NEUTROPHIL COMMUNICATION

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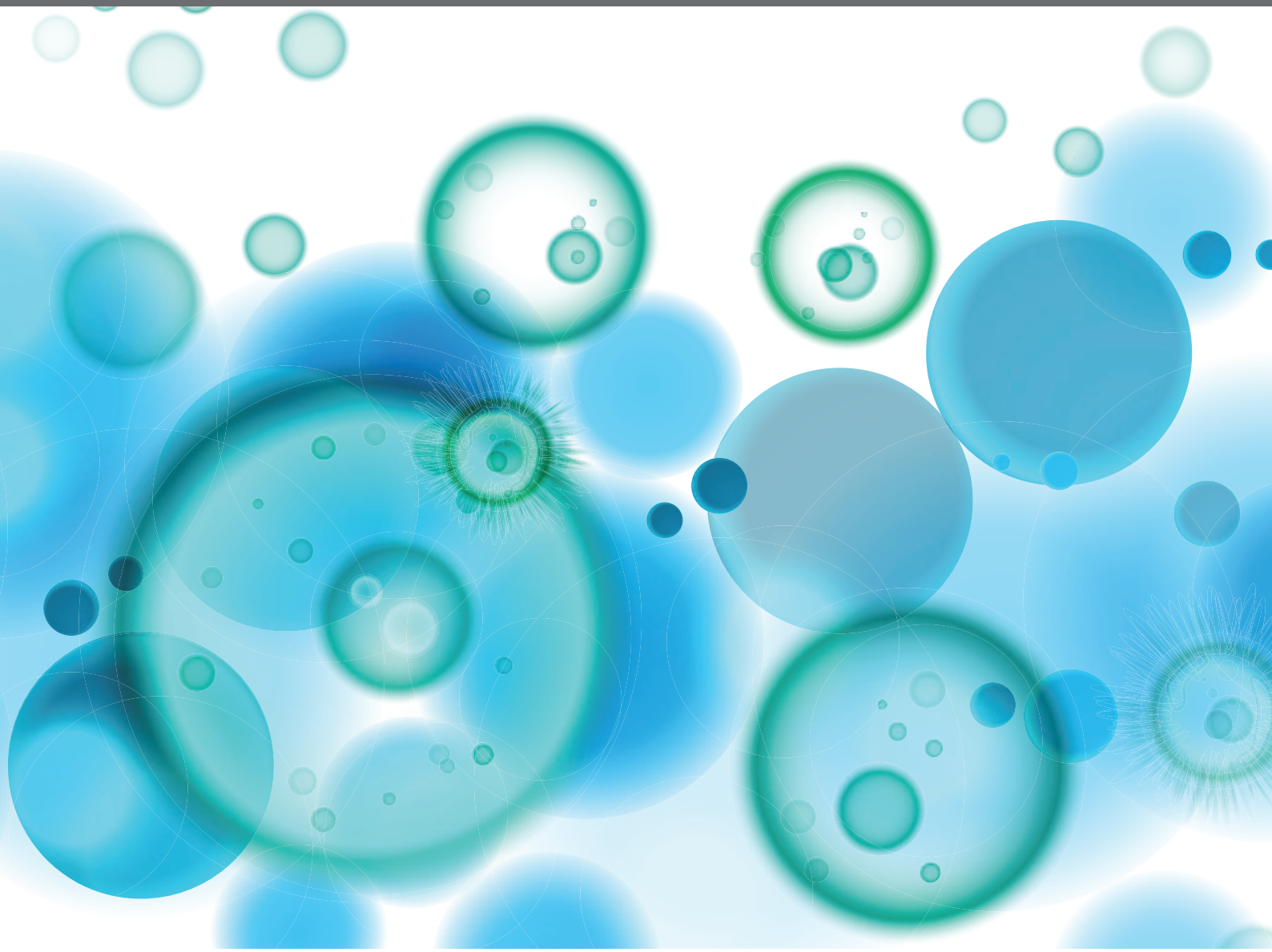

