator of neutrophil abundance in the tissue. PGLYRP-1 levels correlated significantly with secretion of MPO, IL-β, TNF-α and IL-6 but not IL-8 in cultured LPCs from inflamed colon (Fig. 4). This suggests a positive relationship between PGLYRP-1, neutrophil infiltration and colon inflammation as reflected in several proinflammatory cytokines.

Blocking TREM-1 dampens the secretion of proinflammatory cytokines by LPCs from inflamed colon

Given the correlation between PGLYRP-1 and proinflammatory cytokines, we next addressed whether blocking TREM-1, which is the receptor for PGLYRP-1[26], would reduce cytokine secretion. Thus, LPCs from UC and CD patients with ongoing inflammation were stimulated for 24h with PGLYRP-1+PGN to activate TREM-1[26]. This was done in the presence of an anti-TREM-1 antibody or an anti-TNP isotype control and MPO and proinflammatory cytokines were subsequently measured in the

supernatants. Secretion of MPO, TNF-α, IL-1β and IL-8, but not IL-6, were reduced when PGLYRP-1 + PGN-stimulated LPCs from inflamed UC and CD patients were cultured in the presence of the anti-TREM-1 antibody (Fig. 5a). As PGLYRP-1 + PGN stimulation of LPCs is a very potent activator of TREM-1[26], we addressed the affect of anti-TREM-1 when LPCs were stimulated with bacteria as a broader stimuli and the source of PGN. Using this approach, only MPO secretion was reduced when LPCs were stimulated with hk *B. subtilis* (Fig. 5b). In addition, anti-TREM-1 had no effect on the secretion of MPO or proinflammatory cytokines when LPCs were isolated from non-inflamed and control patients (data not shown). Thus, blocking TREM-1 reduces MPO and proinflammatory cytokine secretion when TREM-1 on LPCs is stimulated with PGLYRP-1 + PGN while bacterial stimulation resulted in little effect of the antibody. Overall, the data indicate that the anti-TREM-1 antibody reduces secretion of several proinflammatory cytokines in inflamed tissue.

Blocking TREM-1 more potently reduces proinflammatory cytokine secretion by LPCs from inflamed individuals with high neutrophil influx

Blocking TREM-1 resulted in reduced inflammatory cytokine and MPO secretion when LPCs from inflamed biopsies were potently stimulated with PGLYRP-1+PGN whereas little effect was apparent using bacterial stimuli (Fig. 5). We addressed this further by asking whether an effect of blocking TREM-1 would become apparent with bacterial stimulation of LPCs from individuals with high neutrophil influx as a source of PLGYRP-1. The data in Fig. 6a revealed a subgroup of inflamed UC and CD patients with very high levels of both PGLYRP-1 (>1250 pg/ml) and MPO (>50 ng/ml), suggesting that they had high neutrophil influx and were highly inflamed. Using LPCs from this subgroup of patients (Fig. 6a), a reduced secretion of MPO,

TNF-α and IL-1β, the 3 factors where anti-TREM-1 had an effect using PGLYRP-1 + PGN-stimulated LPCs (Fig. 5a), was apparent after *ex vivo* stimulation with hk *E. coli* compared to the inflamed patients with lower MPO and PGLYRP-1 (Fig. 6b, compare "+ cutoff" to "- cutoff"). Thus, the anti-TREM-1 antibody reduces proinflammatory cytokine secretion by LPCs stimulated with hk *E. coli* in individuals with high neutrophil influx as reflected in high levels of MPO and PGLYRP-1.

Discussion

TREM-1 is selectively expressed on neutrophils and monocytes/macrophages, is upregulated by LPS, bacteria or during septic shock and potently amplifies inflammation[19-23]. However, little is known about the role of TREM-1 in human IBD. Consistent with previous data that showed increased TREM-1^+ $CD68^+$ or $CD33^+$ macrophages in the LP of IBD patients[28], we found a higher frequency of TREM-1^+HLA-DR^{int} macrophages, which are recruited to inflamed colon[29], relative to non-inflamed colon of IBD patients or controls. TREM-1^+ neutrophils were also higher in inflamed LP relative to non-inflamed in both UC and CD. Thus, the paucity of TREM-1^+ myeloid cells in non-inflamed colon LP is increased in inflamed colon of IBD patients and is accounted for by increased TREM-1^+ neutrophils and recruited HLA-DR^{int} macrophages while HLA-DR^{hi} resident macrophages contribute to a lesser extent.

In light of the increase in TREM-1-expressing cells in inflamed LP of IBD patients, and the potential role of TREM-1 in amplifying inflammation during IBD, we addressed if interfering with the activation of TREM-1 would dampen the secretion of proinflammatory cytokines and possibly lead to a new therapeutic option. To investigate this, we quantitated the level of the recently identified TREM-1 ligand, the antimicrobial peptide PGLYRP-1[26], in inflamed versus non-inflamed colon LP. We found increased levels of PGLYRP-1 in inflamed LP of both UC and CD patients, and neutrophils were largely responsible for the increase. Moreover, increased PGLYRP-1 in inflamed LP correlated with increased secretion of TNF-α, IL-β, IL-6 and MPO but not, for example, with the neutrophil recruiting cytokine IL-8

We next addressed whether interfering with the binding of PLGYPR-1 to TREM-1 using an anti-TREM-1 antibody would dampen the release of

proinflammatory cytokines. However, mulitmerization of PLGYPR-1 is crucial for it to become a functional TREM-1 ligand, and PLGYPR-1+PGN multimers are a potent TREM-1 agonist[26]. We thus performed initial blocking experiments using LPCs stimulated with PGLYRP-1+PGN to ensure robust TREM-1 ligation. Using this stimulus we found that the anti-TREM-1 antibody reduced secretion of TNF-α, IL-β, IL-8 as well as MPO by LPCs from inflamed colon. No reduction was seen in the secretion of IL-6, suggesting that the effect of the anti-TREM-1 antibody has on the IL-6 secretion by neutrophils and TREM-1^+ macrophages is most likely overcome by IL-6 secreted from cell types lacking TREM-1 expression. We next analyzed more physiological conditions and stimulated LPCs with heat-killed *E. coli* or *B. subtilis*. This resulted in a poor blocking effect of the anti-TREM-1 antibody, with a reduction found only in MPO secretion by *B. subtilis*-stimulated LPCs from inflamed colon. The poor blocking effect using LPCs stimulated with hk bacteria could be that too little PGLYRP-1 is released by bacteria-neutrophil interaction. This would result in insufficient PGLYRP-1 released by the neutrophils and poor PGLYRP-1 multermerization (with PGN). This, in turn, would inefficiently trigger TREM-1 and a blocking effect of the antibody would not be apparent due to poor TREM-1 ligation.

