

ARTICLE

Impaired neutrophil migration underpins host susceptibility to infectious colitis

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Citrobacter rodentium models infection with enteropathogenic *Escherichia coli* and ulcerative colitis (UC). While C57BL/6 (C57) mice recover, C3H/HeN (C3H) mice succumb to infection, partially due to increased colonic neutrophil elastase activity, also seen in UC patients; however, the underlying cause was unknown. Here, we found that bone marrow, blood, and colonic C57 neutrophils expressed (CD)11b^{Hi} and reached the infected colonic lumen, where they underwent productive NETosis. In contrast, while the number of C3H neutrophils increased in the bone marrow, blood, and colon, they remained CD11b^{Lo} and got trapped in the submucosa, away from *C. rodentium*, where they underwent harmful NETosis. CD11b^{Lo} neutrophils in C3H mice infected with CR₁₉, which triggers expression of neutrophil chemoattractants, reached the colonization site, resulting in host survival. UC patient neutrophils also displayed decreased levels of the activation/differentiation markers CD16/CXCR4. These results, suggesting that neutrophil malfunction contributes to exacerbated colitis, provide insight for future therapeutic prospects.

Mucosal Immunology (2024) 17:939–957; <https://doi.org/10.1016/j.mucimm.2024.06.008>

INTRODUCTION

Infectious and noncommunicable colitis are major public health concerns^{1,2}. The former is caused by enteric bacterial pathogens, and the latter by non-resolving chronic inflammation, most commonly presenting as inflammatory bowel diseases (IBDs), including ulcerative colitis (UC).

Citrobacter rodentium (CR) is an extracellular, mouse-adapted enteric pathogen used to model infection with the human pathogens enteropathogenic and enterohemorrhagic *Escherichia coli*, as well as sharing several features of UC (e.g. localized colonic inflammation, marked hyperplasia, diarrhea, and increased fecal calprotectin)³. The severity of CR infection is dependent on the mouse's genetic background. While in C57BL/6 (C57) and BALB/c mice CR causes mild self-limiting infection, C3H/HeN (C3H) and FVB mice succumb between 8–12 and 12–20 days post infection (DPI), respectively^{4,5}.

CR colonizes and replicates in the gut through injection of type III secretion system (T3SS) effectors into intestinal epithelial cells (IECs). The effectors form intracellular signaling networks that mediate intimate bacterial attachment to the apical surface and subvert mucosal inflammation⁶. CR infection triggers tissue regeneration and IEC proliferation, manifested as colonic crypt hyperplasia (CCH)^{7,8}. The CR infection cycle in C57 mice is divided into four phases: establishment (up to 4 DPI), expansion (4–8 DPI), steady state (8–12 DPI), and clearance (13–20 DPI). Clearance is mediated by complement⁹- and pathogen-specific

immunoglobulin G (IgG)-mediated opsonophagocytosis by neutrophils^{3,10}. C3H mice succumb to CR as they fail to enter the clearance phase. The increased susceptibility of C3H mice has been associated with disparities in the microbiome, genetic factors^{11,12}, and excess colonic neutrophil elastase (NE) activity, which leads to accumulation of dead colonic IECs¹³.

Neutrophils are derived from a common myeloid progenitor in the bone marrow (BM) and are post mitotic in the bloodstream, with a half-life of less than a day¹⁴. Neutrophils respond to chemotactic signals via surface cytokine receptors [e.g. interleukin (IL)-8/CXCL1-CXCR2 and granulocyte colony-stimulating factor (G-CSF)-G-CSF-R] and adhesion molecules (e.g. integrins) that mediate their recruitment, migration, and extravasation into infected sites^{15,16}. Neutrophils are the first responders to infection and directly eliminate microbes through phagocytosis, generation of reactive oxygen species, release of proteases via degranulation, and formation of highly destructive neutrophil extracellular traps (NETs)¹⁵, which consist of extruded DNA decorated with antimicrobial peptides (e.g. calprotectin – S100A8/A9) and proteases [e.g. NE or myeloperoxidase (MPO)]¹⁷. Neutrophils are activated by pathogen and damage-associated molecular patterns, cytokines, and cell-to-cell interactions¹⁸ and have been shown to play a key role in the maintenance of intestinal homeostasis^{19–23}. However, if deregulated, activated neutrophils can cause chronic inflammation, and neutrophil accumulation in the gut is associated with mucosal injury and ulcerating IBD^{16,24,25}.

We have recently shown that C3H mice could be rescued from CR infection by neutralizing NE activity *in vivo*¹³. To inves-

Keywords: *Citrobacter rodentium*; Ulcerative colitis; Neutrophils; Netosis; Host susceptibility.

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tigate the aberrant behavior of neutrophils during CR infection of C3H mice, we compared BM, blood, and colonic neutrophils from CR-infected C3H and C57 mice. A combination of deep proteomics, morphological, and phenotypic analyses revealed that C57 BM neutrophils undergo reprogramming upon CR infection, with increased expression of neutrophil differentiation markers including integrins such as cluster of differentiation (CD)11b (CD11b^{Hi}), corresponding to efficient recruitment of C57 neutrophils to the colonic lumen. In contrast, C3H BM, blood, and colonic neutrophils were not fully activated and presented low expression of CD11b (CD11b^{Lo}) and the activation marker Ly6G. This correlated with pathological accumulation of C3H neutrophils in the colonic submucosa, where they underwent harmful NETosis. Importantly, C3H mice survived infection with CRi9, a CR strain engineered to trigger a hyperinflammatory state, which restored migration of the CD11b^{Lo} neutrophils to the gut lumen. Clinically, UC patients in flare also display decreased expression of the activation/differentiation markers CD16 and CXCR4 compared to healthy controls. Taken together, these results suggest that malfunctioning neutrophils contribute to exacerbated colitis.

RESULTS

The BM neutrophil phase in response to CR infection

We aimed to investigate the mechanism underpinning neutrophil-mediated C3H susceptibility to CR infection. In homeostasis, mature neutrophils migrate from the BM into the circulation, where they patrol the body in search of signals that will direct them into an infected site. We therefore first investigated the BM neutrophil phase following CR infection.

Quantification of BM neutrophils in uninfected (UI) and CR-infected mice (gating strategy is shown in [Supplementary Fig. 1A](#)) revealed that the proportion of Ly6G⁺ cells within the white blood cell (WBC) population did not change and remained at 25 % in CR-infected C57 mice ([Fig. 1A](#)). Of note, while in UI C57 mice the proportion of BM CD11b^{Hi} neutrophils is 12.6 %, this increases to 25.9 % upon infection ([Fig. 1B](#) and [Supplementary Fig. 1C](#)). Conversely, despite similar colonic infection burden ([Supplementary Fig. 1B](#)), the proportion of neutrophils within the BM WBCs increased from 10.7 % to 47.7 % in CR-infected C3H mice ([Fig. 1A](#) and [B](#)). However, despite showing an increase from what was seen in UI mice, the proportion of CD11b^{Hi} neutrophils in infected C3H is 11.5 % ([Fig. 1B](#) and [Supplementary Fig. 1C](#)), suggesting that while proliferating, these BM neutrophils are not fully activated. The higher proportion of neutrophils in the BM of infected C3H mice correlated with an increased level of serum G-CSF, the major growth factor stimulating production of neutrophils in the BM ([Fig. 1C](#)).

To further determine the differentiation and activation state, we compared neutrophil morphology²⁶ in BM smears. Uninfected C57 BM smears revealed myelocytes, metamyelocytes, and banded and mature neutrophil morphologies, the latter two more frequently seen upon infection ([Fig. 1D](#)). In contrast, similar BM neutrophil morphologies were seen in UI and CR-infected C3H mice, with most cells presenting myelocyte and metamyelocyte morphologies, with only few banded neutrophils. These results suggest that following CR infection BM neutrophils in C3H mice divide but fail to fully differentiate.

To determine the molecular basis underpinning the observed differences, we compared the C57 and C3H BM neutrophil proteomes. We quantified a total of 3438 unique proteins that mapped to the *Mus musculus* proteome ([Supplementary Table 1](#))

[peptide false discovery rate (FDR) of <1 %]. Principal component analysis and hierarchical clustering of the proteins showed clear separation between neutrophils isolated from UI C3H and C57 mice, suggesting significant difference in homeostasis ([Fig. 1E](#) and [Supplementary Fig. 1D](#)). While we observed close clustering of neutrophils purified from UI and CR-infected C3H mice, C57 neutrophils showed clear separation between UI and infected mice ([Fig. 1E](#)), which is corroborated by increased abundance of the transcription factors Spi1 and Cebpd and the receptors Csf3r and Tlr2, which are implicated in the final stages of differentiation ([Fig. 1F](#) and [Supplementary Fig. 1D](#)).

1D enrichment analysis performed on values normalized to the those obtained from neutrophils purified from the corresponding UI mouse strain revealed that processes related to neutrophil effector functions such as phagocytosis, N-glycan biosynthesis, protein export, degranulation, chemotaxis, and oxidative phosphorylation are enriched in C57 neutrophils ([Fig. 1G](#) and [Supplementary Fig. 1D](#) and [E](#)), indicating that these processes are up-regulated following infection. In contrast, neutrophils purified from infected C3H mice displayed negative enrichment of pathways related to cell migration (e.g. integrins such as Itgam/CD11b) ([Fig. 1G](#) and [H](#)) upon infection and positive enrichment of processes involved in cell cycle progression ([Fig. 1G](#) and [Supplementary Fig. 1F](#)), which correlated with increased abundance of the proliferation marker Ki67 ([Supplementary Fig. 1D](#), cluster 17). Nonetheless, the abundance of acute phase reactants and antimicrobial proteins (e.g. S100A8) was higher in the C3H BM neutrophils ([Fig. 1G](#) and [Supplementary Fig. 1D](#) and [G](#)).

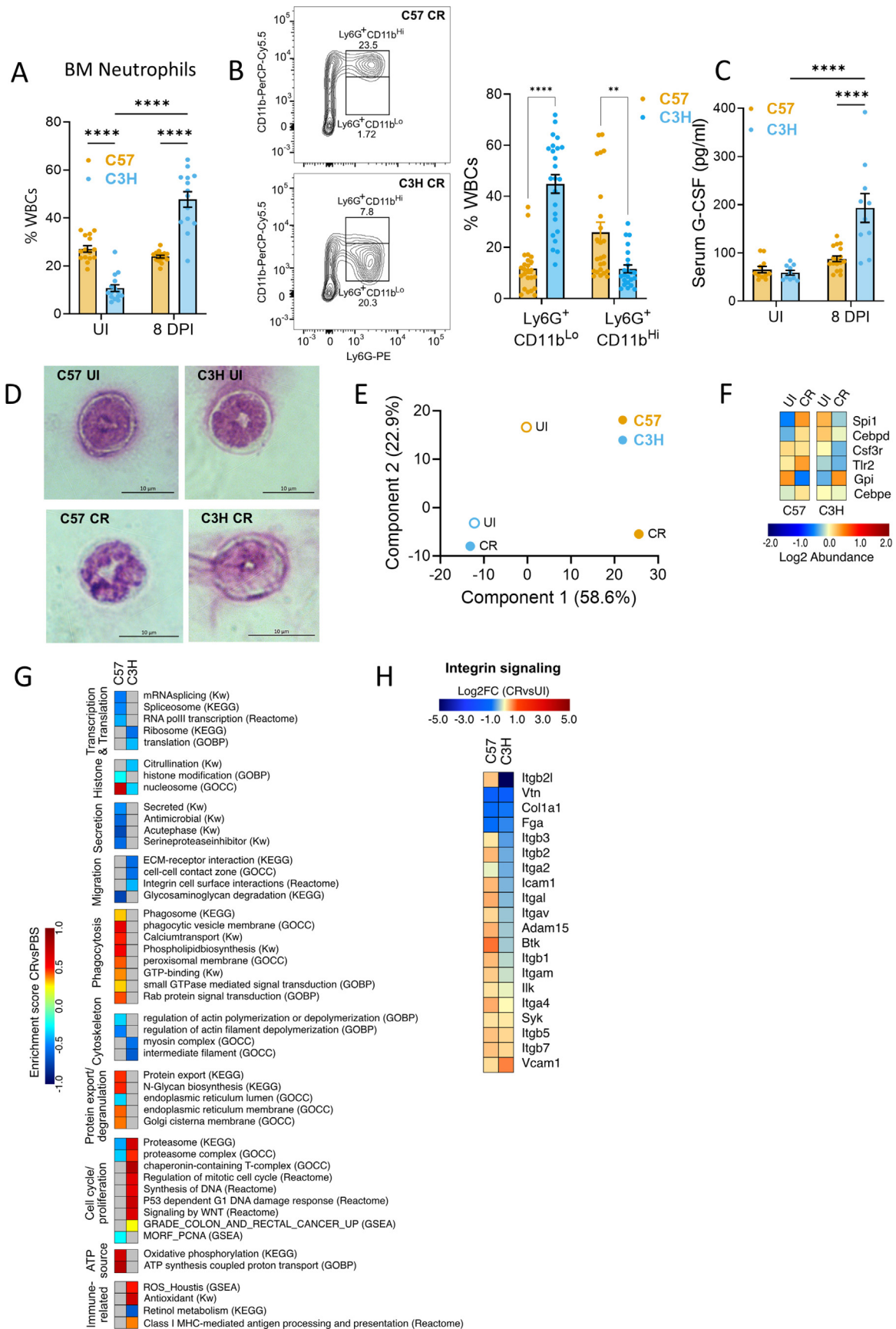
Taken together, these results suggest that following CR infection C57 BM neutrophils undergo late-stage differentiation, while C3H BM neutrophils present little proteomic and morphological changes while continuing to divide and upregulating inflammatory markers (e.g. S100A8).