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NEUTROPHIL COMMUNICATION

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EDITORIAL
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Editorial: Neutrophil Communication

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Editorial on the Research Topic

Neutrophil Communication

Neutrophils are key immune cells that participate in host defense through a variety of mechanisms including phagocytosis, the generation of reactive oxygen species (ROS), release of granular contents, and the formation of neutrophil extracellular traps (NETs). The capacity of neutrophils to orchestrate inflammatory and immune responses is dependent on their release of neutrophil-derived molecules, including cytokines, alarmins, and NETs, as well as their capacity to interact with and direct other innate and adaptive immune cells. Over the past decade, the field of neutrophil biology has exploded, with remarkable discoveries highlighting neutrophils as indispensable players in immune regulation. While neutrophils are recognized to be prominent phagocytes involved in the clearance of pathogens and cell debris, they are beginning to emerge as essential communicators crucial in shaping immune response.

In this Research Topic, a series of articles provides comprehensive insights into the current view of neutrophil biology, highlighting neutrophil interactions with platelets, infectious agents, and tumor cells, as well as neutrophil generation of inflammatory mediators such as NETs.

In the review by Wirestam et al., we are reminded of the devastating role of neutrophils in rheumatic diseases, including systemic lupus erythematosus (SLE) and anti-phospholipid syndrome (APS) wherein neutrophils recognize circulating immune complexes promoting ROS-dependent NET formation. Intriguingly, SLE patients have impaired NOX2, relying on mitochondrial ROS for NET formation. This results in concomitant mitochondrial extrusion and a qualitatively distinct NETs with inflammatory oxidized mitochondrial DNA as an important cargo. Takeda et al. highlighting the important role for immune complexes, namely bactericidal/permeability-increasing protein (BPI) immune complexes, in mediating NET formation in systemic anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. The regulation and role of the main immune complex receptor, Fc gamma R, in steady state and inflammatory conditions, is comprehensively covered in an excellent review by Wang and Jönsson. Beyond immune complexes, large aggregates, including monosodium urate (MSU) crystals are known to activate macrophages and neutrophils, though the underlying signaling events have not been well-characterized. In an elegant study by Tatsiy et al., performing transcriptomic analyses, the authors demonstrate the novel finding that neutrophils, upon engulfment of MSU crystals, are programmed to attract monocytes in an NF-κB- and CCL4-dependent manner. This observation is particularly interesting as neutrophils are essential in licensing macrophages for cytokine production in atherosclerosis, indicating a pathophysiological role for their crosstalk (1).

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Furthermore, the authors find that TAK1 and Syk are early mediators, upstream of MAPK and Akt, partaking in NET formation, and as such, representing a potential therapeutic target for gouty arthritis. A similar transcriptomic approach was undertaken by Miralda et al. to assess neutrophil signaling pathways regulated by the bacteria *Filifactor alocis*. Among the many pathways being regulated by *F. alocis*, the authors made the intriguing finding that the bacteria blunted TNF-induced MAPK activation in neutrophils, suggesting that this may be a bacterial strategy to subvert innate immunity.

Another study investigating the underlying requirements for NET formation (Gößwein et al.), made the novel observation of a crosstalk between a protease, calpain, and peptidylarginine deiminase 4 (PAD4) in nuclear decondensation, with PAD4-mediated citrullination facilitating proteolytic cleavage of nuclear proteins. This observation markedly advances our understanding of the molecular events during NET formation and may allow for additional therapeutic strategies to prevent exacerbated NETosis. In the final NET-related paper included in this Research Topic, Agarwal et al. demonstrate that NETs are not only a product of neutrophil activation, but also a potent stimulus for secondary NETosis, inducing NET formation through TLR9-dependent mechanisms. This mechanism may be particularly important in post-traumatic inflammation, when NETs disrupted by mechanical strain may amplify inflammation and prevent physiological wound healing.