Moreover, stimulating LPCs with killed bacteria is a broader stimulation engaging more receptors and signaling pathways than PGLYRP-1 + PGN. It is thus likely pathways other than those mediated by TREM-1 will be activated with bacterial stimulation that can mask an effect of blocking the TREM-1 pathway. PGN in bacteria is also sequestered in the cell wall, which may make it less available to effectively bind PGLYRP-1 and result in insufficient multerimers to potently trigger TREM-1. Interestingly, the one significant blocking effect seen using hk bacteria-stimulated cells (MPO) was observed using Gram positive *B. subtilis*, which has a

higher fraction of its cell membrane composed of PGN relative to *E. coli*. Alternatively, the PGN present in the cell wall of the added *E. coli* and *B. subtilis* could interact with PLGYRP-1, depleting its availability to multerimerize and effectively bind TREM-1. Indeed, adding increased numbers of hk *E. coli* and *B. subtilis* to a fixed concentration of PLGYRP-1 resulted in reduced PLGYRP-1 remaining in the supernatant (data not shown). This indicates that PGLYRP-1 may preferentially bind bacteria or lysed bacterial products in the environment, leaving insufficient PGLYRP-1 to multimerize and stimulate TREM-1. Support for this comes from our data showing that a significant blocking effect of the anti-TREM-1 antibody was seen in a subgroup of individuals with both high MPO, indicating high neutrophil infiltration, and high PGLYRP-1, which comes from the neutrophils. In contrast, anti-TREM-1 did not have any effect on the release of TNF-α or IL-β when LPCs from patients with low MPO (reflecting low neutrophil influx) and low PGLYRP-1 were stimulated with hk *E. coli*. Thus, patients with high neutrophil influx and high PGLYRP-1 are a subgroup that could be suitable for anti-TREM-1 blocking therapy.

Taken together, we show that an anti-TREM-1 antibody can down-regulate the secretion of proinflammatory cytokines by LPCs from inflamed colon of IBD patients. We also show that high levels of the TREM-1 ligand, PGLYRP-1, correlate with increased proinflammatory cytokines in the LP and that high levels of PGLYRP-1 and neutrophil markers can predict a blocking effect of the anti-TREM-1 antibody. Indeed, we demonstrate that a subgroup of inflamed IBD patients with a high neutrophil influx respond better to the anti-TREM-1 antibody than patients with a lower neutrophil influx. These studies open the possibility for a new treatment for

IBD and offer insight into PGLYRP-1, combined with a neutrophil marker such as MPO, as biomarkers to predict patients that would benefit from anti-TREM-1 therapy.

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Figure legends

Figure 1. The frequency of TREM-1-expressing neutrophils is increased in inflamed colon of UC and CD patients. a) Dot plots show the gating strategy to identify neutrophils as live $CD45^+CD66b^+CD15^{hi}$ cells in the indicated patient groups. TREM-1 expression on neutrophils of representative patients from the indicated groups is show in the histogram. b) The number of $TREM\text{-}1^+CD66b^+CD15^{hi}$ neutrophils among 10^5 $CD45^+$ cells from inflamed and non-inflamed colon of UC and CD patients as well as controls is shown. The horizontal lines indicate the median. c) The percent of TREM-1-expressing $CD66b^+CD15^{hi}$ neutrophils among $CD66b^+CD15^{hi}$ cells from paired colon biopsies from inflamed and non-inflamed regions of the same patient taken during the same colonoscopy. Statistical significance was analyzed using the Mann Whitney U test with a Bonferroni correction for multiple comparisons (b) and Wilcoxon matched pairs signed rank test was used for paired samples in (c).

Figure 2. The frequency of TREM-1-expressing HLA-DR^{int} macrophages is increased in inflamed colon of UC and CD patients. a) The gating strategy used to identify HLA-DR^{int} and HLA-DR^{hi} macrophages in colon biopsies from UC and CD patients is shown. The dot plots are representative CD patients and a control. TREM-1 expression on the gated macrophage populations is displayed in the histograms. b) The number of $TREM\text{-}1^+$HLA-DR^{int} macrophages among 10^5 $CD45^+$ cells from inflamed and non-inflamed UC and CD patients as well as controls is shown. The median is indicated with a horizontal line. c) The number of TREM-1-expressing HLA-DR^{int} macrophages among 10^5 $CD45^+$ cells from paired colon biopsies from

inflamed and non-inflamed regions of the same patient taken at the same colonoscopy is shown. The median is indicated with a horizontal line. d) The frequency of TREM-1 expressing HLA-DR^{int} macrophages among HLA-DR^{int} macrophages from paired colon biopsies from inflamed and non-inflamed regions of the same patient taken at the same colonoscopy is shown. e) The number of TREM-1^{+}HLA-DR^{hi} macrophages among 10^5 CD45^{+} cells from inflamed and non-inflamed UC and CD patients as well as controls is shown. The median is indicated with a horizontal line. f) The number of TREM-1 expressing HLA-DR^{hi} macrophages among 10^5 CD45^{+} cells from paired colon biopsies from inflamed and non-inflamed regions of the same patient taken at the same colonoscopy is shown. For b) and e), statistical significance was assessed using the Mann Whitney U test with a Bonferroni correction for multiple comparisons, while samples Wilcoxon matched pairs signed rank test was used for paired samples in c), d) and f). ns = not significant ($p>0.05$)

Figure 3. Neutrophils are the primary source of increased PGLYRP-1 in inflamed colon of UC and CD patients. Total LPCs were isolated from biopsies of inflamed and non-inflamed colon of UC and CD patients as well as controls. Cells were incubated at 37°C for 24 hrs and the concentration of PGLYRP-1 in the supernatants was analyzed by ELISA. a) The left two panels show the PGLYRP-1 concentration in supernatants from the indicated patients groups. Dotted lines indicate the detection limit and horizontal bars indicate the median. The right panels show the PGLYRP-1 concentration in supernatants from non-inflamed and inflamed colon tissue taken from the same patient and at the colonoscopy. b) LPCs were isolated from inflamed colon biopsies from a UC patient and a fraction of the LPCs were depleted of CD66b^{+} cells. LPCs (With Neutrophils; grey bar) and CD66b^{+} depleted

LPCs (Without neutrophils; black bar) were then incubated at 37°C for 24 hrs and PGLYRP-1 was quantitated.