Blood C3H neutrophils maintain their CD11b^{Lo} profile

We next investigated the blood neutrophil phase. In contrast to the BM, blood smears showed mature neutrophil morphology in both C57 and C3H mice with segmented nuclei in the shape of a ring and a pale cytoplasm, with increased numbers apparent only in infected C3H mice ([Fig. 2A](#) and [Supplementary Fig. 2A](#) and [B](#)). Flow cytometry analysis (Gating strategy [Supplementary Fig. 2C](#)) revealed that the percentage of blood neutrophils stayed at around 20 % in UI and CR-infected C57 mice. In contrast, the proportion of neutrophils increased ~4-fold, to 39 %, in infected C3H mice ([Fig. 2B](#) and [Supplementary Fig. 2D](#)). Of note, the proportion of circulating CD11b^{Lo} neutrophils in C3H mice increased upon infection from 37.6 %, comparable to that seen in C57 mice, to 75.4 % while also exhibiting lower Ly6G expression ([Fig. 2C](#) and [D](#)). Importantly, the CD11b expression level in the circulation, as quantified by the median fluorescence intensity, correlated with worse outcomes in C3H mice ([Fig. 2E](#)).

To investigate the impact of the expanded neutrophil population in the circulation, we quantified the levels of MPO and calprotectin (S100A8) in the peripheral blood. This revealed that the level of both MPO and S100A8 increased in infected C3H mice, while they remain unchanged in C57 mice ([Fig. 2F](#)).

Taken together, these results show that while presenting lower expression of CD11b and Ly6G, the number of circulating neutrophils and the levels of blood calprotectin and MPO increase in infected C3H mice.



Neutrophils are trapped in the submucosa of C3H infected mice

To study the colonic neutrophil phase, we profiled temporal colonic neutrophil recruitment (Gating strategy [Supplementary Fig. 3A](#)). This revealed that the percentage of colonic myeloid cells, other than neutrophils, was similar between CR-infected C3H and C57 mice at all time points ([Supplementary Fig. 3B](#)). The percentage of C57 neutrophils increased at 4 DPI and expanded moderately at 6 DPI, at which point they plateaued. In contrast, the percentage of C3H neutrophils increased significantly from 3 DPI and continued to expand daily up to 8 DPI ([Fig. 3A](#)). The increase of colonic neutrophils in C3H mice paralleled earlier and greater disruption of cellular junctions and tissue damage response, indicated by decreased E-cadherin staining and body weight, increased CCH ([Fig. 3B](#) and [Supplementary Fig. 3C–E](#)) and earlier and higher levels of IL-22 ([Fig. 3C](#) and [Supplementary Table 2](#)). Correlating with increased neutrophil recruitment earlier during infection were higher levels of IL-1 β , a strong inducer of chemotactic lipid expression which can also be released by neutrophils,²⁷ and the canonical neutrophil chemoattractants CXCL1 and IL-18. However, by 8 DPI the colonic cytokine levels were similar between C57 and C3H mice ([Fig. 3C](#) and [Supplementary Table 2](#)).

We next compared the colonic neutrophil proteomes in C3H and C57 mice. Due to low number of colonic neutrophils in homeostasis, we only used neutrophils isolated from infected C3H and C57 mice at 8 DPI, which were sorted with a purity of ~80–98 % ([Supplementary Fig. 3F](#)). We quantified a total of 2399 unique proteins that mapped to the *Mus musculus* proteome (peptide FDR of <1 %) ([Supplementary Table 3](#)). 1D enrichment analysis revealed that both apoptosis, thought to favor resolution of inflammation, and focal adhesion, including proteins involved in cell-to-cell interactions, were negatively enriched in the C3H neutrophils compared to C57 ([Supplementary Fig. 3G](#)). While the abundance of most integrins increased by the time the C3H neutrophils reached the colon, components of the integrin complex Mac-1, CD11b, and Itgb2, as well as other proteins involved in migration such as SIRPA, remained low or unchanged ([Supplementary Fig. 3H](#)). Moreover, the abundance of several pro-inflammatory proteins, such as Casp1 and Gsdmd, was higher in the colonic C3H neutrophils ([Fig. 3F](#) and [Supplementary Fig. 3I](#)). Importantly, we detected a similar abundance of proteins involved in degranulation and the antimicro-

bial proteins NE (Ela), MPO, LCN-2, and metalloproteases in neutrophils of C3H and C57 mice ([Fig. 3D](#)). Of note, while the level of fecal S100A8 and LCN-2 levels were similar in C3H and C57 mice, the level of fecal MPO was higher in the former ([Fig. 3E](#) and [Supplementary Fig. 3J](#)).

Effector neutrophil mechanisms include several forms of cell death including apoptosis, pyroptosis, and NETosis; the latter is required for CR clearance²⁸. As we detected lower abundance of proteins involved in NETosis in C3H neutrophils ([Supplementary Fig. 3K](#)), we compared neutrophil NETosis in infected C3H and C57 mice. To this end, we first quantified neutrophils in the colonic lumen, mucosa, and submucosa ([Fig. 4A](#) and [B](#); example of a healthy neutrophil in [Supplementary Fig. S4A](#) and delimitation between areas in colonic tissue in [Supplementary Fig. S4B](#)). This revealed similar number of neutrophils in the mucosa between the two mouse strains, while the number of neutrophils in the lumen was higher in C57 mice. However, while CR was mainly seen in the lumen and the mucosal surface, we observed extensive accumulation of neutrophils in the submucosa of C3H mice starting at 4 DPI (~25 neutrophils/mm² compared to 6.1 in UI) and reaching 254 neutrophils/mm² at 8 DPI ([Fig. 4B](#) and [C](#)). Taken together, these results suggest that in C3H-infected mice neutrophils are trapped within the submucosa away from the colonizing CR.

We next quantified colonic NETosis by immunostaining. This revealed that the citrullinated H3 histone stain, mainly overlapping with DNA (example in [Supplementary Fig. 4A](#)), was observed in the lumen in C57 mice; this was significantly lower in C3H mice, both in total number and when comparing the proportion of neutrophils releasing NETs ([Fig. 4D](#) and [Supplementary Fig. 4C](#) and [D](#)). However, significantly more NETosing neutrophils were seen in the mucosa and submucosa of infected C3H mice ([Fig. 4D](#) and [Supplementary Fig. 4C](#)), which is consistent with the higher levels of fecal and serum MPO in these mice ([Figs. 2F](#) and [3E](#)).

We next profiled expression of CD11b and Ly6G in colonic C3H and C57 neutrophils. This revealed a higher proportion of CD11b^{Lo} neutrophils in infected C3H mice, which also expressed lower levels of Ly6G when compared to their C57 counterparts ([Fig. 4E](#) and [F](#)). Staining of colonic sections with Ly6G and CD11b revealed homogenous high levels of expression of CD11b in C57 mice. In contrast, this type of staining was only seen in the minority of C3H neutrophils displaying a mature

Fig. 1 The BM neutrophil compartment in CR-infected C57 and C3H mice (related to [Supplementary Fig. 1](#)). (A) Percentage of BM neutrophils within the WBC population of CR- or mock-infected (UI) C57 and C3H mice (8 DPI). (B) Representative flow cytometry plots (left) and quantification of Ly6G⁺ and CD11b^{Lo} or CD11b^{Hi} cells at 8 DPI (right). (C) Levels of G-CSF in the serum of CR-infected (8 DPI) and UI C57 and C3H mice. (D) Representative Wright-Giemsa stained BM smear samples. <scale bar: 10 μ m> (E) PCA based on neutrophil proteins with quantitative values of CR or UI mice. (F) Heatmap representation of the abundance of proteins involved in myeloid cell and neutrophil maturation. (G) 1D enrichment scores of selected KEGG, Kw, GSEA, Reactome, and GOBP and GOCC significantly up-regulated (positive enrichment score) or down-regulated (negative enrichment score) (Benjamini/Hochberg FDR is <0.02). (H) Heatmap displaying the relative abundance (CR-infected normalized to mock-infected) of BM neutrophil proteins involved in integrin signaling. (A, C) Bars represent means $\pm$ SEM for $n = 3$ biologically independent repeats with 10–18 mice per treatment and strain. (B) Bars represent means $\pm$ SEM for $n = 5$ biologically independent repeats with 23–25 mice per strain. (D) Images are representative of $n = 3$ biological repeats. (A–C) Statistical significance was determined by two-way ANOVA corrected for multiple comparisons with Sidak's multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. 1D = one-dimensional; ANOVA = analysis of variance; ATP = adenosine triphosphate; BM = bone marrow; CR = *Citrobacter rodentium*; DPI = days post infection; FC = fold change; FDR = false discovery rate; G-CSF = granulocyte colony-stimulating factor; GOBP = Gene Ontology biological processes; GOCC = Gene Ontology cellular components; GSEA = Gene Set Enrichment Analysis; KEGG = Kyoto Encyclopedia of Genes and Genomes; Kw = keyword; PCA = principal component analysis; SEM = standard error of the mean; UI = uninfected; WBC = white blood cell.

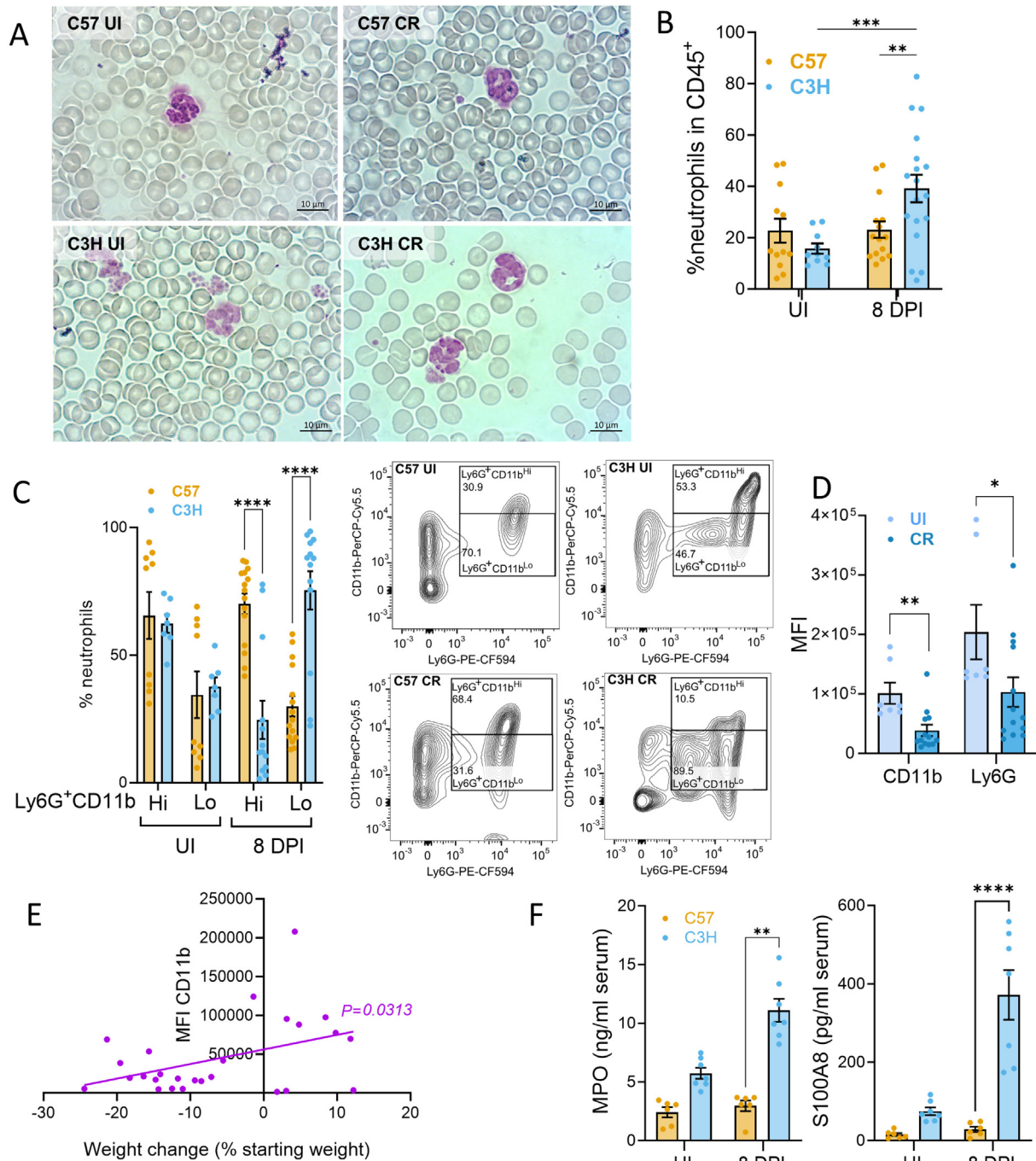
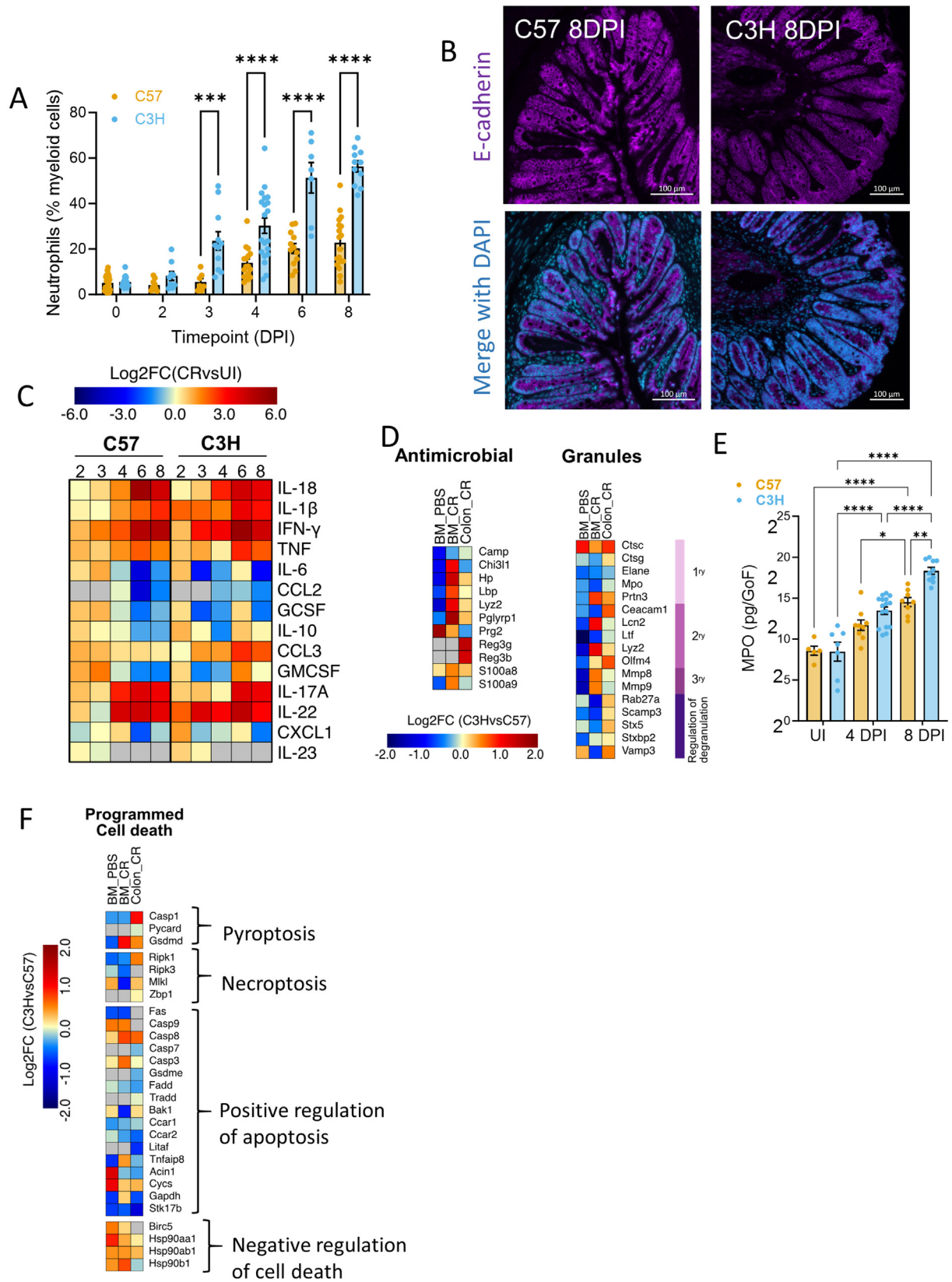