Two interesting papers in this collection suggest a critical role for platelets as orchestrators of neutrophil recruitment to diseased organs. Psoriasis is a systemic inflammatory disease characterized by intense leukocyte infiltration into the skin. Herster et al. undertook an unbiased screen of neutrophil surface markers in individuals with psoriasis and found upregulation of CD41 and CD61 (two classic platelet glycoproteins). These surface markers were attributable to increased platelet-neutrophil aggregates in the blood of patients with psoriasis (Herster et al.). The aggregates also appeared to infiltrate the skin, where platelets could be found in close proximity to both intact neutrophils and neutrophil remnants suggestive of NETs (Herster et al.). Intriguingly, platelet depletion was protective against neutrophil infiltration and overall skin inflammation in a mouse model of psoriasis (Herster et al.).

Systemic inflammatory response syndrome (SIRS) puts many organ systems at risk including the lungs, where it may lead to acute respiratory distress syndrome. Hook et al. characterized a model of SIRS triggered by administration of zymosan. This model has previously been shown to be exacerbated by NADPH oxidase 2 (Nox2) deficiency. In this new study (Hook et al.), Nox2-deficient mice demonstrated upregulation of platelet-derived chemokines such as CXCL4 and CXCL7 in the bronchoalveolar fluid. Neutrophils strongly expressing P-selectin glycoprotein ligand-1 were found at increased levels in the same fluid (Hook et al.). While Nox2 deficiency may protect against NETosis in some contexts, that was not the case in this model where exaggerated NETosis appeared to be driven by platelets in a PAD4-dependent fashion (Hook et al.). In summary, these studies shine a light on the role of platelets in recruiting neutrophils into inflamed tissues, and suggest the potential for anti-platelet agents to have efficacy in neutrophil-dependent diseases.

A further group of articles have focused on some of the functional crosstalk that neutrophils are known to engage in with other leukocytes. Firstly, Bernson et al. extended our knowledge on the molecular basis underlying the capacity of natural killer (NK) cells to trigger neutrophil apoptosis under inflammatory conditions, by performing a comprehensive characterization of neutrophil expression of ligands for NK cell receptors. Ultimately, the authors demonstrated that inflammatory neutrophils, including either blood neutrophils stimulated *in vitro* or *in vivo*-transmigrated neutrophils, become more susceptible to NK cell-mediated apoptosis than resting neutrophils. Furthermore, their enhanced sensitivity is associated with a pronounced downregulation of HLA class I expression, which is known to release activating signaling by NK cells (Bernson et al.). Secondly, Aarts et al. compared the immunosuppressive capacity of circulating neutrophils from healthy donors (HDs) with those displayed by autologous bone marrow (BM) myeloid cell fractions at various differentiation stages. They show that, unlike "early mature" BM neutrophils, immature BM neutrophils, *per se*, are not efficient in suppressing T cells when activated with physiological stimuli (Aarts et al.). The authors therefore speculate that, for instance in cancer patients, functional granulocyte-myeloid-derived suppressor cells (g-MDSCs) require neutrophil differentiation from immature populations in the bloodstream, which are unable to perform any immunosuppressive activity in their own right (Aarts et al.). In another article, Oberg et al., first, exhaustively review current knowledge on the reciprocal interactions between neutrophils, tumor cells, and particularly gamma/delta T cells. They then provide data on the modulation of anti-tumor cytotoxicity of short-term expanded human gamma/delta T cell lines by autologous freshly isolated neutrophils. Their results demonstrate that, under certain circumstances, the presence of neutrophils can enhance, rather than inhibit, the killing capacity of gamma/delta T cells, by increasing their release of cytotoxic mediators (Oberg et al.). Finally, the role of neutrophils in malignancy was extensively reviewed by Lecot et al., highlighting the heterogeneity of neutrophils, and their effector functions contributing to cancer suppression as well as progression. The authors also reviewed different strategies aimed at targeting neutrophils in malignancy, ranging from neutrophil depletion to succinct targeting of recruitment or other key effector functions.