Figure 4. The level of pro-inflammatory cytokines correlates with the PGLYRP-1 level in inflamed colon. Total LPCs were isolated from biopsies of inflamed colon of UC and CD patients. Cells were incubated at 37°C for 24 hrs in the absence of stimulus and the indicated cytokines as well as PGLYRP-1 were quantitated in the same supernatant by CBA or ELISA, respectively. Cytokine concentrations were plotted against the PGLYRP-1 levels measured in the same supernatant and correlations were evaluated using a Spearman correlation coefficient.

Figure 5. Anti-TREM-1 reduces pro-inflammatory cytokine secretion by LPCs from inflamed colon stimulated with PGLYRP-1 + PGN. Total LPCs were isolated from biopsies of inflamed colon of UC and CD patients. Cells were incubated at 37°C for 24 hrs with either a) PGLYRP-1 + PGN or b) hk *E. coli* or hk *B. subtilis* in the presence of either anti-TREM-1 or anti-TNP antibody as indicated. Cytokine secretion in supernatants was measured by CBA. Circles are biopsies from inflamed colon of UC patients and squares indicate biopsies from inflamed colon of CD patients. The line connecting symbols indicate the cytokine level in stimulated cells incubated with anti-TNP relative to a parallel culture incubated with anti-TREM-1. *p* values where blocking with anti-TREM-1 resulted in a significant reduction compared to blocking with anti-TNP are indicated for the paired biopsies. All other comparisons were non-significant. A Wilcoxon matched pairs signed rank test was used and a *p* value <0.05 was considered statistically significant. ns = not significant ($p>0.05$).

Figure 6. Anti-TREM-1 reduces secretion of pro-inflammatory cytokines by LPCs from a subset of inflamed UC and CD patients stimulated with hk *E. coli*. a) The MPO vs. PGLYRP-1 plot from Figure 5 is shown with indicated cutoff values used for MPO (50 ng/ml) and PGLYRP-1 (1250 pg/ml). b) The level of the indicated cytokine detected in parallel cultures incubated with anti-TREM-1 or anti-TNP after 24h of stimulation with hk *E. coli,* expressed as "TREM-1/TNP ratio", is shown on the y-axis. Patients with expression levels below the 50 ng/ml and 1250 pg/ml cutoff values for MPO and PGLYRP-1, respectively, are indicated as "-cutoff" while patients with expression levels above both cutoff values (circled in panel a) are indicated as "+cutoff". Each dot represents a single individual indicated by a distinct color; the same color in the different plots represents the same individual. Circles indicate UC, squares indicate CD patients and triangles indicate the samples that are below the cutoff limit.

Figure S1. Gating strategy for distinguishing neutrophils and eosinophils. a) Total LPCs were isolated from colon biopsies and analyzed by flow cytometry to distinguish neutrophils and eosinophils. The dot plot shows the gating strategy used to identify two populations of $CD66b^{+}$ cells among live $CD45^{+}$ LPCs that differ in the level of CD15 expression. The histogram shows CCR3 expression on the indicated $CD15^{low}$ and $CD15^{high}$ populations.

Figure S2. The fraction of Trem-1^{+} neutrophils in peripheral blood is not altered by intestinal inflammation and TREM-1 expression is highest on $CD14^{++}CD16^{-}$ monocytes in peripheral blood. Total lymphocytes were isolated from the peripheral blood of UC and CD patients as well as controls and analyzed by flow cytometry. a)

The dot plot shows the gating strategy to identify $CD66b^{+}CD15^{hi}$ neutrophils from a CD patient with active inflammation. TREM-1 expression on the gated cells is shown in the histogram. b) The number and c) the percent of $CD66b^{+}CD15^{hi}$ neutrophils among $CD45^{+}$ cells in peripheral blood from controls, UC patients with an inflamed or non-inflamed colon and inflamed CD is shown. d) The gating strategy depicting $CD14^{++}CD16^{-}$ (classical), $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}CD16^{++}$ (non-classical) monocytes from peripheral blood from an inflamed CD patient is shown in the dot plot and TREM-1 expression on these monocyte populations is shown in the histogram.

Figure S3. Little if any change in the number of TREM-1-expressing $CD1c^{+}CD103^{+}$ and $CD141^{+}CD103^{+}$ DCs is apparent in inflamed colon. a) The gating strategy used to identify $CD1c^{+}CD103^{+}$ and $CD141^{+}CD103^{+}$ DCs in colonic biopsies is shown. The dot plot shows a representative CD patient with an inflamed colon. Cells were pregated as indicated in the left dot plot and $CD11c^{+}$HLA-DR^{+} cells were separated into $CD1c^{+}$ and $CD141^{+}$ populations followed by analysis of CD103 expression. The gates defining $CD1c^{+}CD103^{+}$ and $CD141^{+}CD103^{+}$ DCs are shown to the right with the lower panel being control staining for CD103 using HLA-DR^{hi} macrophages. b) The histogram shows TREM-1 expression on $CD1c^{+}CD103^{+}$ and $CD141^{+}CD103^{+}$ DCs from a non-inflamed region of a CD patient. c) - d) The number of TREM-1^{+} $CD1c^{+}CD103^{+}$ (left) and TREM-1^{+} $CD141^{+}CD103^{+}$ (right) DCs among 10^{5} $CD45^{+}$ cells from colon biopsies from inflamed and non-inflamed intestinal tissue of UC (c) and CD (d) patients as well as controls. The median is indicated with a horizontal line. Statistical significance was assessed using the Mann

Whitney U test with a Bonferroni correction for multiple comparisons. ns = not significant ($p>0.05$).

Table 1 Patient demographics

	UC	*CD*	*Controls*
Total number of patients	45	26	17
Male/female	29/16	15/11	10/7
Age	42 (19-73)	37,5 (22-70)	56 (26-78)
Smoking habit (active/ex smoker/never)	2/5/38	1/4/21	4/2/11
Disease duration, years	14 (0-58)	14 (1-41)	NA
Disease status (active inflammation/remission)	25/20	14/12	NA
Samples obtainted (colon/blood)	45/21	26/9	15/10
Treatments:			
5-ASA	20	8	0
5-ASA, anti-TNF	0	1	0
5-ASA, corticosteroids	1	1	0
5-ASA, thiopurines	8	1	0
Anti-TNF	0	3	0
Corticosteroids	4	0	0
None	6	6	17
Thiopurines	4	5	0
Thiopurines, corticosteroids	1	1	0