Fig. 2 Characterization of the blood neutrophil phase (related to [Supplementary Fig. 2](#)). (A) Representative Wright-Giemsa stained blood smears of C57 and C3H mice. <scale bar: 10 μ m> See [Supplementary Fig. 2B](#) for additional images. (B) Proportion of neutrophils within the CD45⁺ population in the blood at 8 DPI in CR- and mock-infected mice. (C) Proportion of blood neutrophils displaying high or low levels of CD11b at 8 DPI. Representative flow cytometry plots are shown on the right. (D) The MFI for Ly6G and CD11b is reduced in blood neutrophils from infected C3H mice. (E) Graph showing the correlation between weight change during infection and the level of CD11b expression in blood neutrophils. (F) Levels of serum S100A8 and MPO at 8 DPI of C57 and C3H mice. (A) Images are representative of $n = 3$ biological repeats; (B, C, and D) Graph shows means $\pm$ SEM for $n = 3$ biologically independent repeats (total of 7–18 mice per treatment and strain); (F) Data shows means $\pm$ SEM for $n = 2$ biologically independent repeats (total of 7 mice treatment and strain). (B, C) Statistical significance was determined by two-way ANOVA corrected for multiple comparisons with Sidak's multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (D) Statistical analysis was performed by Mann-Whitney test to compare UI and CR-infected C3H mice values within each marker. * $p < 0.05$; ** $p < 0.01$. (E) Correlation was determined by calculation of Pearson's correlation coefficient using data from three biological repeats. (F) Statistical significance was determined by one-way ANOVA and Tukey's multiple-comparison test. ** $p < 0.01$; **** $p < 0.0001$. ANOVA = analysis of variance; CD = cluster of differentiation; CR = *Citrobacter rodentium*; DPI = days post infection; MFI = median fluorescence intensity; MPO = myeloperoxidase; SEM = standard error of the mean; UI = uninfected.



morphology (i.e. clearly lobed nucleus) reaching the mucosa or the lumen (Fig. 4G and Supplementary Fig. 4E).

In order to confirm that the accumulation of neutrophils in the submucosa in C3H mice is due to a migration defect, we performed transepithelial migration assays (TEM) assays using BM neutrophils purified from UI or CR-infected mice; BM neutrophil from C57 mice were used as a control (purity ~80–99 %; Supplementary Fig. 5A). C3H neutrophils (from UI and infected mice) presented lower migration in response to N-Formylmethionine-leucyl-phenylalanine (fMLP) compared to C57 mice (Fig. 5A and Supplementary Fig. 4B). In contrast, migration in response to CXCL1 was significantly lower in C3H neutrophils from CR-infected mice compared to UI mice, indicating that BM reprogramming in response to CR infection makes C3H neutrophils less responsive to CXCL-1. Conversely, C57 neutrophils from UI and CR-infected mice migrated similarly in response to CXCL1, with a trend to higher migration levels in neutrophils from CR-infected mice (Fig. 5B and C and Supplementary Fig. 5C).

To confirm that other neutrophil activities were not affected by the changes in the BM, we assessed phagocytosis. Despite the reduced abundance of proteins involved in phagocytosis (Fig. 1G and Supplementary Fig. 1E), neutrophils from CR-infected C3H were able to phagocytose CR to the same degree as neutrophils from UI C3H mice and C57 neutrophils (Fig. 5D). We also performed immunofluorescence staining to distinguish intra- and extracellular bacteria. This revealed that most CR bacteria were internalized, with only few neutrophils associated with extracellular CR (Fig. 5E). In addition to killing via phagocytosis by targeting intracellular bacteria in phagosomes with antimicrobial proteins such as MPO, both C57 and C3H neutrophils were also able to respond to CR by extruding their cellular contents via NETosis, indicating they are capable of mounting an effective response against CR (Supplementary Fig. 5D).

Taken together, our data suggest that C3H neutrophils from CR-infected mice maintain their CD11b^{Lo} profile as they pass through the BM, blood, and colonic phases while maintaining other antimicrobial functions. While a minority of C3H neutrophils expressing CD11b^{Hi} reach the colonic mucosal surface, those expressing CD11b^{Lo} remain in the submucosa, unable to migrate across the epithelium in response to chemoattractants, and are thus unable to perform their antimicrobial functions at the site of infection.

C3H mice survive infection with CR₁₉

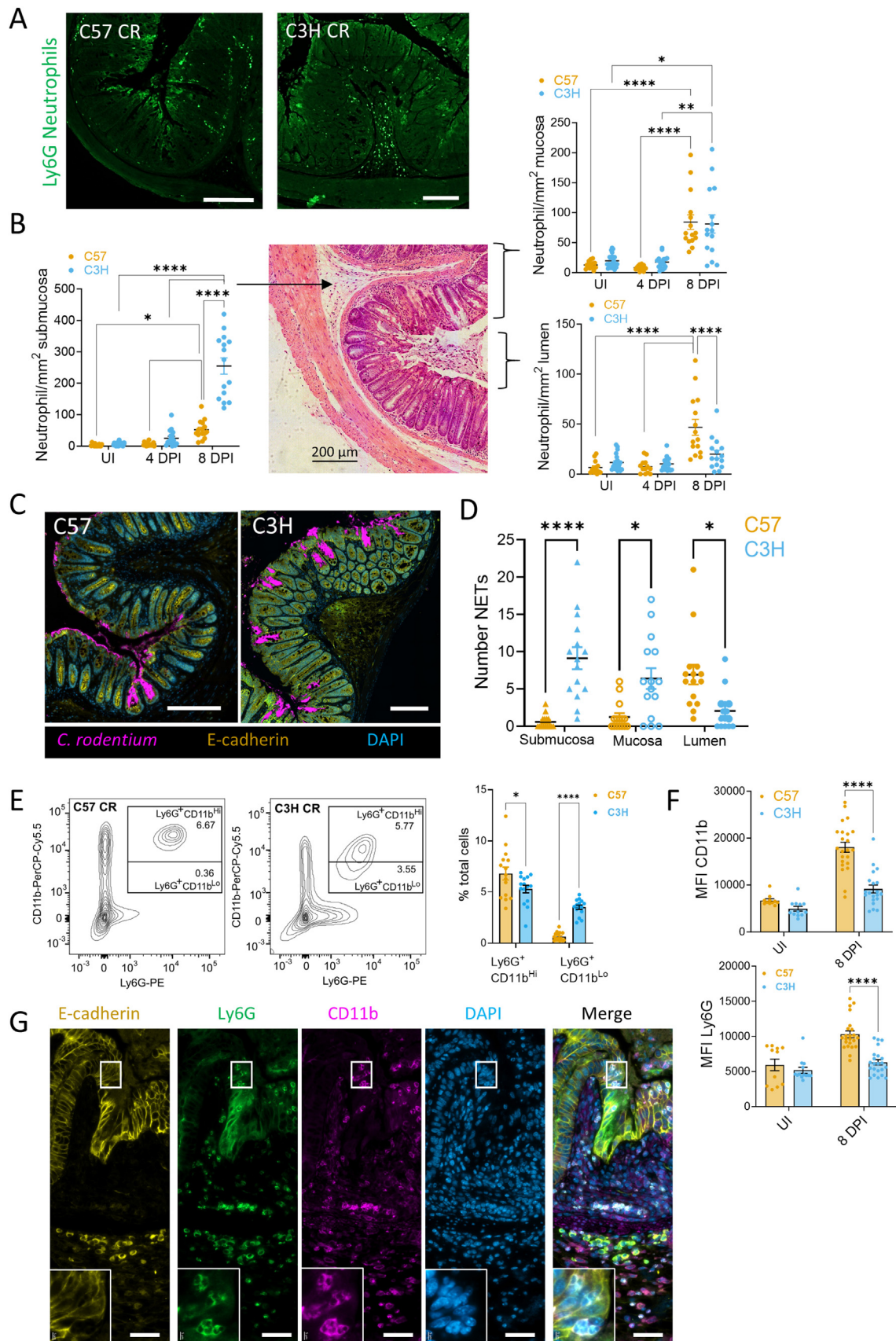
We hypothesized that expression of higher levels of neutrophil chemoattractants in the C3H colonic environment could overcome the migration impediment seen in infected C3H neutrophils. To test this hypothesis, we infected C3H mice with CR₁₉, which triggers a hyperinflammatory colonic state²⁹. Following infection, we found that wild-type CR (CR_{WT}) and CR₁₉ similarly colonized C3H mice (Supplementary Fig. 6A and B). However, unlike CR_{WT}, CR₁₉ did not cause persistent weight loss, and the mice cleared the pathogen at around 30 DPI (Fig. 6A and Supplementary Fig. 6A) as they mount a similar serum IgG levels to those seen in C57 mice (Supplementary Fig. 6A and C). Analyzing BM neutrophils revealed similar proportion of neutrophils in the BM between CR_{WT} and CR₁₉-infected C3H mice (Supplementary Fig. 6E); however, expression of CD11b and Ly6G was higher in the latter (Supplementary Fig. 6E).

Colonic tissue CXCL1, IL-1 β , IL-17A, and IL-22 (significant), as well as IL-18 and G-CSF (trend), were higher in CR₁₉- compared to CR_{WT}-infected mice, while their levels in the blood were similar (Fig. 6B and Supplementary Fig. 6D). Unexpectedly, lower numbers of circulating and colonic neutrophils were seen in CR₁₉- compared to CR_{WT}-infected C3H mice (Fig. 6C and D). Importantly, while the level of CD11b was not significantly different between the colonic neutrophils in CR_{WT} and CR₁₉-infected mice, the latter migrated more efficiently from the submucosa toward the site of infection in the mucosal surface and lumen (Figs. 6E and Supplementary Fig. 6G). Functionally, NETs were mostly seen in the lumen in CR₁₉-infected colons, making the distribution of NETosing neutrophils more like that of infected C57 mice (Fig. 6G and Supplementary Fig. 6H). Taken together, these data suggest that heightened local release of chemoattractants triggered migration of CD11b^{Lo} neutrophils to the gut lumen. Moreover, CD11b^{Lo} neutrophils are functional if activated at the right anatomical site but harmful when undergoing NETosis in the submucosa.