In conclusion, neutrophils are heterogeneous cells with a huge range of effector functions partaking in shaping our immune system. The current papers included in this Research Topic highlights the emerging role of neutrophils as communicators in health and disease with promises of future therapies targeting these pathways.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Whole Transcriptome Analysis Reveals That *Filifactor alocis* Modulates TNFα-Stimulated MAPK Activation in Human Neutrophils

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Periodontitis is an irreversible, bacteria-induced, chronic inflammatory disease that compromises the integrity of tooth-supporting tissues and adversely affects systemic health. As the immune system's first line of defense against bacteria, neutrophils use their microbicidal functions in the oral cavity to protect the host against periodontal disease. However, periodontal pathogens have adapted to resist neutrophil microbicidal mechanisms while still propagating inflammation, which provides essential nutrients for the bacteria to proliferate and cause disease. Advances in sequencing technologies have recognized several newly appreciated bacteria associated with periodontal lesions such as the Gram-positive anaerobic rod, *Filifactor alocis*. With the discovery of these oral bacterial species, there is also a growing need to assess their pathogenic potential and determine their contribution to disease progression. Currently, few studies have addressed the pathogenic mechanisms used by oral bacteria to manipulate the neutrophil functional responses at the level of the transcriptome. Thus, this study aims to characterize the global changes at the gene expression level in human neutrophils during infection with *F. alocis*. Our results indicate that the challenge of human neutrophils with *F. alocis* results in the differential expression of genes involved in multiple neutrophil effector functions such as chemotaxis, cytokine and chemokine signaling pathways, and apoptosis. Moreover, *F. alocis* challenges affected the expression of components from the TNF and MAPK kinase signaling pathways. This resulted in transient, dampened p38 MAPK activation by secondary stimuli TNFα but not by fMLF. Functionally, the *F. alocis*-mediated inhibition of p38 activation by TNFα resulted in decreased cytokine production but had no effect on the priming of the respiratory burst response or the delay of apoptosis by TNFα. Since the modulatory effect was characteristic of viable *F. alocis* only, we propose this as one of *F. alocis'* mechanisms to control neutrophils and their functional responses.

Keywords: human neutrophils, periodontitis, emerging oral pathogens, MAPK signaling, neutrophil transcriptome

INTRODUCTION

Periodontitis is a chronic inflammatory disease where inflammophilic pathogenic bacterial communities accumulate at the gingival crevice. These dysbiotic microbial communities induce a severe inflammatory response that fails to control bacterial growth and contributes to the irreversible destruction of tooth-supporting tissues (1). Historically, periodontal research has focused on the pathogenic members of the "red complex," which includes *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia*. However, recent human microbiome studies have revealed many previously uncultured organisms with a strong correlation with periodontal disease (2, 3). One of these newly appreciated species is *Filifactor alocis*, a Gram-positive anaerobic rod with emerging pathogenic potential and contribution to periodontal diseases. *F. alocis* is consistently and abundantly found in periodontal active lesions (4–9). Furthermore, *F. alocis* shares virulence characteristics with other periodontal pathogens such as resistance to oxidative stress, biofilm formation, secretion of proteases, and evasion of the immune system (10–14).

Neutrophils constitute an overwhelming majority of the leukocytes recruited to the oral cavity, where they are essential for maintaining homeostasis of periodontal tissues (15–17). Neutrophils can deploy several strategies to efficiently detect, detain, and destroy microbes. These include phagocytosis, release of antimicrobial enzymes or toxic factors, generation of massive amounts of reactive oxygen species (ROS), and discharge of their nuclear material into neutrophil extracellular traps (NETs) (18). However, oral pathogens have evolved mechanisms to manipulate neutrophil functional responses to prevent being killed while propagating inflammation (17, 19). Previous work from our laboratory has shown that despite efficient phagocytosis by neutrophils, *F. alocis* survives within neutrophils by inducing minimal production of intracellular ROS and curtailing the fusion of antimicrobial granules with its phagosome (20, 21). However, in comparison to the keystone oral pathogen, *P. gingivalis*, and another emerging oral pathogen, *Peptoanaerobacter stomatis*, challenge with *F. alocis* resulted in a mild release of neutrophil-derived pro-inflammatory cytokines, which resulted in limited recruitment of monocytes and other neutrophils (22). Thus, we hypothesize that *F. alocis* may modulate neutrophil signaling events to interrupt pro-inflammatory cytokine production and alter immune cell recruitment and communication.