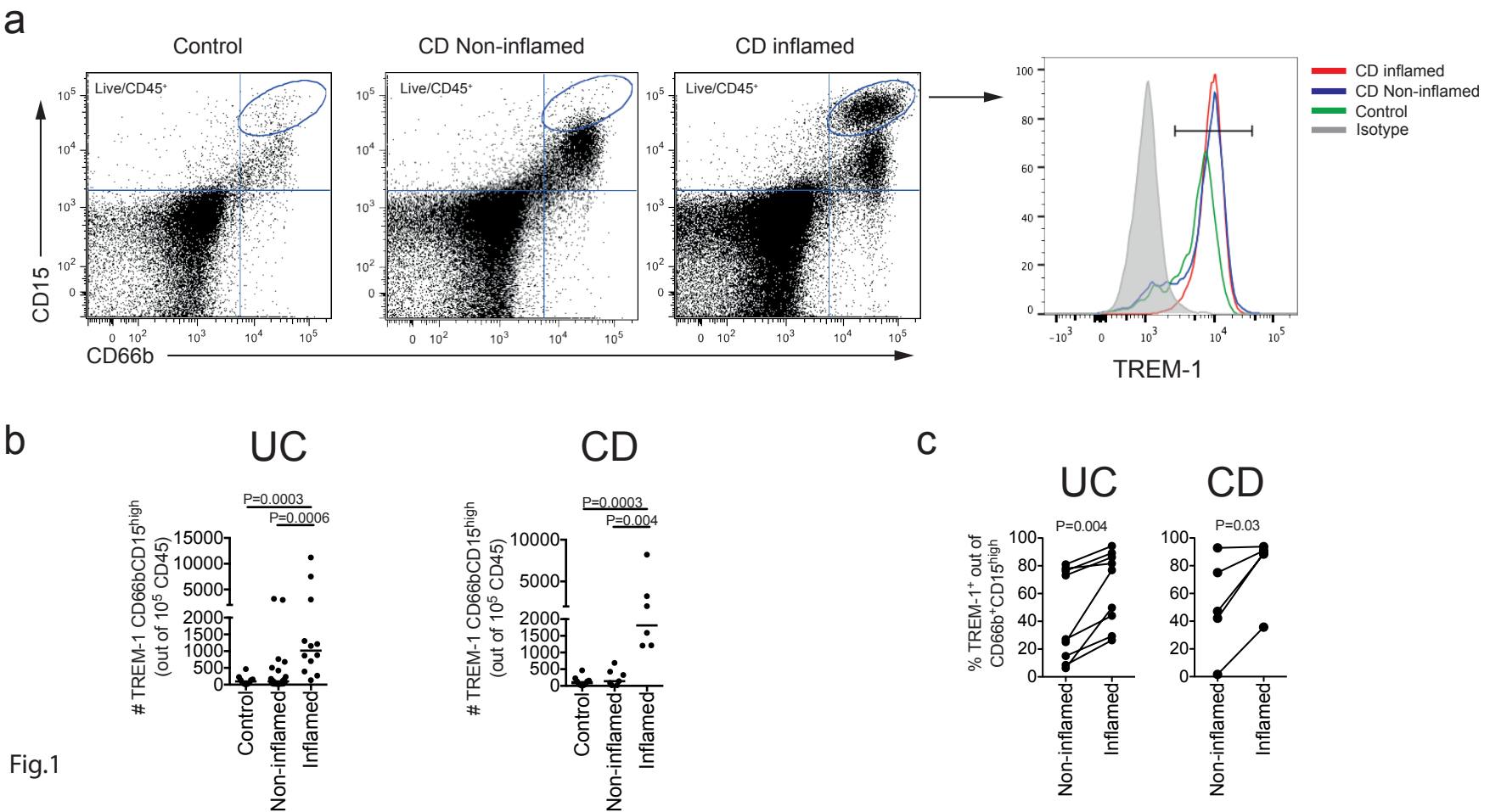

a
Control
CD Non-inflamed
CD inflamed
Live/CD45+
CD15
CD66b
TREM-1
CD inflamed
CD Non-inflamed
Control
Isotype
b
UC
CD
# TREM-1 CD66bCD15high (out of 10^5 CD45)
P=0.0003
P=0.0006
P=0.004
Control
Non-inflamed
Inflamed
c
% TREM-1+ out of CD66b+CD15high
P=0.004
P=0.03
Non-inflamed
Inflamed