Neutrophils from IBD patients display lower levels of differentiation markers

Given that infection of C3H shares multiple characteristics with active UC, we compared blood neutrophils between patients suffering from a flare of UC and healthy controls (Supplementary Table 4; gating strategy Supplementary Fig. 7A). Despite similar numbers of circulating WBCs, UC patients undergoing a flare

Fig. 3 Characterization of colonic neutrophil phase (related to Supplementary Fig. 3). (A) Temporal neutrophil recruitment (represented as the percentage of the total myeloid cells) into the C57 and C3H mouse colons. Each dot represents the mean per mouse. (B) Representative image of the distal colon of C57 and C3H mice at 8 DPI; note the decreased E-cadherin (violet) signal in C3H mice. DAPI was used to stain nuclei. <scale bar: 100 μ m> (C) Cytokine heatmap displaying temporal Log2FC measured in colonic explants comparing CR-infected to mock-infected mice. Raw data values are shown in Supplementary Table 2. (D) Abundance heatmaps of antimicrobial proteins and neutrophil granule proteins in colonic neutrophils, the latter divided into primary, secondary, and tertiary granules, and proteins involved in the degranulation process. (E) Fecal MPO levels in C57 and C3H mice, at 8 DPI. (F) Heatmap of proteins involved in cell death, including pyroptosis (higher abundance in C3H neutrophils), necroptosis, and apoptosis (lower abundance in C3H neutrophils). (A) Bars show means $\pm$ SEM for $n = 2$ (2 and 3 DPI C57 mice), $n = 3$ –5 (all other infected time points) biologically independent repeats. (B) Images are representative of $n = 3$ biologically independent repeats. (C) Values are representative of two (C57 2–3 DPI) and three independent biological repeats. (E) Graphs represent means $\pm$ SEM for $n = 3$ biologically independent repeats. (A) Statistical significance was determined by two-way ANOVA corrected for multiple comparisons with Sidak's multiple comparisons test. *** $p < 0.001$; **** $p < 0.0001$. (E) Statistical significance was determined by one-way ANOVA corrected for multiple comparisons with Tukey's multiple comparisons test. * $p < 0.05$; **** $p < 0.0001$. ANOVA = analysis of variance; BM = bone marrow; CR = *Citrobacter rodentium*; DAPI = 4',6-diamidino-2-phenylindole; DPI = days post infection; FC = fold change; IFN = interferon; IL = interleukin; MPO = myeloperoxidase; SEM = standard error of the mean; TNF = tumor necrosis factor; UI = uninfected.



had a higher proportion of circulating neutrophils (Fig. 7A and Supplementary Fig. 7B). The level of CD11b, known to be expressed at low levels in humans unless they are suffering from an infection and/or sepsis, was similar between the UC and healthy controls. In contrast, the neutrophil activation/differentiation markers CD16 and CXCR4 were lower in neutrophils purified from the UC patients (Fig. 7B and Supplementary Fig. 7C), while other markers such as CD15 and CXCR2 were not significantly different (Supplementary Fig. 7D). We performed Giemsa stains of patient and healthy control blood, which showed that while most of the neutrophils appeared mature (multi-lobed and granulated), in some of the patient samples less granular, more band-like neutrophils were seen (Fig. 7C). Moreover, quantification of serum calprotectin revealed, similarly to CR-infected C3H mice, significantly higher serum levels in the patients (Fig. 7D). These results suggest both CR-infected C3H and UC patients' neutrophils display anomalous functions.

DISCUSSION

In this study, we analyzed the different neutrophil phases during CR infection of resistant C57 mice and susceptible C3H mice. We report that, contrary to C57 neutrophils, neutrophils in CR-infected C3H mice express CD11b^{Lo} and are trapped in the colonic submucosa where they undergo harmful NETosis.

Our analyses show that the proteomes of the BM C3H and C57 mouse neutrophils are different in homeostasis, suggesting the existence of intrinsic differences between the two mouse strains. While the number of C57 mice BM neutrophils did not change following CR infection, they were clearly separated from the mock-infected (UI) control neutrophils, displaying increased differentiation and activation markers (including CD11b^{Hi}). This suggests the existence of a colon-BM axis, which primes BM neutrophils to fight infection at a remote site. In contrast, BM neutrophils from infected and UI C3H mice were clustered together, as they failed to fully differentiate (CD11b^{Lo}) and kept proliferating, likely in response to serum G-CSF. Despite this, *in vitro* assays performed with BM C57 and C3H neutrophils from UI and CR-infected mice revealed that effector functions such as phagocytosis and NETosis were not impacted. However, migration in response to CXCL1 was reduced in C3H neutrophils after CR infection, while C57 neutrophils showed a trend toward increased migration, suggesting reprogramming in the BM

affecting neutrophil migration in the colon. In keeping with the existence in intrinsic differences even in the absence of CR infection, C3H neutrophils from UI mice showed decreased migration in response to fMLP, correlating with the role of CD11b in fMLP-mediated migration³⁰.

Similar numbers of neutrophils were detected in the blood of UI and CR-infected C57. In contrast, the number of circulating blood neutrophils (mainly CD11b^{Lo}) significantly increased in CR-infected C3H mice. However, while in the blood, the C3H neutrophils underwent morphological maturation and released calprotectin and MPO. This suggests that the C3H neutrophils either prematurely lysed or undergo uncontrolled degranulation in the circulation.

CR is an extracellular pathogen that colonizes the apical surface of IECs. For clearance, neutrophils need to migrate to the lumen of the gut where they eliminate the pathogen via opsonophagocytosis (via IgG or complement)^{3,10} or release of their antimicrobial contents, for example via NETosis^{15,17}. We found that this process occurs efficiently in CR-infected C57 mice. In contrast, in infected C3H mice, a high number of CD11b^{Lo} neutrophils are trapped in the submucosa, which, as the proteomics suggests, could also be expressing lower levels of other receptors involved in migration such as SIRPα³¹. It seems that as the neutrophils do not reach the infection site in the gut lumen, a feedback loop is activated, leading to the observed high levels of serum G-CSF that trigger release of more neutrophils from the BM to the circulation. As the trapped neutrophils undergo NETosis in the submucosa, they release the highly destructive enzymes NE and MPO away for the infection site, which leads to tissue damage and inability to mount the clearance phase, leading to mortality. Importantly, this defective response in C3H mice seems to be limited to extracellular gut infection and sterile colitis^{32,33}, indicating a specific defect of neutrophil trafficking in the gut, as these mice are resistance to *Salmonella typhimurium* and *Mycobacterium bovis* as well as viral and parasitic infections^{34–38}.

As the CD11b^{Lo} C3H neutrophils express the proteins involved in and are capable of antimicrobial effector functions, we tested if induction of greater gut inflammation might attract them to the infection site where they could execute productive NETosis. To this end, we infected C3H with CR₁₉, which is missing many T3SS effectors that inhibit several inflammatory processes,

Fig. 4 Neutrophils in CR-infected C3H accumulate in the colonic submucosa (related to Supplementary Fig. 4). (A). Representative images of CR-infected colons from C57 and C3H at 8 DPI; <scale bar: 200 μm> neutrophils are stained in green. (B) Quantification of neutrophils (defined as Ly6G⁺, E-cadherin⁺, and DAPI⁺; see Supplementary Fig. 4A) per mm² of the indicated colonic regions, delimited in Supplementary Fig. 4B. Each dot represents the mean per mouse. (C) Representative images of CR-infected C57 and C3H colons at DPI. CR (violet) is in association with the epithelial layer in both mouse strains; E-cadherin (yellow) and DAPI (cyan) are used to stain epithelial junctions and cell nuclei, respectively. <scale bar: 200 μm> (D) Quantification of NETs observed in the indicated colonic regions at 8 DPI in C57 and C3H mice. Each dot represents the mean per mouse. (E) Representative flow cytometry graphs displaying Ly6G and CD11b expression in CR-infected C57 and C3H (left) and quantification of the proportion of neutrophils within the total colon cell populations, divided into CD11b^{Hi} and CD11b^{Lo}, all at 8 DPI. (F) Colonic neutrophils from CR-infected C3H mice (8 DPI) display lower levels of CD11b (top) and Ly6G (bottom) as determined by the MFI. (G) Representative images of CR-infected C3H colonic tissue at 8 DPI stained with E-cadherin (yellow), Ly6G (green), CD11b (violet), and DAPI (cyan), showing that neutrophils in the mucosa are Ly6G^{Hi}, CD11b^{Hi}. <scale bar: 50 μm> (B, D) Graphs show means ± SEM for *n* = 4 biologically independent repeats. (F, G) Graph represents mean ± SEM from *n* = 3 biological repeats (15 mice per mouse strain). (A, C, E, H) Images are representative of *n* = 3 biologically independent replicates. (B, D) Statistical analysis by nonparametric Kruskal-Wallis test corrected for multiple comparisons by Dunn's post hoc test. (F, G) Statistical significance was determined by two-way ANOVA corrected for multiple comparisons with Sidak's multiple comparisons test. **p* < 0.05; ***p* < 0.01; *****p* < 0.0001. ANOVA = analysis of variance; CD = cluster of differentiation; CR = *Citrobacter rodentium*; DAPI = 4',6-diamidino-2-phenylindole; DPI = days post infection; MFI = median fluorescence intensity; NET = neutrophil extracellular trap; SEM = standard error of the mean; UI = uninfected.

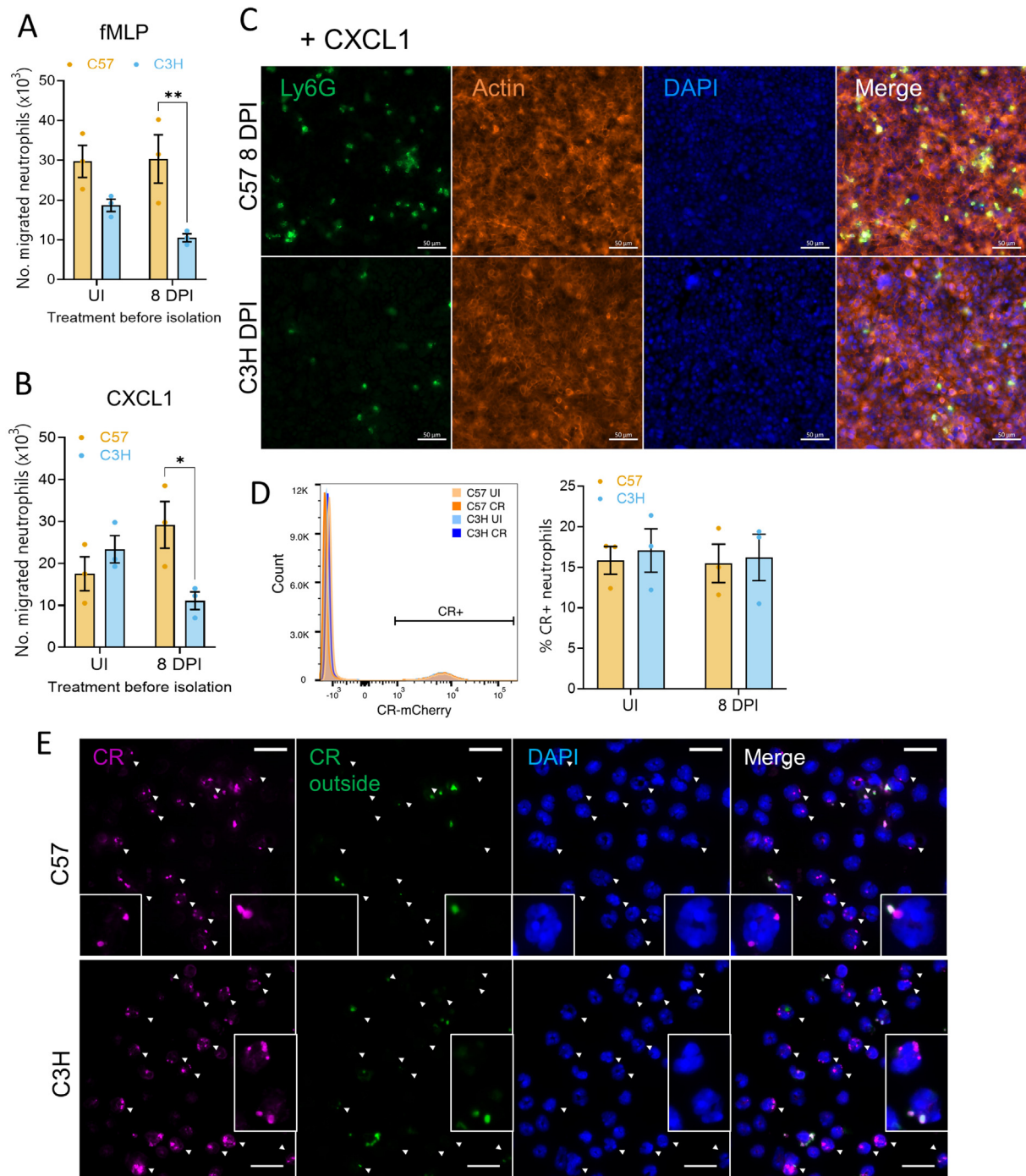


Fig. 5 C3H neutrophils have migration defects in response to chemoattractants (related to [Supplementary Fig. 5](#)). (A and B). Quantification of number of neutrophils from UI and CR-infected (8 DPI) C57 and C3H mice that migrated through CMT-93 monolayers in response to the neutrophil chemoattractant fMLP (A) and CXCL1 (B). (C) Representative microscopy images of the CMT-93 epithelial monolayer 3 h after adding BM neutrophils from the indicated mouse strain and condition to the basolateral side and CXCL1 (20 ng/mL) on the apical side; Phalloidin (orange) and DAPI (blue) are used to stain the actin cytoskeleton and cell nuclei, respectively, Ly6G (green) was used to stain neutrophils. <scale bar: 50 μ m> (D) Quantification of CR⁺ neutrophils after incubating for 45 min at MOI 5; representative histograms are shown below. (E) Representative images of BM C57 and C3H neutrophils infected with CR at MOI 5 for 45 mins; extracellular bacteria are stained in green and all bacteria in violet, DAPI was used to stain nuclei <scale bar: 20 μ m> (arrowheads indicate neutrophils with intracellular CR). (A, B, D) Graphs show means $\pm$ SEM for $n = 3$ biologically independent repeats. (C, E) Images are representative of $n = 3$ biologically independent replicates. (A, B, D) Statistical significance was determined by two-way ANOVA corrected for multiple comparisons with Uncorrected Fisher's LSD test. * $p < 0.05$; ** $p < 0.01$. Graphs with no comparison lines indicate lack of significant differences between the groups compared. ANOVA = analysis of variance; BM = bone marrow; CR = *Citrobacter rodentium*; CR+ = CR positive; DAPI = 4',6-diamidino-2-phenylindole; DPI = days post infection; fMLP = N-Formylmethionine-leucyl-phenylalanine; LSD = least significant difference; MOI = multiplicity of infection; SEM = standard error of the mean; UI = uninfected.