The mitogen-activated protein kinases (MAPKs) are evolutionarily conserved regulators that carry out signal transduction for many cellular functional processes. MAPK activation cascades are well-characterized and usually begin with the ligation of cell surface receptors followed by activation of a relay cascade of phosphorylation of three core kinases: MAP3K, MAP2K (MEK or MKK), and MAPK. Active MAPKs can phosphorylate a variety of intracellular targets including transcription factors, nuclear pore proteins, membrane transporters, cytoskeletal elements, and other protein kinases, so their activation is subjected to spatiotemporal regulation by complex feedback and crosstalk mechanisms (23, 24). In human neutrophils, bacterial lipopolysaccharide (LPS) activates Toll-like receptor (TLR) 4 followed by downstream activation of MAPK signaling pathways and the transcription factor regulator nuclear factor (NF)-κB, both of which can independently regulate the production of inflammatory cytokines and chemokines (25, 26). Both p38 MAPK and ERK pathways control transcription and translation of inducible cytokines in neutrophils stimulated with LPS or TNFα (27). Due to the relevant role that MAPK signaling plays in regulation of immune responses, it is not surprising that some pathogens have developed mechanisms to hijack this signaling cascade on immune cells (28, 29). For example, *Mycobacterium tuberculosis* acetylates a MAPK phosphatase, DUSP16, to increase phosphatase activity on Janus kinase (JNK) and limit inflammatory cytokine production by bone marrow-derived macrophages (30). Prior work from our group showed that *F. alocis* initially activates both p38 MAPK and ERK1/2 through TLR2 (20); however, it is unknown what the MAPK response is after *F. alocis* stimulation for longer time points or how the cells respond to secondary stimuli after *F. alocis* challenge.

Few sequencing studies have tracked transcriptome changes in human neutrophils during challenge with a bacterial pathogen (31–34). Even fewer studies have measured changes in the neutrophil transcriptome associated with the challenges of putative oral pathogens. Thus, we sought to characterize global changes at the gene expression level in human neutrophils during infection with *F. alocis*. Analysis of whole-transcriptome by RNA-based next-generation sequencing (RNAseq) shows that *F. alocis* challenge alters the human neutrophil transcriptome by inducing significant changes in the expression of genes involved in various neutrophil effector functions. One of the findings of our RNA-seq screen was that *F. alocis* challenge affected the expression of components in both the TNF and MAPK kinase signaling pathways. This resulted in decreased p38 MAPK activation by secondary stimuli TNFα but not by fMLF. Moreover, only live *F. alocis* limited the TNFα-stimulated production of IL-8, demonstrating that this is one of the mechanisms actively induced by the oral pathogen to control neutrophil functional responses.

MATERIALS AND METHODS

Human Neutrophil Isolation

Human donor recruitment, blood draws, and the use of the materials required for this procedure were done in accordance with the guidelines approved by the Institutional Review Board of the University of Louisville. Neutrophils were isolated from venous blood of healthy donors using plasma-Percoll gradients as described previously (35). Neutrophil populations were further enriched to obtain highly pure cells (>99%) by negative magnetic selection using the Easy Eights EasySep Magnet and human neutrophil enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) as previously described (36). Cell purity was assessed by simultaneously staining with FITC-conjugated anti-CD66b (clone G10F5; BioLegend, San Diego, CA, USA) and APC-conjugated anti-CD16 (clone CB16; eBioscience, San Diego, CA, USA) antibodies and determining the percentage

of $CD66b^+CD16^+$ cells using a BD Celesta flow cytometer (BD Biosciences, San Jose, CA, USA). Both pure (>90–95%) and highly pure (>99%) neutrophils were cultured in complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 5% human serum (Atlanta Biologicals, Flowery Branch, GA, USA).