Fig.1

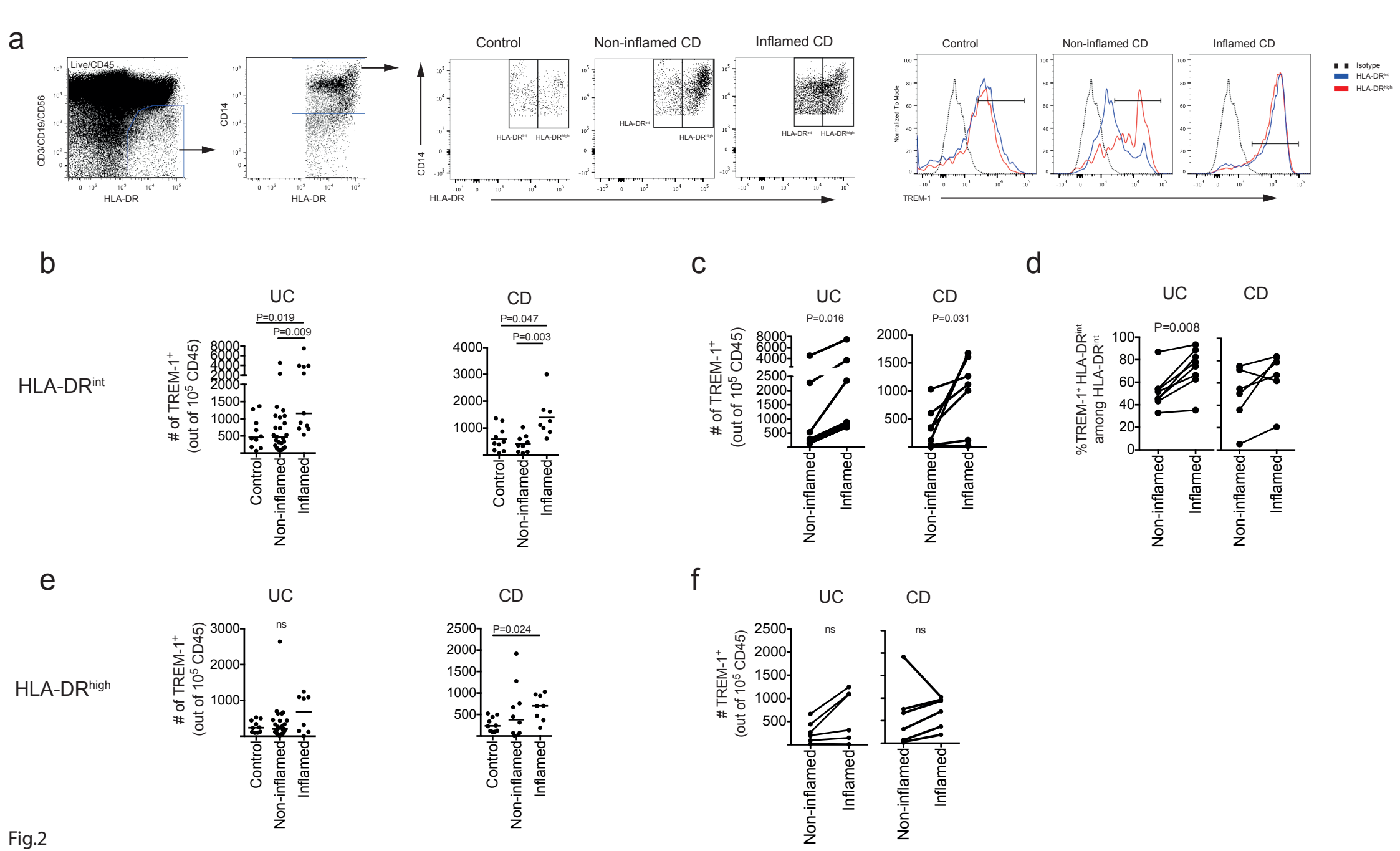

a
Live/CD45
CD3/CD19/CD56
HLA-DR
CD14
Control
Non-inflamed CD
Inflamed CD
HLA-DRint
HLA-DRhigh
Normalized To Mode
TREM-1
Isotype
b
UC
CD
HLA-DRint
# of TREM-1+ (out of 10^5 CD45)
P=0.019