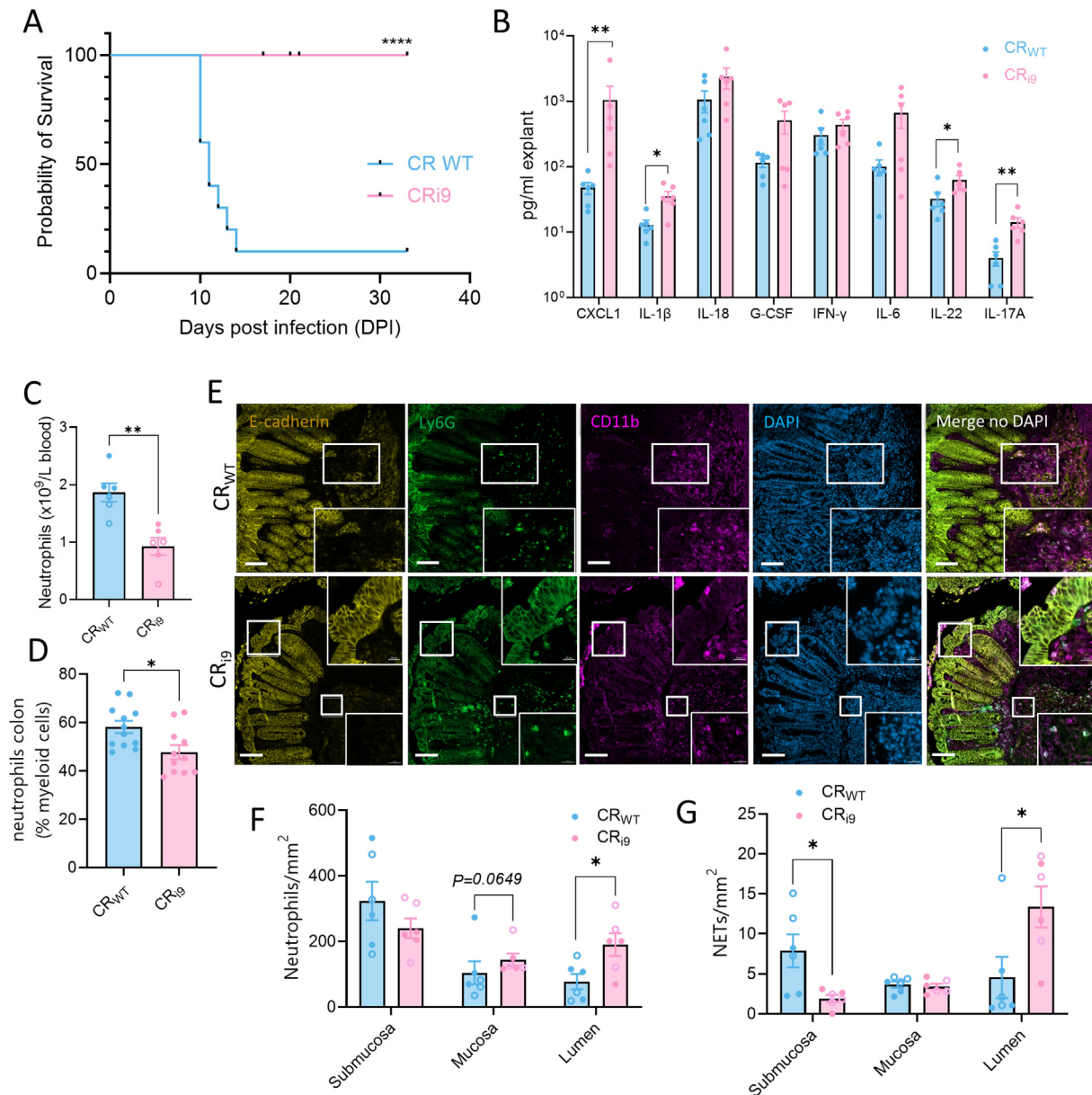


Fig. 6 C3H mice survive infection with CR_{i9} (related to [Supplementary Fig. 6](#)). (A) Survival curve for C3H infected with CR_{WT} (blue) and CR_{i9} (pink). (B) Cytokines levels in explants from CR_{i9}- and CR-infected C3H mice at 8 DPI. (C) Blood neutrophil numbers in C3H mice infected with CR_{WT} or CR_{i9} at 8 DPI. (D) Proportion of neutrophils within the myeloid cell population recruited into the colon of CR_{i9}- and CR_{WT}-infected C3H mice at 8 DPI. (E) Representative images of colonic tissue of CR_{i9}- and CR_{WT}-infected C3H mice at 8 DPI stained with E-cadherin (yellow), Ly6G (green), CD11b (violet), and DAPI (cyan) showing more neutrophils reaching the mucosa in CR_{i9}-infected mice. <scale bar: 100 μ m> (F and G) Quantification of the number of neutrophils (Ly6G⁺, CD11b⁺, DAPI⁺ E-cadherin⁺) per mm² (F) and NETs (Ly6G⁺, H3cit⁺, DAPI⁺ E-cadherin⁺) (G) of the indicated regions of C3H transversal colonic cuts. (A) Graphs represent data from three biologically independent repeats (10–11 mice per strain). (B, C, F, and G) Graph represent means $\pm$ SEM. from two biological repeats. (D) Bar graphs represent means $\pm$ SEM from three biological repeats. (E) The images are representative of $n = 2$ biologically independent repeats. Statistical significance was determined by log-rank (Mantel-Cox) test (A) and by unpaired, two-tailed t test comparing CR_{WT} with CR_{i9} infected mice; (B, C, D, and G). In (F) statistical significance was determined using unpaired t test for lumen and submucosa and Mann-Whitney test for the mucosa. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. CR = *Citrobacter rodentium*; DAPI = 4',6-diamidino-2-phenylindole; DPI = days post infection; G-CSF = granulocyte colony-stimulating factor; IFN = interferon; IL = interleukin; NET = neutrophil extracellular trap; WT = wild-type.

including NF- κ B, JNK and Caspase-11²⁹. Indeed, the exacerbated inflammation induced by CR_{i9} resulted in high expression of neutrophil chemoattractants. As a consequence, this led to the otherwise trapped neutrophils reaching the infection site, contributing to bacterial clearance and host survival.

CR-induced colitis shares multiple features with UC, including intestinal dysbiosis^{39,40}, inflammation of the distal colonic mucosa, goblet cell depletion, CCH, and increased fecal calprotectin. CR-infected C3H mice show further similarities with UC, including diarrhea, mucoid and bloody stools, extended surface

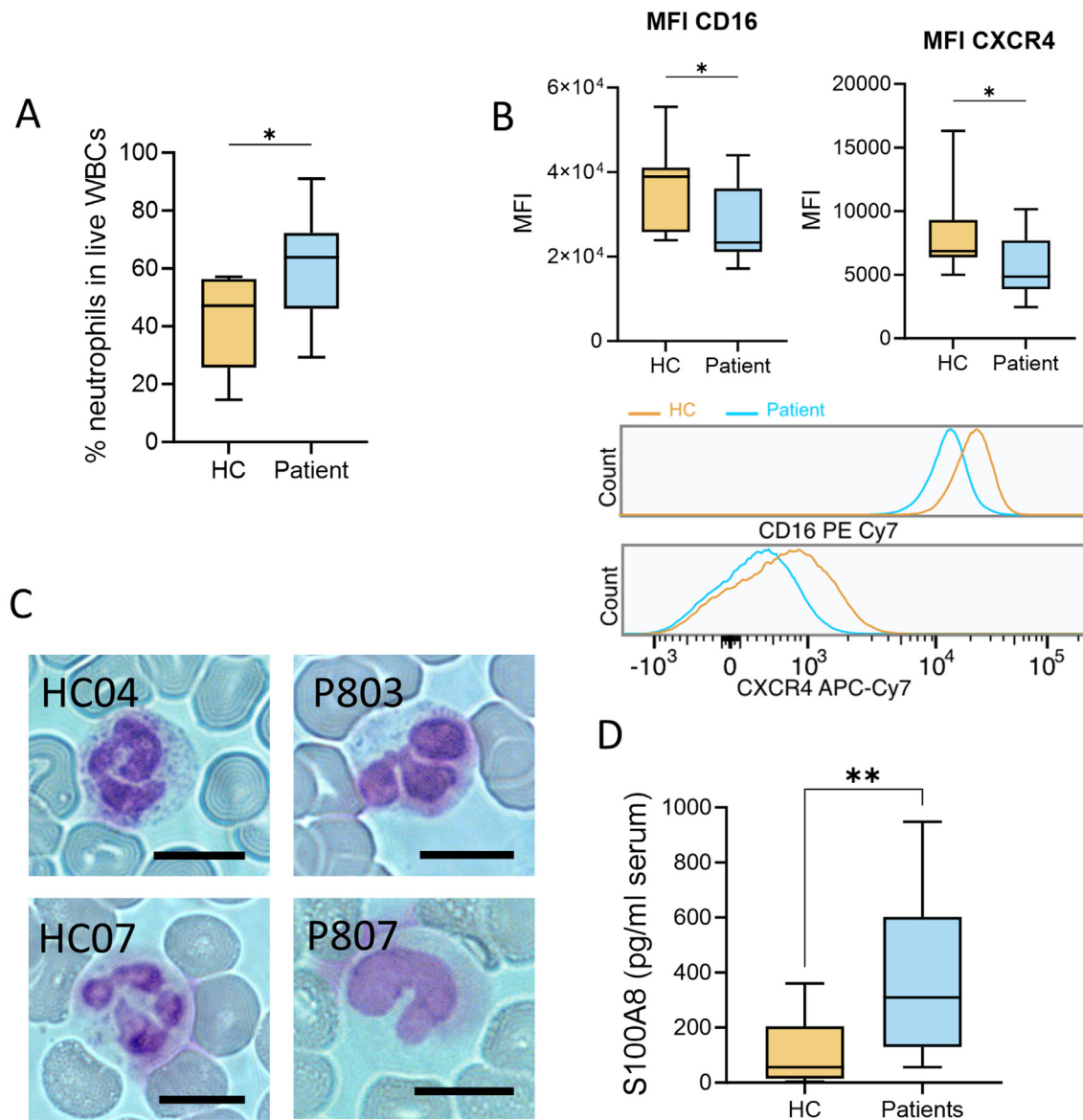


Fig. 7 UC patient neutrophils display lower levels of the activation marker CD16 and CXCR4 (related to [Supplementary Fig. 7](#)). (A) The % of neutrophils in blood from UC patients compared to HC. (B) MFI for CD16 (upper left) and CXCR4 (upper right) in neutrophils from UC patients and HC. Representative histograms are shown below the graphs. (C) Representative Wright-Giemsa stained blood smears from two HC and two patient blood samples (P) <scale bar: 10 μ m>; images are representative of eight patient samples and seven HC. (D) Plasma calprotectin (S100A8) levels from HC and UC patient samples as determined by ELISA. (A, B, and D) Box and whiskers graphs show median line with boxes from 25th to 75th percentile, where the whiskers go from the minimum to the maximum value, from 13 UC patients and 11 healthy controls. Statistical significance was determined using unpaired *t* test (A and B) and Mann-Whitney test (D) **p* < 0.05; ***p* < 0.01. ELISA = enzyme-linked immunosorbent assay; HC = healthy control; MFI = median fluorescence intensity; UC = ulcerative colitis; WBC = white blood cell.

erosion, and presence of crypt abscesses as well as increased apoptotic foci in the mucosa^{3,13,41}. Moreover, we have previously shown that, like CR-infected C3H mice, UC patients with active disease display high level of fecal NE activity compared to patients in remission¹³. This prompted us to characterize circulating neutrophils in UC patients presenting a colitis flare. Similarly to CR-infected C3H mice, we have observed that UC patients presented increased numbers of neutrophils, which displayed decreased expression of the differentiation markers CD16 and CXCR4. While the pathophysiology underpinning UC is not well understood,⁴¹ we hypothesize that, in UC, an initial insult

can lead to rapid recruitment of neutrophils to the colon where they remain, contributing to exacerbated inflammation. Moreover, the sera of UC patients showed higher levels of calprotectin compared to control, suggesting premature lysis or degranulation. Importantly, CXCR4 is involved in reverse migration of neutrophils to the BM⁴² where they can be cleared, indicating that the premature release of their contents might also be due to an inability of these neutrophils to return to the BM.

UC flares are characterized by increased serum and mucosal levels of pro-inflammatory mediators including IL-8 (murine CXCL1), and increased presence of neutrophils in the lamina pro-

pria and crypts is associated with disease^{41,43,44}. However, pre-clinical studies have shown that depleting neutrophils and/or neutrophil functions can be deleterious in IBD^{45–47}. Furthermore, no IBD treatment targets granulocyte migration (only T cell homing⁴¹), indicating that, analogously to C3H CR infection, neutrophil presence is required but needs to be adequately modulated.

Taken together, our data suggest that defective neutrophil activation and migration underpin infectious and noncommunicable colitis. The fact the modulation local inflammatory responses in the gut enables the trapped neutrophils to reach the submucosa opens up new therapeutic avenues for tackling infectious and noncommunicable colitis.