Bacterial Strains and Growth Conditions

F. alocis ATCC 38596 was cultured in brain heart infusion (BHI) broth supplemented 5 mg/mL yeast extract, L-cysteine (0.05%), and arginine (0.05%) for 7 days anaerobically at 37 °C as previously described (20, 37). Serum opsonization was performed by incubating *F. alocis* at 37°C for 20 min in 10% normal human serum (Complement Technology, Inc., Tyler, TX, USA). Heat-killed *F. alocis* was generated by incubation at 90°C for 60 min. Non-viability was confirmed by incubation in culture media at the same conditions used for the live organism.

F. alocis Challenge and RNA Isolation

Highly pure (>99%) human neutrophils (10–20 × 10^6 cells/mL) were unstimulated or challenged with opsonized *F. alocis* at a multiplicity of infection (MOI) of 10, for 1, 3, or 6 h. The infection was synchronized by centrifugation at 14°C for 4 min at 600×g. After each time point, the cells were harvested using Trizol (Life Technologies, Carlsbad, CA, USA) and stored at −80°C until RNA extraction. RNA was isolated from unstimulated and *F. alocis*-challenged human purified neutrophils using the hybrid method of Trizol and RNeasy minikit (Qiagen, Venlo, Netherlands). The aqueous phase containing RNA was loaded on the Qiagen column for further purification of RNA. The purified RNA quality was measured by running the sample on Bioanalyzer.

Library Preparation

The isolated RNA was checked for integrity using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and quantified using a Qubit fluorometric assay (Thermo Fisher Scientific, Waltham, MA, USA). Total RNASeq libraries were prepared following Illumina's TruSeq Stranded Total RNA LT with Ribo-Zero Gold library preparation protocol (Illumina Inc., San Diego, CA, USA, Cat# RS-122-2301). After depletion of ribosomal RNA, all samples were ligated with Illumina adapters and individually barcoded. The absence of adapter dimers and a consistent library size of approximately 300 bp was confirmed using the Agilent Bioanalyzer 2100. Library quantitation was performed by qPCR using the KAPA Library Quantitation Kit (Kapa Biosystems, part of Roche Sequencing and Life Science, Wilmington, MA, USA) for Illumina Platforms.

Sequencing Run

1.8 pM of the library pool was loaded with 1% PhiX spike-in on two NextSeq 500/550 75 cycle High Output Kit v2 sequencing flow cells. Sequencing was performed on the Illumina NextSeq 500 sequencer targeting 50M 1 × 75bp reads per sample.

Bioinformatic Analysis

Each of four single-end raw FASTQ files for each replicate was concatenated into one single end FASTQ file using the Unix cat command. A total of sixteen files representing four independent donors and four experimental conditions were generated. Quality control (QC) of the raw sequence data was performed using FastQC (version 0.10.1). The interquartile range remained above 30 (99.9% base call accuracy) across the reads. The concatenated sequences were directly aligned to the *Homo sapiens* reference genome assembly (hg38.fa) using tophat2 (version 2.0.13) (38), generating alignment files in BAM format. The alignment rate ranged from 88 to 93 percent across the samples. Differential expression analysis between each treatment condition (1, 3, and 6 h) and the control condition was performed using Cufflinks-Cuffdiff2 (version 2.2.1) (39, 40). A q-value cutoff ≤ 0.05 with an absolute $|\log_2 FC| \geq 1$ was used to determine differential expression.

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR)

Total RNA isolated from the different experimental conditions was followed by reverse-transcription into cDNA using a High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA, USA), while qPCR was carried out using SYBR®Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems StepOnePlus cycler with StepOne software V2.2.2. Sequences of the gene-specific primers (Integrated DNA Technologies, Skokie, IL, USA) used in this study are listed in **Table 1**. Data were calculated and expressed as mean normalized expression (MNE) units after GAPDH normalization as previously described (41).