P=0.009
P=0.047
P=0.003
Control
Non-inflamed
Inflamed
c
P=0.016
P=0.031
d
%TREM-1+ HLA-DRint among HLA-DRint
P=0.008
e
HLA-DRhigh
ns
P=0.024
f

Fig.2

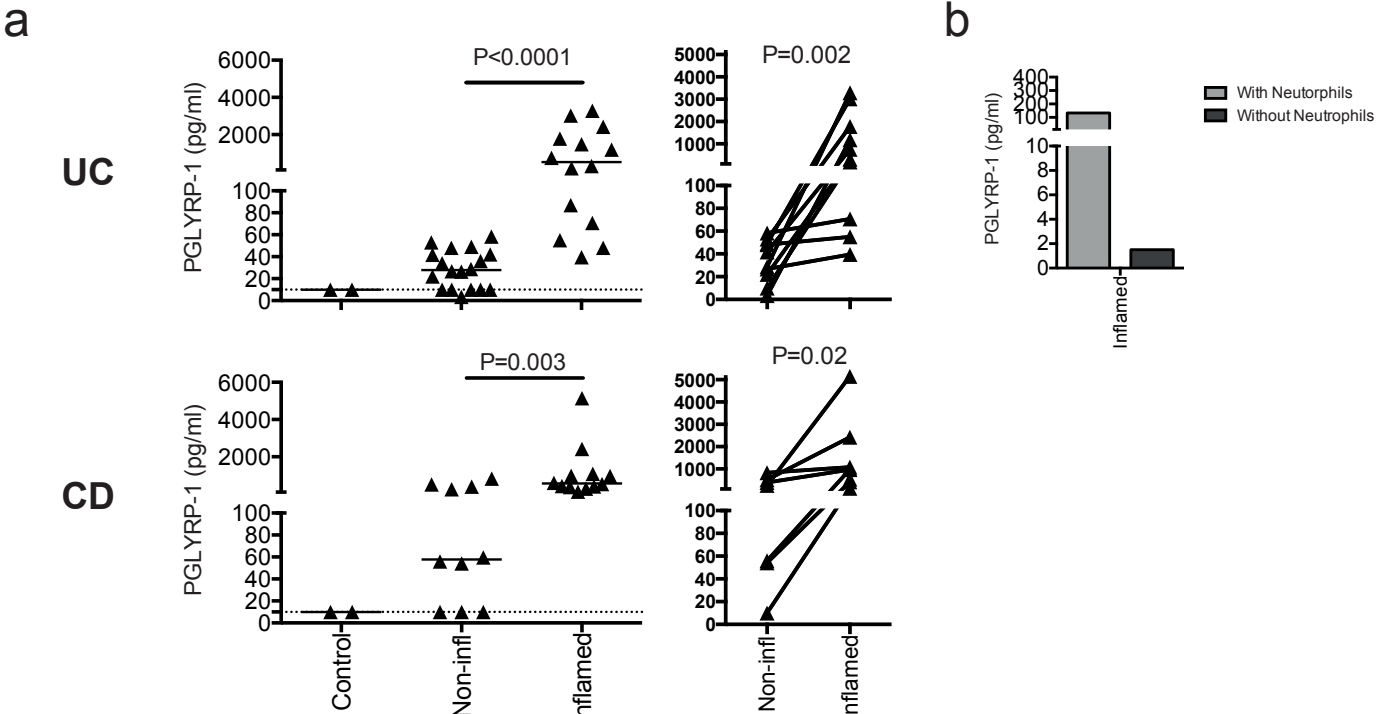
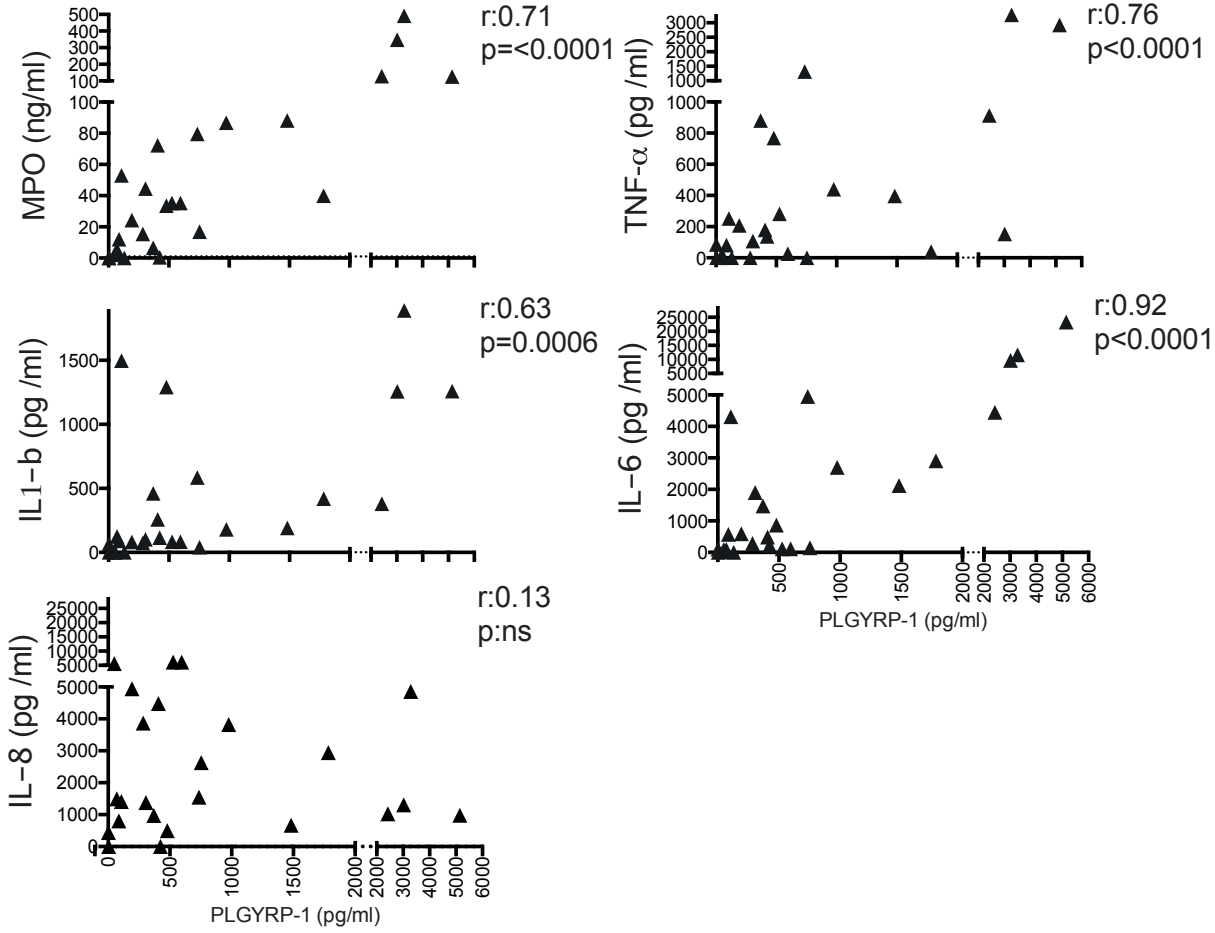