METHODS

All the materials and key resources can be found in [Supplementary Table 5](#).

Experimental model details

Pathogen-free 8–10-week-old female C3H/HeN mice and pathogen-free C57BL/6 18–20-g female mice were purchased from Charles River Laboratories and housed in groups of 3 to 5 in individually HEPA-filtered cages with bedding, nesting, and free access to food and water. The temperature, humidity, and light cycles were kept within the UK Home Office code of practice, with the temperature between 20 °C and 24 °C, the room humidity at 45–65 %, and a 12 h/12 h light cycle with a 30-minute dawn and dusk period to provide a gradual change. Where mice were purchased from a supplier, we used female mice only to avoid the logistical issues and stress to animals associated with male in-cage fighting.

For each experiment, mice were randomly assigned to experimental groups. Investigators were not blind to the allocation. Treatments and measurements were always performed following the same cage order. We typically house mice in groups of five and perform 2–3 biological replicates, with five mice per group, as power calculations using the means and standard deviation from previous and preliminary studies indicates this group size will give us enough power (0.8) to obtain significance. For example, calculating the effect size using colonic IL-22 values from WT and CRi9-infected mice from a previous study²⁹, the CR_{WT} and CRi9-infected groups have mean $\pm$ standard deviation of 84.08 ± 65.47 and 467.2 ± 514 pg/mL, respectively, converting to an effect size (d) of 1.181203, which translates into a sample size of 13 mice per group to reach a power of 0.8110747.

Mice that lost >20 % of their starting weight or became moribund during the experimental period were culled and excluded from all analyses except for the survival analysis. For the experiments described in [Fig. 6A](#) and [Supplementary Fig. 6A](#) and B, mouse infections were followed until humane endpoint or total clearance of the bacteria, defined as two consecutive CR negative stool samples. Tail vein sampling was then performed to obtain ~50 μ L of blood for determination of serum IgG levels, and mice were then reinfected with CR_{WT} strain ICC169 to follow bacterial shedding for an additional 8 days.

CR infection

CR was grown in lysogeny broth (LB) at 37 °C and with 200-rotation per minute (rpm) shaking overnight. To induce colitis with CR, mice were orally gavaged with 1×10^9 colony forming unit (CFU)/mL CR strain; control (UI) mice were mock-infected via oral gavage with 200 μ L sterile phosphate-buffered saline

(PBS)⁴⁸. CR inoculum was retrospectively confirmed by serial dilution and quantification of CFU growth on LB agar supplemented with nalidixic acid at 50 μ g/mL. In all animal experiments except those in [Fig. 6](#) and [Supplementary Fig. 6](#), mice were infected with ICC169 (CR_{WT}); for the experiments in [Fig. 6](#) and [Supplementary Fig. 6](#) mice were infected with either ICC169 (CR_{WT}) or CR_{i9}. Body weight and CFU counts were assessed as previously described⁴⁸. Briefly, analysis of CFUs was determined via serial dilutions of mechanically homogenized fecal pellets on the indicated days after CR infection, typically day 3 and 8, which were plated on LB agar plates supplemented with nalidixic acid at 50 μ g/mL.

Enzyme-linked immunosorbent assay of fecal markers

Stool samples were homogenized in 10 mL/g PBS with 0.1 % Tween 20 using a vortex machine for 15 min. Samples were centrifuged at 16,000 rpm for 10 min, and the supernatant was extracted and stored at –80 °C. The LCN-2 concentration was determined using a DuoSet mouse lipocalin-2 enzyme-linked immunosorbent assay (ELISA) (Bio-Techne) according to the manufacturer's instructions. Similarly, the concentration of S100A8 and MPO was determined using the DuoSet S100A8 and MPO ELISAs from Bio-Techne following the manufacturer's instructions. Readings were obtained using a FLUOstar Omega microplate reader (BMG biotech).

Preparation of colonic cells for flow cytometry (intraepithelial and lamina propria)

The terminal 3 cm of mouse colon was cut longitudinally to remove feces, washed in HBSS, and cut transversally into 0.5 cm pieces to then be transferred into a c-Mac (Miltenyi Biotec) tube containing RPMI supplemented with 10 % fetal bovine serum (FBS), penicillin and streptomycin solution (final concentration of 100 U and 100 μ g/mL, respectively), HEPES (10 mM), and sodium pyruvate (1 mM), all from Sigma (hereafter called complete RPMI) and kept cold until the next step. Complete RPMI supplemented was with Liberase TM (0.13 mg/mL final) and DNaseI (10 μ g/mL) was added to reach a total of 4 mL of media per tube. A first mild homogenization step was carried out using a gentleMACS dissociator (Miltenyi Biotec), followed by enzymatic digestion for 40 min on a shaker at 37 °C. A second homogenization step with the gentleMACS dissociator was performed after addition of 10 mM EDTA to the samples. The cells were filtered through a 70- μ m cell strainer, centrifuged, and resuspended in complete RPMI. From this point forward, steps were carried out on ice or at 4 °C to preserve the cells.

Blood cell preparation

For analysis of blood cell populations from mice, mice were first anesthetized via intra-peritoneal injection with 100 mg/kg ketamine/1 mg/kg medetomidine. Blood is then extracted via cardiac puncture and collected into a potassium EDTA (K3 EDTA) blood collection tube to avoid coagulation. Blood is then transferred into a falcon, diluted with PBS, and centrifuged 500g 10 min. Pellets are resuspended in red blood cell (RBC) lysis buffer (10 mM potassium bicarbonate, 155 mM ammonium chloride, 0.1 mM EDTA, and 5 % FBS in water) and incubated for 5 mins at room temperature (RT). After a second centrifugation at 4 °C, the WBC pellet was checked for traces of RBCs (a second RBC lysis was performed if necessary), cells were counted using a hemocytometer, and $\sim 5 \times 10^5$ cells per mouse were transferred

to a 96 well V-bottom plate for staining. All steps from here onwards were done on ice or at 4 °C.

BM cell preparation

For neutrophil analysis in the BM, tibia, femur, and hip bones were extracted and cleaned to then be crushed in PBS with 2 % FBS. The cells were filtered through a 40- μ m strainer, depleted of red blood cells using the RBC lysis buffer, and resuspended to be transferred to a 96 well V-bottom plate for staining. All steps from here onwards were done on ice or at 4 °C.

Staining for flow cytometry

For staining, $\sim 5 \times 10^5$ cells from each sample were added per well in a 96-well V-bottom plate. Dead cells were routinely excluded with Zombie Aqua or UV Fixable Dead Cell Stain (ThermoFisher Scientific). Single-cell suspensions were incubated with Fc block in fluorescence-activated cell-sorting (FACS) buffer [1 bovine serum albumin (BSA), 2 mM EDTA], followed by staining with the conjugated antibodies indicated in the Key Resources Table in FACS buffer, all at 4 °C in the dark; FMOs were always included as controls. For the initial characterization of colonic myeloid cell populations, neutrophils were defined as CD11b⁺ Ly6G⁺ cells; monocytes/macrophages as CD11b⁺ F4/80⁺ CD64⁺ cells; conventional dendritic cells cDCs as CD11b⁺ CD64⁺ CD11c⁺ cells; eosinophils as CD11b⁺ CD11c⁺ siglecF⁺ (gating strategy shown in [Supplementary Fig. 13A](#)). In the characterization of blood cell populations, leukocytes were defined as CD45⁺, and within these populations, B cells were broadly defined as B220⁺ and CD3⁻ and T cells as CD3⁺ B220⁻ (both populations also FSC^{Lo} and SSC^{Lo}). B220⁻ and CD3⁻ cells (Lin⁻) were further divided into neutrophils (Ly6G⁺, CD11b⁺, FSC^{Hi}, and SSC^{Hi}), CD11c⁺ DCs (Ly6G⁻ and CD11c⁺), inflammatory monocytes (Ly6G⁻, CD11c⁻, and Ly6C^{Hi}), and more resident-like monocytes (Ly6G⁻, CD11c⁻, Ly6C^{Lo}, and F4/80^{Hi}) (gating strategy in [Supplementary Fig. 32C](#)).

Cells were then washed and fixed for 20 min with 1 % PFS in PBS; after fixing the cells were kept in the dark at 4 °C until analysis. Single stain controls for compensation were prepared using VersaComp beads (Beckman Coulter) and ArC Amine Reactive Compensation beads (ThermoFisher Scientific) and flow cytometry analysis on at least 50,000 live cells was performed on a BD LSRFortessa cell analyzer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star); absolute numbers of cells in the sample were calculated using CountBright absolute beads (ThermoFisher Scientific).

FACS

When isolating colonic immune cells for neutrophil isolation via FACS, the colonic cell preparation protocol was altered to minimize changes in the neutrophils and prevent the excessive neutrophil cell death seen during sorting. To this end, we added 1 % paraformaldehyde (PFA) during the digestion step in the incubator to fix the cells before staining, as Ly6G and CD11b can also be detected after compromising membrane integrity due to fixation (gating shown in [Supplementary Fig. 1A](#)). Neutrophils, defined as Ly6G⁺ CD11b⁺ compared to the FMOs, were sorted on a BD FACSAria III cell sorter. No separation was done of neutrophils displaying higher or lower levels of these markers.

Sample preparation and tandem mass tag labeling

Isolated neutrophils from four to five mice per group from three biological repeats were pooled and solubilized using probe son-

ication in lysis buffer (100 mM triethylammonium bicarbonate, 1 % sodium deoxycholate, 10 % isopropanol, and 50 mM sodium chloride) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific), boiled for 30 min at 90 °C, and sonicated once more. The protein concentration of the BM neutrophil samples was determined with a Quick Start Bradford protein assay (BioRad) according to the manufacturer's protocol. Equal amounts of protein from each mouse from each group were combined. A total of 36.6 μ g of protein per BM sample was reduced with 5 mM Tris-2-carboxyethyl phosphine for 1 h, followed by alkylation with 10 mM iodoacetamide for 30 min, and then digested by adding trypsin (Pierce) at a final concentration of 75 ng/ μ L to each sample and incubating the samples for 18 h at RT. Peptides were labeled with tandem mass tag (TMT) multiplex reagent (Thermo Scientific) according to manufacturer instructions. TMT-labeled peptides were combined at equal amounts, and sodium deoxycholate was precipitated with formic acid (FA) at a final concentration of 2 % (vol/vol) and centrifugation for 5 min at 10,000 rpm. The supernatant containing TMT-labeled peptides was dried with a centrifugal vacuum concentrator. Due to the limited numbers of neutrophils purified from infected colons, the protein concentration in the neutrophil colon samples was too low to be determined using the Bradford protein assay; instead, we reduced and digested the whole sample as above and ran a premix ratio test to determine the correct ratio for equal mixing before pooling the samples for the remainder of the protocol.

High-pH reversed-phase peptide fractionation

TMT-labeled peptides were reconstituted in 0.1 % ammonium hydroxide and fractionated using a high-pH reversed-phase Waters XBridge C18 column (2.1 by 150 mm, 3.5 μ m) on a Dionex Ultimate 3000 high-performance liquid chromatography system. Mobile phase A was 0.1 % ammonium hydroxide, and mobile phase B was 100 % acetonitrile and 0.1 % ammonium hydroxide. A gradient elution at 200 μ L/min was used to separate the peptides in the following steps: isocratic for 5 min at 5 % phase B, gradient for 40 min to 35 % phase B, gradient to 80 % phase B in 5 min, isocratic for 5 min, and reequilibration to 5 % phase B. A total of 65 fractions were collected every 42 s into a 96-well plate, dried, and concatenated into 28 fractions upon reconstitution in 0.1 % FA.

Liquid chromatography-mass spectrometry analysis

All data were acquired using an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 system. The mobile phases were 0.1 % FA (phase A) and 80 % acetonitrile in 0.1 % FA (phase B). Samples were loaded onto a C18 trapping column (Acclaim PepMap 100, 100 μ m by 2 cm, 5 μ m, 100 Å) at a 10- μ L/min flow rate, followed by low-pH gradient elution on a nanocapillary reversed-phase column (Acclaim PepMap C18, 75 μ m by 50 cm, 2 μ m, 100 Å) at 45 °C. A flow rate of 300 nL/min was applied to separate the peptides over a 90-minute gradient from 5 % to 38 % phase B followed by 10 min up to 95 % phase B, isocratic for 5 min at 95 % phase B, reequilibration to 5 % phase B in 5 min, and isocratic for 10 min at 5 % phase B. Mass spectrometry analysis was performed as previously described in Sialana et al.⁴⁹.

Protein identification and TMT-based quantification

Proteome Discoverer 2.3 (Thermo Scientific) using the SEQUEST-HT algorithm was employed to search MS/MS spectra against

UniProt annotated reference proteomes of *Mus musculus* and CR. Searches were performed with a 20-ppm precursor mass tolerance, a 0.02-Da fragment ion mass tolerance, and trypsin digestion with up to two missed cleavages. The static modifications were specified as carbamidomethylation of Cys residues (+57.02146) and TMT modification of peptide N-terminal and Lys residues (+229.16293), while dynamic modifications were specified as oxidation of Met (+15.99491) and deamidation of Asp and Glu residues. The confidence of peptide discovery was estimated at a 1 % FDR with the percolator node based on the q value and decoy database search. The reporter ion quantifier node included a TMT 10-plex quantification method with an integration window tolerance of 15 ppm and an integration method based on the most confident centroid peak at the MS2 level. Protein groups with only unique peptides were used for quantification.