a
UC
CD
PGLYRP-1 (pg/ml)
P<0.0001
P=0.002
P=0.003
P=0.02
Control
Non-infl
Inflamed
b
With Neutorphils
Without Neutrophils
Inflamed

Fig. 4


MPO (ng/ml)
r:0.71
p=<0.0001
TNF-α (pg /ml)
r:0.76
p<0.0001
IL1−b (pg /ml)
r:0.63
p=0.0006
IL−6 (pg /ml)
r:0.92
p<0.0001
IL−8 (pg /ml)
r:0.13
p:ns
PLGYRP-1 (pg/ml)
PLGYRP-1 (pg/ml)

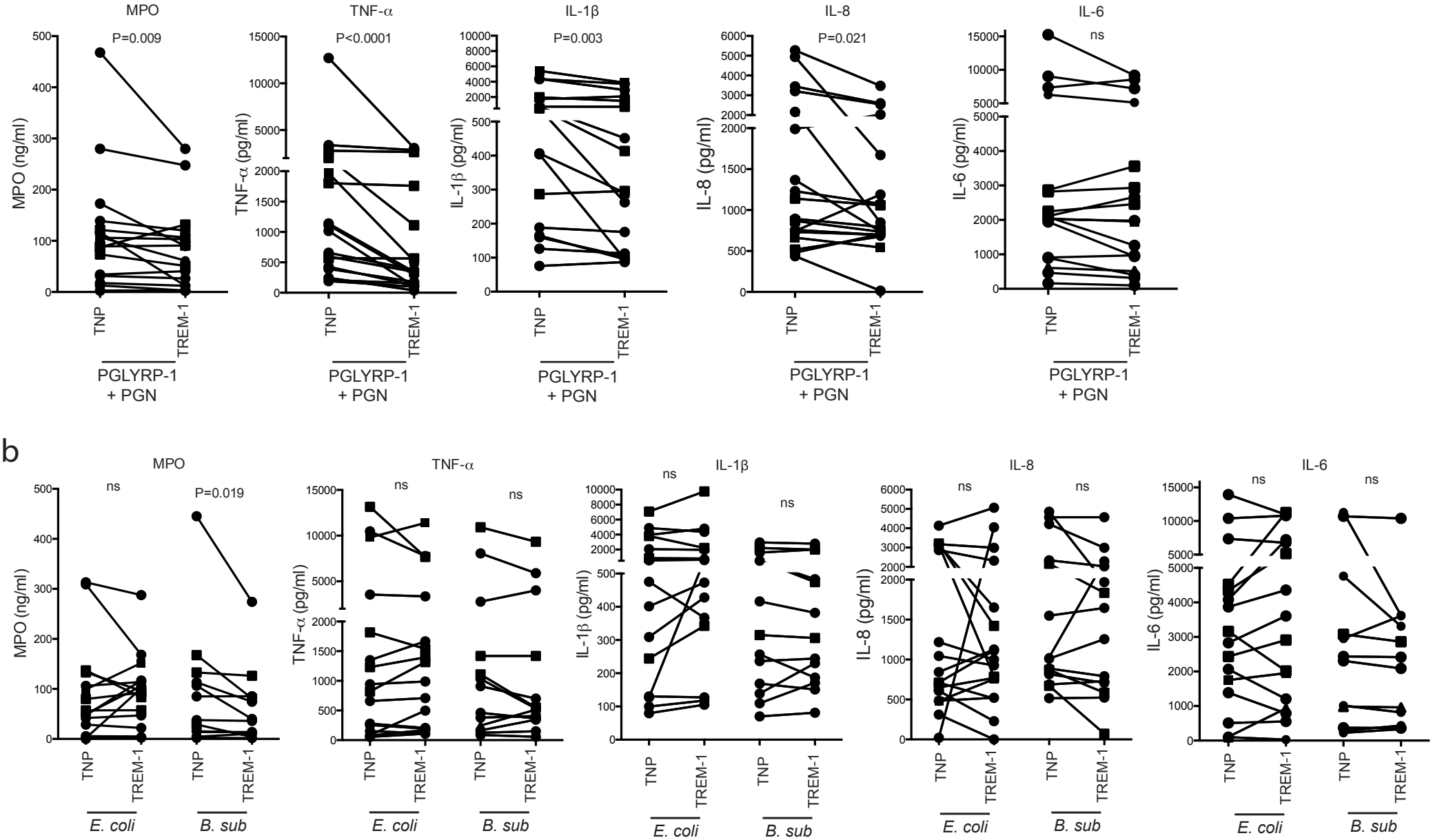
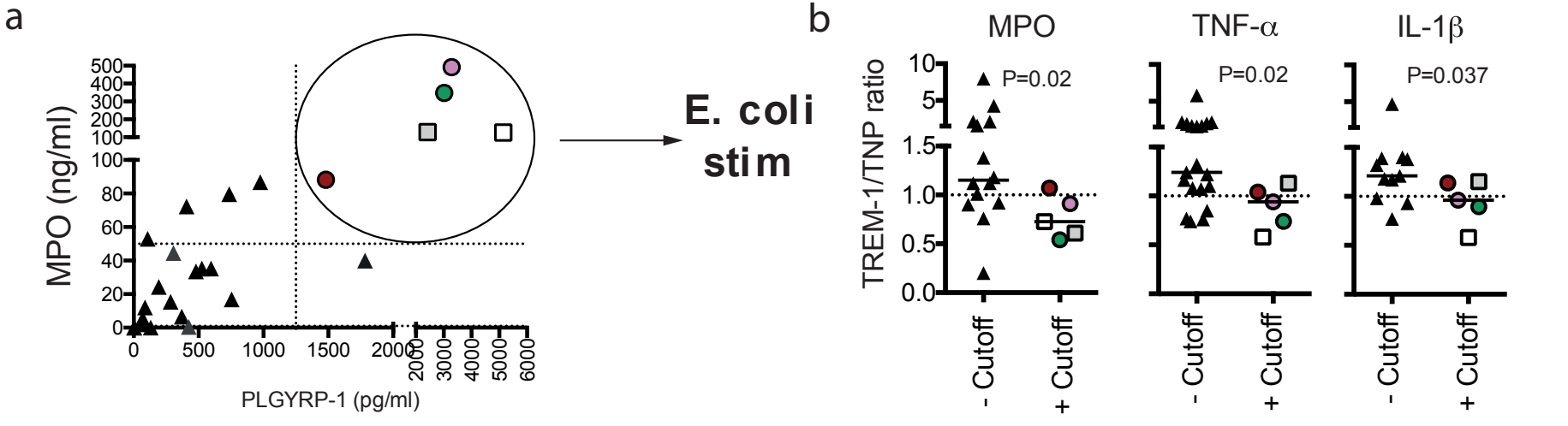

MPO
P=0.009
MPO (ng/ml)
TNF-α
P<0.0001
TNF-α (pg/ml)
IL-1β
P=0.003
IL-1β (pg/ml)
IL-8
P=0.021
IL-8 (pg/ml)
IL-6
ns
IL-6 (pg/ml)
TNP
TREM-1
PGLYRP-1 + PGN
b
MPO
ns
P=0.019
TNF-α
ns
ns
IL-1β
ns
ns
IL-8
ns
ns
IL-6
ns
ns
E. coli
B. sub

Fig. 6


a
MPO (ng/ml)
PLGYRP-1 (pg/ml)
E. coli stim
b
MPO
TNF-α
IL-1β
TREM-1/TNP ratio
P=0.02
P=0.02
P=0.037
- Cutoff
+ Cutoff

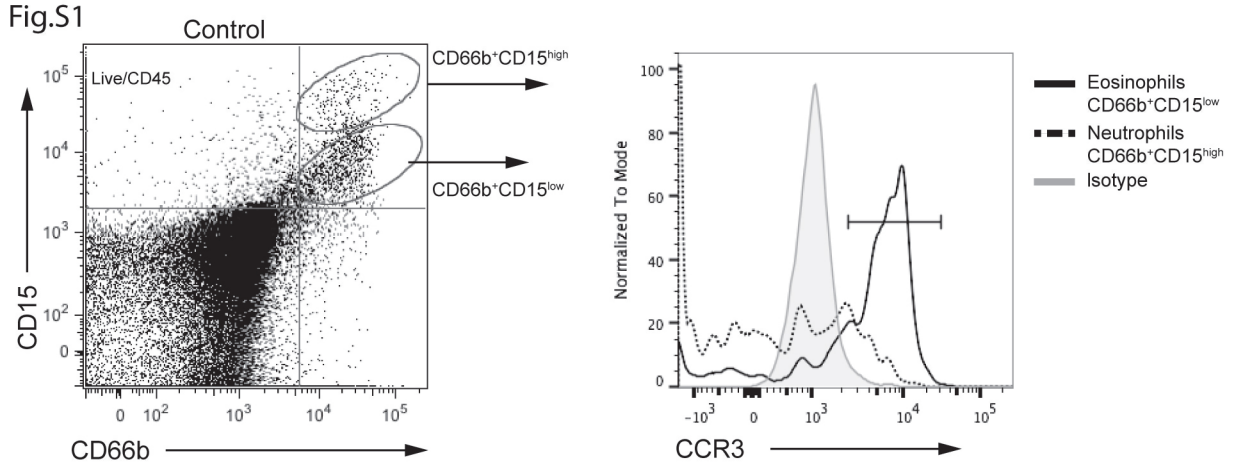


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Fig.S2

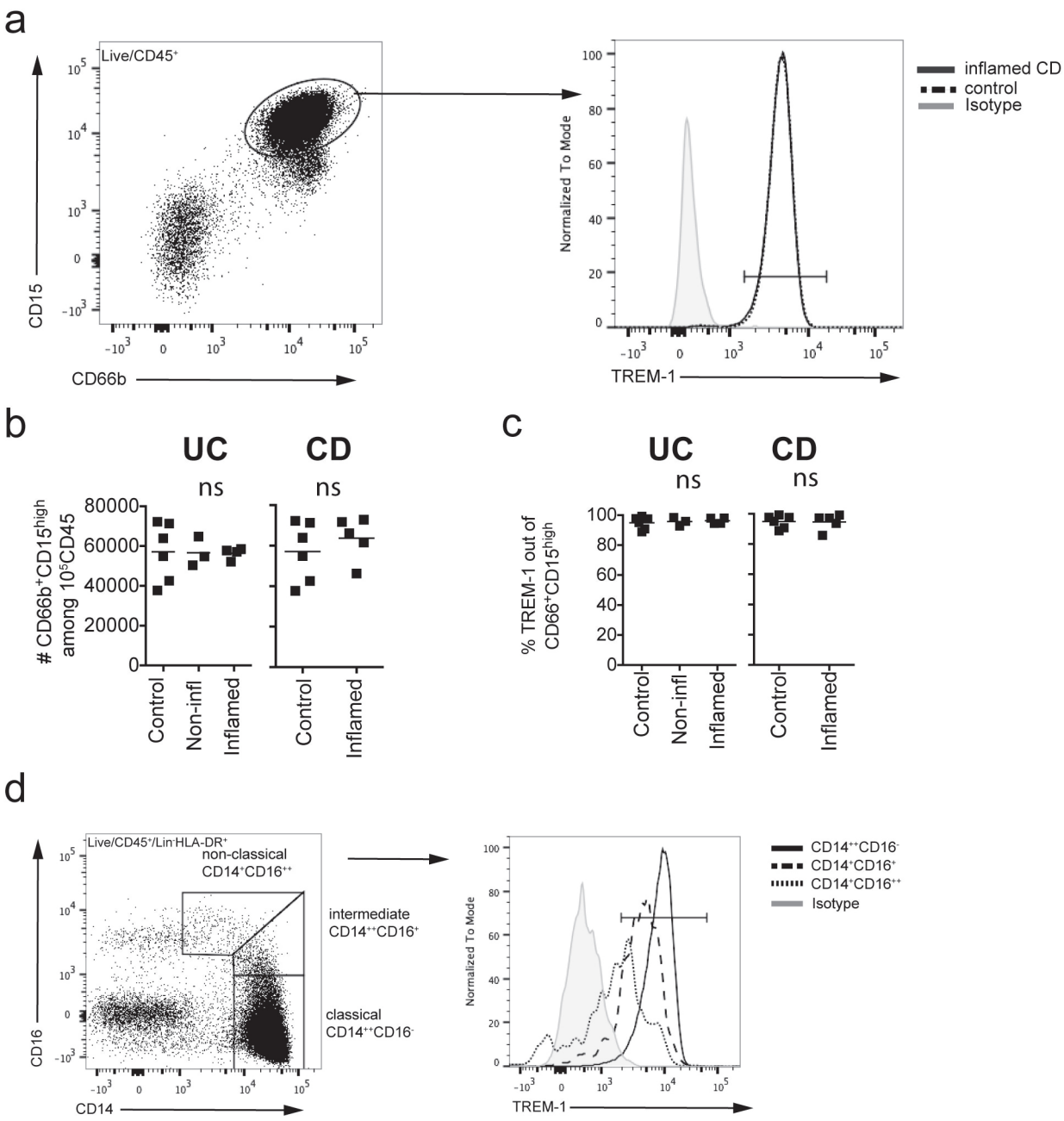

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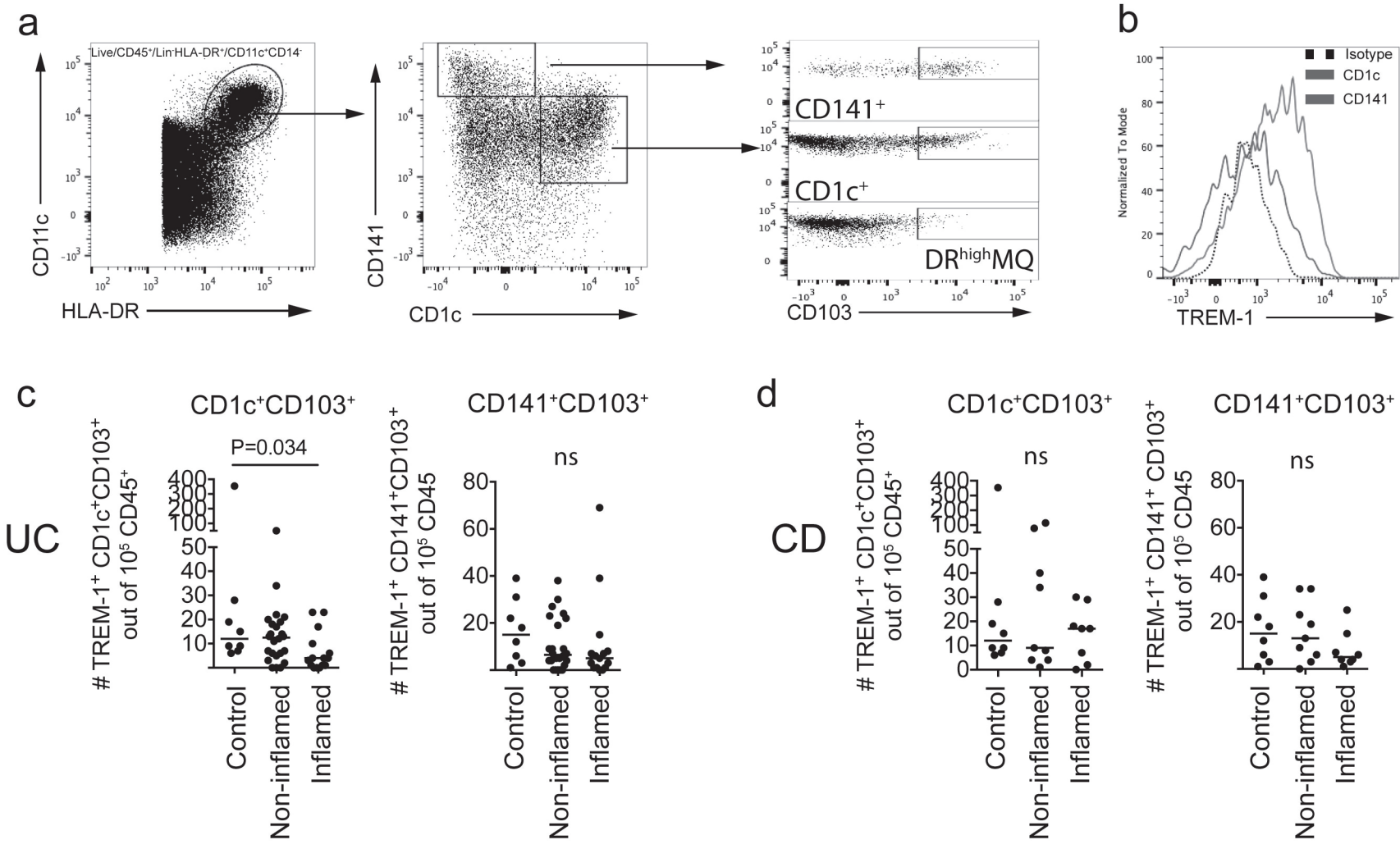
Fig.S3



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