Proteomics data normalization, analysis, and visualization

To compare the results between sample batches, the datasets were normalized in various steps. For proteins with at least 60 % of the TMT channels present, the remaining missing values were replaced with the minimum value in the TMT batch. The normalization factor for each condition was calculated by the sum of all raw abundances in each TMT channel and dividing it by the maximum sum within given TMT-plex. For %CV calculations and principal component analysis, each biological group was centered to its mean within their respective TMT batch. Cluster normalization was also applied to BM samples. For further analysis, the normalized data were used to compute log2 ratios versus the UI samples or the C57 samples.

For further analysis, the technical replicates of the colonic neutrophil sample were averaged. Protein annotations were obtained with Gene ontology, Kyoto Encyclopedia of Genes and Genomes, keywords, Reactome, and Gene Set Enrichment Analysis; enrichment analysis was performed with one-dimensional-annotation enrichment method⁵⁰ in the Perseus software⁵¹. One-dimensional enrichment for BM neutrophils was performed on Log2-transformed fold change values calculated from neutrophils obtained from CR-infected mice normalized to values from those obtained from UI mice of the same mouse strain. Enrichment analysis of colon neutrophils was performed on Log2 ratios of C3H versus C57 neutrophils. The data were visualized using the online Phantasus tool⁵²). Terms with significant positive enrichment were addressed as up-regulated and those with negative enrichment as down-regulated. All terms were filtered for a Benjamini-Hochberg FDR of <0.05.

TEM assay

The mouse cell line CMT-93 was grown in Dulbecco's modified Eagle's medium (DMEM) (4500 mg of glucose per liter) supplemented with 10 % FBS and 1 % glutaMAX (complete DMEM) at 37 °C in a 5 % carbon dioxide (CO₂) (v/v) incubator. CMT-93 cells were seeded in complete DMEM on the underside of a 24 well PET transwell insert (pores of 3-μm diameter; #83.3932.300, Sarstedt) for 4 h at 37 °C, 5 % CO₂ (v/v), and then flipped and incubated for 2 days until a stable monolayer is formed. Media was changed on the day prior to the TEM assay.

BM cells were obtained as described above, and BM neutrophils were purified using Histopaque density gradients-1077 and -1119. Briefly, BM cells from mock-infected and CR-infected (8 DPI) mice were overlaid over layers of Histopaque 1119 (density, 1.119 g/mL) and Histopaque 1077 (density,

1.077 g/mL) in a 15-mL conical centrifuge tube and centrifuged at 870g for 30 min at RT using no brake. Neutrophils were collected at the interface of the Histopaque 1119 and Histopaque 1077 layers and washed twice in HBSS with calcium, magnesium, no phenol red (HBSS++) and counted using a hemocytometer; viability was assessed by trypan blue exclusion. Purity was determined by flow cytometry and was shown to be 80–99 % Ly6G⁺ neutrophils.

Prior to the migration assays, the CMT 93 monolayers were washed once with HBSS++, and the transwells were placed in a new ultra-low binding 24 well plate. A total of 350 μL of HBSS+ containing (or not) the chemoattractants were added to the bottom chamber (apical side), and 5 × 10⁵ neutrophils in HBSS++ were added to the top (basolateral side) of each Transwell. The chemoattractants/cytokines tested were the following: 100 nM fMLP and 20 ng/mL CXCL1/Kc. Control wells only had HBSS++ added to the bottom chamber. After incubation for 3 h at 37 °C, the plates were cooled on ice, and the medium of the lower chamber was centrifuged at 1000g for 10 min to pellet the neutrophils that migrated across the epithelial sheet. The neutrophils were washed and resuspended in cold PBS for counting using a hemocytometer and trypan blue exclusion. No migration was observed in the absence of chemoattractants.

The epithelial cell monolayer and neutrophils remaining on the PET transwell membrane post neutrophil migration were visualized using fluorescence microscopy. Membranes were fixed with 1 % (v/v) PFA for 10 min at RT, washed three times in PBS, and kept at 4 °C until staining. Membranes were removed from the transwell using a scalpel, and cells were incubated in PBS plus 50 mM ammonium chloride as a quenching agent for 10 min, washed and permeabilized for 10 min in 0.1 % Triton X-100, washed thrice in PBS, and blocked in PBS plus 3 % BSA for 1 h before staining with primary antibodies in 1 % BSA-PBS for 1 h. Membranes were washed three times in PBS and incubated with secondary antibodies, Phalloidin TRITC, and DAPI in 1 % BSA-PBS for 1 h (details about the reagents and antibodies used can be found in the Key Resources Table); washed twice in PBS; and once in water before being mounted in Gold ProLong Antifade medium and analyzed with a Zeiss AxioVision Z1 microscope (Zeiss).

CR *in vitro* neutrophil infections

Overnight cultures of WT CR or CR pULTRA-mCherry were grown in LB with Nalidixic acid (50 μg/mL) and Nalidixic acid with Gentamicin (10 μg/mL), respectively, at 37 °C with 200 rpm agitation.

To perform phagocytosis assays, BM neutrophils were seeded at 5 × 10⁵ cells/well in a u-bottom 96 well plate in HBSS with 0.1 % glucose and 1 % BSA. Neutrophils were then mock-infected or infected with CR pULTRA-mCherry at a multiplicity of infection (MOI) of 5, confirmed by retrospective plating. The mixtures were incubated at 37 °C, 5 % (v/v) CO₂, shaking for 45 min, then placed on wet ice for 10 min. The neutrophils were transferred to a V-bottom plate, spun down, and resuspended in PBS containing Zombie UV live/dead dye. After staining for 15 min. in the dark, cells were incubated with Fc block in 2 % FBS-PBS and stained with Ly6G-FITC for 20 min, washed, and fixed in 1 % PFA-PBS. Flow cytometry analysis was performed on a BD LSR II with BD FACSDiva software and analyzed using FlowJo software.

For immunofluorescence analysis, cells were seeded out on glass coverslips in 24-well plates at a concentration of 1 × 10⁶ cells/well in serum-free media and either mock-infected or

infected with MOI 5 or as indicated. Plates were centrifuged at 700g for 10 min at RT and incubated for 45 min or 2 h at 37 °C in a 5 % CO₂ atmosphere. The infection was stopped by placing the plate on ice and washing two times with cold PBS. If differentially staining intra- and extracellular bacteria, rabbit anti-CR (1:100) was added to the cells and incubated for 15 mins, followed by three washes with ice-cold PBS. Cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 10 min at RT and washed three times in PBS. The staining protocol was the same as detailed above for the membranes used in the TEM assay, details about the reagents used and antibodies can be found in the figure legends and Key Resources Table.

Cytokine profiling explants

A 0.5-cm distal colonic tissue sample without feces was weighed and washed for 2 h in RPMI 1640 medium with glutamine (Sigma) with 100 µg/mL of streptomycin and 100 µg/mL of penicillin to remove residual cytokines present on the tissue surface. Explants were then rinsed and cultured in complete RPMI medium at 1 mL/0.1 g tissue (RPMI 1640 medium with glutamine supplemented with 10 % heat-inactivated FBS, 1 mM sodium pyruvate, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES for 24 h at 37 °C with 5 % CO₂). The supernatant was extracted, centrifuged for 10 min at 3,000g to remove cellular debris, and stored at −80 °C until further analysis.

Explant cytokine levels were assessed from the corresponding samples via LEGENDplex (custom-made mouse panel 13-plex; BioLegend) kit per manufacturer's instructions. The panel included TNF, IFN-γ, IL-22, IL-17A, CXCL1, IL-23 or CCL2, IL-6, G-CSF, IL-18, IL-1β, GM-CSF, CCL3, and IL-10. Cytokine levels were acquired using a FACSCalibur flow cytometer (BD Biosciences), and analyses were performed using LEGENDplex data analysis software (BioLegend).

For each cytokine, a biological repeat was considered valid only if at least 90 % of the samples were above the limit of detection; any values below the detection limit within a repeat such as this were shown as the lowest value detectable by the assay.

Serum/plasma ELISAs

For analysis of serum cytokines, mice were anesthetized as indicated above, and blood was extracted via cardiac puncture. Blood was collected in BD Microtainer Tubes with Microgard Closure with BD SST Amber Clot Activator/Gel Gold Cap. Serum was separated via centrifugation and stored in aliquots at −80 °C until analysis. Serum cytokine levels were determined following the manufacturer's instructions using the following: Mouse G-CSF DuoSet ELISA kit (Bio-Techne), Mouse CXCL1/Kc DuoSet ELISA kit (Bio-Techne), or the LEGENDplex kit (custom-made mouse panel 13-plex). Levels of the marker for decreased kidney function and glomerular filtration rate, cystatin C, were measured using the mouse Cystatin C DuoSet ELISA, and the levels of serum amyloid A were determined using the Mouse Serum Amyloid A DuoSet ELISA (both from Bio-Techne). Levels of human calprotectin (S100A8) were determined in plasma from healthy controls and UC patients undergoing a flare using Human S100A8 DuoSet ELISA according to the manufacturer's instructions.

Anti-CR IgG ELISA

For serum measurement of IgG, blood was extracted from mice as indicated above, and serum was stored at −80 °C. To prepare

heat-killed CR, ICC169 was grown to saturation in LB at 37 °C at 200 rpm, diluted 1:50 in low-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma), and grown overnight at 37 °C with 5 % CO₂ to stimulate T3SS expression. The culture was then normalized to an optical density (at 600 nm) of 0.4, heat inactivated in the presence of cOmplete™ Protease Inhibitor Cocktail at 60 °C for 1 h, and finally diluted 20-fold in PBS. ELISA clear flat-bottom maxisorp immuno 96-well plates were incubated with 50 µL/well heat-killed CR for the samples or 100 µL/well goat anti-mouse IgG antibody for the standards (2 µg/µL) overnight at 4 °C. Plates were washed three times with PBS plus 0.1 % Tween 20, blocked for 1 h at RT with PBS plus 0.1 % Tween 20 and 5 % BSA, and incubated with samples or serial dilutions of purified mouse serum IgG as the standard overnight at 4 °C. Plates were washed as described above and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) (1:5000 dilution) for 2 h at RT. Plates were washed as described above and developed by the addition of 100 µL 3,3',5,5'-tetramethylbenzidine for 5 to 10 min before quenching with 2N orthophosphoric acid. The absorbances at 450 and 540 nm were measured in a plate reader (FLUOstar Omega; BMG Labtech), and anti-CR IgG was quantified in samples by comparison to a mouse IgG standard curve.

Histological analysis and immunofluorescence staining

The distal 0.5 cm of the colon was fixed in 4 % PFA for 2.5 h and then submersed in 70 % ethanol. Fixed tissues were processed, paraffin embedded, and sectioned at 5 µm. The sections were then stained with either hematoxylin and eosin (H&E) or processed for immunofluorescence. Crypt hyperplasia was determined by measuring the lengths of at least 10 well-oriented crypts from a H&E-stained section of each mouse, and the mean of each mouse was plotted for statistical analysis.

For immunofluorescence staining, sections were dewaxed by submersion in Histo-Clear solution twice for 10 min, 100 % ethanol twice for 10 min, 95 % ethanol twice for 10 min, 80 % ethanol twice for 3 min, and PBS-0.1 % Tween 20–0.1 % saponin (PBS-TS) twice for 3 min. Subsequently, sections were heated for 30 min in demasking solution (0.3 % trisodium citrate-0.05 % Tween 20 in distilled water, pH 6.0). Once cooled, slides were first blocked in PBS-TS supplemented with 10 % normal donkey serum for at least 1 h in a humid chamber before being incubated with primary antibody diluted in PBS-TS with 10 % normal donkey serum overnight at 4 °C. Sections were incubated with the antibodies indicated in the figure legends. Slides were rinsed twice for 10 min each time in PBS-TS, followed by incubation with secondary antibodies (1:100) and DAPI (1:1000) (details about the reagents used and antibodies can be found in the Key Resources Table). Washing steps were repeated before slides were mounted with ProLong Gold antifade mountant and left to cure overnight in the dark. When staining for Ly6G in the 488 nm channel, an autofluorescence quencher (Vector Tru-VIEW) was used before mounting to reduce autofluorescence from red blood cells.

Images were acquired with a Zeiss AxioVision Z1 microscope, using an AxioCam MRm camera for H&E and a Hamamatsu microscope camera for immunofluorescence, and processed using Zen 2.3 Blue Version (Carl Zeiss MicroImaging GmbH, Germany). Neutrophil numbers per mm² were quantified by counting the number of Ly6G⁺ E-cadherin⁺ DAPI⁺ cells (representative images in [Supplementary Fig. 4A](#)) in each of the delimited regions indicated in [Supplementary Fig. 4B](#) (lumen, mucosa,

and submucosa) and dividing the number by the total area of the region, which was calculated using the Zen software.

Patient sample collection

Blood samples (1–2 mL) were obtained from patients with confirmed IBD; inclusion criteria were an SCCAI (Simple Colitis Clinical Activity Index) of >5 and a raised fecal calprotectin (>100 µg/g) or endoscopy confirming active disease (all within one month) (Supplementary Table 4). They were collected in trisodium citrate (0.109 M, 3.2 %) BD Vacutainer blood collection tubes. Samples collected were processed as indicated below within 2–3 h of collection, including blood smears, staining for cell profiling, and plasma separation via centrifugation.

Human immune cell profiling

For characterization of the human blood cell population, with a focus on neutrophils, blood samples were diluted in DPBS, centrifuged, and depleted of RBCs using the RBC lysis buffer. Cells were then resuspended in DPBS, stained with Zombie Aqua fixable dye, washed, incubated with human Fc block, and then stained with the relevant antibodies (Key Resources Table; gating strategy in Supplementary Fig. 7A). Cells were fixed with 1 % PFA. Single stain controls for compensation were prepared using VersaComp beads (Beckman Coulter) and ArC Amine Reactive Compensation beads (ThermoFisher Scientific). Flow cytometry analysis on at least 100,000 live cells was performed on a BD LSRFortessa cell analyzer; FMOs were used as controls. Data were analyzed using FlowJo software, and absolute numbers of cells in the sample were calculated using CountBright absolute beads.

Wright-Giemsa blood and BM smears

For cytologic analysis of blood cells, smears were performed with 5 µL of uncoagulated blood on a VWR SuperFrost BS 7011, Microscope Slide. For BM smears, the WBC pellet obtained after RBC lysis was resuspended in 100 µL of PBS, and the smear was done using 5 µL of this sample. Smears were then fixed in ice-cold methanol for 5 min, washed, and air-dried. The Wright-Giemsa stain was prepared according to the manufacturer's instructions, and slides were then submerged in Wright-Giemsa stain for 5 min, washed with PBS, and rinsed in distilled water to then be air dried. Slides were mounted using hardset vectashield and imaged 2 h later with a Zeiss AxioVision Z1 microscope using an AxioCam MRm camera. Images were processed using Zen 2.3 Blue Version.

Data analysis

No statistical methods were used to determine sample size. For ELISAs, two technical replicates were used to estimate experimental mean. Statistical significance between two normally distributed groups was performed by Student's *t* test; when normality had not been achieved, a nonparametric Mann-Whitney test was performed. When more than two normally distributed groups were being assessed for statistical significance, a one-way analysis of variance was performed. Statistical analysis of proteomics data is detailed in the section above (Proteomics data normalization, analysis, and visualization). When data were not normally distributed (based on D'Agostino-Pearson or Shapiro-Wilk normality tests), a logarithm transformation was applied, and they were then analyzed by parametric statistical methods (analysis of variance). When normality was not achieved by transformation, a nonparametric test was applied

(Kruskal-Wallis test followed by Dunn's test). When more than three comparisons were made, *p* values were adjusted for multiple comparisons as indicated in the figure legends (Dunnett's or Tukey's post hoc test). Statistical analysis was only performed with datasets where each group analyzed had two or more biological repeats. Data plotting and statistical analysis were performed using Prism 10.0.0 (GraphPad software Inc). Statistical details of experiments are described in the figure legends. A *p* value <0.05 is considered statistically significant.

Study approval

Mouse experiments were performed in accordance with the Animals Scientific Procedures Act of 1986⁵³ and UK Home Office guidelines and were approved by the Imperial College Animal Welfare and Ethical Review Body. Experiments were designed in agreement with the ARRIVE guidelines⁵⁴ for the reporting and execution of animal experiments, including sample randomization and blinding.

Patients were recruited under the ethics of the study "Multi-omic Phenotyping of Inflammatory Bowel Disease" (IRAS ID 243310, REC reference 18/LO/1207), providing written informed consent. Samples of human blood from healthy donors were collected after receiving signed informed consent from the participant, approved by the Regional Ethics Committee and Imperial College Healthcare National Health Service Trust Tissue Bank.

FUNDING

This study was supported by grants from The Wellcome Trust (107057/z/15/z and 224282/Z/21/Z) and the MRC (MR/R02671).

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Julia Sanchez-Garrido: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Yasaman Naemi Baghshomali:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Prashant Kaushal:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Zuza Kozik:** Formal analysis, Methodology, Writing – review & editing. **Robert W. Perry:** Investigation, Methodology, Writing – review & editing. **Horace R.T. Williams:** Investigation, Supervision, Writing – review & editing. **Jyoti Choudhary:** Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Gad Frankel:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE⁵⁵ partner repository with the dataset identifier PXD049327. Additional data are available in the [supplementary material](#), in the source data values files deposited in Mendeley data (<https://data.mendeley.com/preview/wtxn2m9g8p?a=d7ce498b-1745-49f3-bd4e-9ce843e5c384>) and from the corresponding author upon reasonable request.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENTS

We thank Dr Joshua L. C. Wong for helping with blood extractions and Lorraine Lawrence for performing histological staining. We also thank the Flow Cytometry Facility at Imperial College London, in particular Dr Jessica Rowley and Dr Larissa Zárate García, for performing the FACS and always being so willing to help, and the Institute for Cancer Research for core support.

APPENDIX A. SUPPLEMENTARY MATERIAL

Supplementary material to this article can be found online at <https://doi.org/10.1016/j.mucimm.2024.06.008>.

References

- Levine, M. M. et al. Diarrhoeal disease and subsequent risk of death in infants and children residing in low-income and middle-income countries: analysis of the GEMS case-control study and 12-month GEMS-1A follow-on study. *Lancet Glob Health* **8**, e204–e214 (2020).
- GBD 2017 Inflammatory Bowel Disease Collaborators The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol Hepatol* **5**, 17–30 (2020).
- Mullineaux-Sanders, C. et al. *Citrobacter rodentium*–host–microbiota interactions: immunity, bioenergetics and metabolism. *Nat Rev Microbiol* **17**, 701–715 (2019).
- Vallance, B. A., Deng, W., Jacobson, K. & Finlay, B. B. Host susceptibility to the attaching and effacing bacterial pathogen *Citrobacter rodentium*. *Infect Immun* **71**, 3443–3453 (2003).
- Borenshtein, D., Nambiar, P. R., Groff, E. B., Fox, J. G. & Schauer, D. B. Development of fatal colitis in FVB mice infected with *Citrobacter rodentium*. *Infect Immun* **75**, 3271–3281 (2007).
- Sanchez-Garrido, J., Ruano-Gallego, D., Choudhary, J. S. & Frankel, G. The type III secretion system effector network hypothesis. *Trends Microbiol* **30**, 524–533 (2022).
- Zindl, C. L. et al. A nonredundant role for T cell-derived interleukin 22 in antibacterial defense of colonic crypts. *Immunity* **55**, 494–511.e11 (2022).
- Silberger, D. J., Zindl, C. L. & Weaver, C. T. *Citrobacter rodentium*: a model enteropathogen for understanding the interplay of innate and adaptive components of type 3 immunity. *Mucosal Immunol* **10**, 1108–1117 (2017).
- Wu, M. et al. Gut complement induced by the microbiota combats pathogens and spares commensals. *Cell* **187**, 897–913.e18 (2024).
- Kamada, N. et al. Humoral immunity in the gut selectively targets phenotypically virulent attaching-and-effacing bacteria for intraluminal elimination. *Cell Host Microbe* **17**, 617–627 (2015).
- Papapietro, O. et al. R-spondin 2 signalling mediates susceptibility to fatal infectious diarrhoea. *Nat Commun* **4**, 1898 (2013).
- Ghosh, S. et al. Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression. *Am J Physiol Gastrointest Liver Physiol* **301**, G39–G49 (2011).
- Barry, R. et al. Faecal neutrophil elastase-antiprotease balance reflects colitis severity. *Mucosal Immunol* **13**, 322–333 (2020).
- Wigerblad, G. & Kaplan, M. J. Neutrophil extracellular traps in systemic autoimmune and autoinflammatory diseases. *Nat Rev Immunol* **23**, 274–288 (2023).
- Burn, G. L., Foti, A., Marsman, G., Patel, D. F. & Zychlinsky, A. The neutrophil. *Immunity* **54**, 1377–1391 (2021).
- Cassatella, M. A., Östberg, N. K., Tamassia, N. & Soehnlein, O. Biological roles of neutrophil-derived granule proteins and cytokines. *Trends Immunol* **40**, 648–664 (2019).
- Burgener, S. S. & Schroder, K. Neutrophil extracellular traps in host defense. *Cold Spring Harb Perspect Biol* **12**.
- Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**, 519–531 (2011).
- Cremin, M. et al. TRPV1 controls innate immunity during *Citrobacter rodentium* enteric infection. *PLoS Pathog* **19**, e1011576.
- Spehlmann, M. E. et al. CXCR2-dependent mucosal neutrophil influx protects against colitis-associated diarrhea caused by an attaching/effacing lesion-forming bacterial Pathogen1. *J Immunol* **183**, 3332–3343 (2009).
- Lebeis, S. L., Bommarius, B., Parkos, C. A., Sherman, M. A. & Kalman, D. TLR signaling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*. *J Immunol* **179**, 566–577 (2007).
- Danne, C., Skerniskyte, J., Marteyn, B. & Sokol, H. Neutrophils: from IBD to the gut microbiota IBD. *Nat Rev Gastroenterol Hepatol* **21**, 184–197 (2024).
- Fournier, B. M. & Parkos, C. A. The role of neutrophils during intestinal inflammation. *Mucosal Immunol* **5**, 354–366 (2012).
- Carlson, M. et al. Human neutrophil lipocalin is a unique marker of neutrophil inflammation in ulcerative colitis and proctitis. *Gut* **50**, 501–506 (2002).
- Friedrich, M. et al. IL-1-driven stromal–neutrophil interactions define a subset of patients with inflammatory bowel disease that does not respond to therapies. *Nat Med* **27**, 1970–1981 (2021).
- O’Connell, K. E. et al. Practical murine hematopathology: a comparative review and implications for research. *Comp Med* **65**, 96–113 (2015).
- Metzemaekers, M., Gouwy, M. & Proost, P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. *Cell Mol Immunol* **17**, 433–450 (2020).
- Saha, P. et al. PAD4-dependent NETs generation are indispensable for intestinal clearance of *Citrobacter rodentium*. *Mucosal Immunol* **12**, 761–771 (2019).
- Ruano-Gallego, D. et al. Type III secretion system effectors form robust and flexible intracellular virulence networks. *Science* **371**, eabc9531.
- Blake, K. M., Carrigan, S. O., Issekutz, A. C. & Stadnyk, A. W. Neutrophils migrate across intestinal epithelium using beta2 integrin (CD11b/CD18)-independent mechanisms. *Clin Exp Immunol* **136**, 262–268 (2004).
- Liu, Y. et al. Signal regulatory protein (SIRPα), a cellular ligand for CD47, regulates neutrophil transmigration*. *J Biol Chem* **277**, 10028–10036 (2002).
- Elson, C. O. et al. Hapten-induced model of murine inflammatory bowel disease: mucosa immune responses and protection by tolerance. *J Immunol* **157**, 2174–2185 (1996).
- Mähler, M. et al. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am J Physiol* **274**, G544–G551 (1998).
- Tregone, J. S. et al. Genetic susceptibility to the delayed sequelae of neonatal respiratory syncytial virus infection is MHC dependent. *J Immunol* **185**, 5384–5391 (2010).
- Minnich, L. L. & Ray, C. G. Variable susceptibility of mice to group B coxsackievirus infections. *J Clin Microbiol* **11**, 73–75 (1980).
- Bradley, D. J. Regulation of Leishmania populations within the host. II. Genetic control of acute susceptibility of mice to Leishmania donovani infection. *Clin Exp Immunol* **30**, 130–140 (1977).
- Töttemeyer, S., Foster, N., Kaiser, P., Maskell, D. J. & Bryant, C. E. Toll-like receptor expression in C3H/HeN and C3H/HeJ mice during Salmonella enterica serovar Typhimurium infection. *Infect Immun* **71**, 6653–6657 (2003).
- Gros, P., Skamene, E. & Forget, A. Genetic control of natural resistance to Mycobacterium bovis (BCG) in mice. *J Immunol* **127**, 2417–2421 (1981).
- Berger, C. N. et al. *Citrobacter rodentium* subverts ATP flux and cholesterol homeostasis in intestinal epithelial cells in vivo. *Cell Metab* **26**, 738–752.e6 (2017).
- Lloyd-Price, J. et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* **569**, 655–662 (2019).
- Kobayashi, T. et al. Ulcerative colitis. *Nat Rev Dis Primers* **6**, 74 (2020).
- de Oliveira, S., Rosowski, E. E. & Huttenlocher, A. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat Rev Immunol* **16**, 378–391 (2016).
- Daig, R. et al. Increased interleukin 8 expression in the colon mucosa of patients with inflammatory bowel disease. *Gut* **38**, 216–222 (1996).
- Rodríguez-Perálvarez, M. L. et al. Role of serum cytokine profile in ulcerative colitis assessment. *Inflam Bowel Dis* **18**, 1864–1871 (2012).
- Zhou, G. et al. CD177⁺ neutrophils as functionally activated neutrophils negatively regulate IBD. *Gut* **67**, 1052–1063 (2018).
- Leppkes, M. et al. Neutrophils prevent rectal bleeding in ulcerative colitis by peptidyl-arginine deiminase-4-dependent immunothrombosis. *Gut* **71**, 2414–2429 (2022).
- Kühl, A. A. et al. Aggravation of different types of experimental colitis by depletion or adhesion blockade of neutrophils. *Gastroenterology* **133**, 1882–1892 (2007).
- Crepin, V. F., Collins, J. W., Habibzay, M. & Frankel, G. *Citrobacter rodentium* mouse model of bacterial infection. *Nat Protoc* **11**, 1851–1876 (2016).
- Sialana, F. J. et al. SimPLIT: Simplified Sample Preparation for Large-Scale Isobaric Tagging Proteomics. *J Proteome Res* **21**, 1842–1856 (2022).

50. Cox, J. & Mann, M. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. *BMC Bioinformatics* **13**(Suppl. 16), S12 (2012).
51. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* **13**, 731–740 (2016).
52. Zenkova D, Kamenev V, Sablina R, Artyomov M, Sergushichev A. Phantasm: visual and interactive gene expression analysis, (2018).
53. Bleby, J. Animals (Scientific Procedures) Act 1986. *Vet Rec* **119**, 22 (1986).
54. Percie du Sert, N. et al. Reporting animal research: explanation and elaboration for the ARRIVE Guidelines 2.0. *PLoS Biol* **18**:e3000411.
55. Perez-Riverol, Y. et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res* **50**, D543–D552 (2022).