Western Blotting

Neutrophils (10 × 10^6 cells/mL) were cultured at 37°C, 5%CO_2 in RPMI-1640 with 5% heat-inactivated human serum and left unstimulated, stimulated with FSL (100 ng/mL), challenged with either live or heat-killed *F. alocis* for 1, 3, 6, or 10 h followed by stimulation with fMLF (300 nM, 1 min) or TNF-α (10 ng/ml, 15 min). After the different experimental procedures,

TABLE 1 | qPCR primer sequences used to validate RNAseq results.

Gene	qPCR primer sequence
Galectin 3	Forward 5′- CAGAATTGCTTTAGATTTCCAA-3′
	Reverse 5′-TTATCCAGCTTTGTATTGCAA-3′
NCF-1	Forward 5′-AAGATGGCAAGAGTACCGC-3′
	Reverse 5′-TCTCGTAGTTGGCAATGGC-3′
GAPDH	Forward 5′-CTTTGGTATCGTGGAAGGACTC-3′
	Reverse 5′-GTAGAGGCAGGGATGATGTTC-3′
CXCL5	Forward 5′-TCTGCAAGTGTTCGCCATAG-3′
	Reverse 5′-CAGTTTTCCTTGTTTCCACCG-3′
CCL5	Forward 5′-TGCCCACATCAAGGAGTATTT-3′
	Reverse 5′-TTTCGGGTGACAAAGACGA-3′
ASC	Forward 5′-CTCCTCAGTCGGCAGCCAAG-3′
	Reverse 5′-ACAGAGCATCCAGCAGCCAC-3′
NOD2	Forward 5′-CTGAAGAATGCCCGCAAGGT-3′
	Reverse 5′-GTCTCTTGGAGCAGGCGGATG-3′

cells were centrifuged at 6,000 × g for 30 s and lysed for 30 min on ice in ice-cold lysis buffer [20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40, 20 mM NaF, 20 mM NaVO3, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride [PMSF], 21 μg/ml aprotinin, 5 μg/ml leupeptin, and 4 mM Diisopropyl fluorophosphates [DFP]]. After protein estimation using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), samples were adjusted to a concentration of 2 μg/μL. 16–20 μg/μL of total cell lysates were separated by 12% SDS-PAGE and immunoblotted with antibodies for phospho-ERK1/2, total ERK1/2, phospho-p38 MAPK, total p38 MAPK, phospho-AKT, total AKT, phospho-S6 (Cell Signaling Danvers, MA, USA), p47phox, or p67phox (gift from Dr. William M. Nauseef), all at 1:1,000 dilution. The appropriate secondary antibodies were used at 1:2,000 dilution (Cell Signaling, Danvers, MA, USA). The ECL System (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) or the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA) was used to visualize antigen-antibody reactions. Densitometric values of each band were calculated using Image Lab Software (BioRad, Hercules, CA, USA).

Superoxide Generation and Priming

Superoxide anion release was measured spectrophotometrically at 550 nm as the superoxide dismutase-inhibitable reduction of ferricytochrome c as previously described (35). Briefly, neutrophils (4×10^6cells/ml) were cultured in RPMI supplemented with 5% heat-inactivated human serum and left untreated or pre-treated with p38 inhibitor BIRB-796 (75 nM, added to media 60 min before 6- and 10-h time points; Sigma, St. Louis, MO, USA), or with opsonized *F. alocis* (MOI 10) for 6 and 10 h at 37°C in a shaking water bath. After this first pre-treatment, TNFα (10 ng/ml, 10 min) was added to all the samples. Samples were run in duplicate, with one duplicate used to detect basal superoxide levels in the presence or absence of each pre-treatment and the other duplicate used to measure TNF-priming by further challenge with fMLF (300 nM) for 5 min. After stimulation of superoxide production, the samples were centrifuged for 10 min at 600 × g, 4°C, supernatants were collected, and optical densities were read.

IL-8 Cytokine Measurement and Apoptosis

Neutrophils (10×10^6 cells/mL) were cultured in RPMI + 5% heat-inactivated human serum and left untreated, or pre-treated with TAK1 inhibitor (5Z)-7-Oxozeanol (3 μM, added 30 min before 6- and 10-h time points; Cayman, Ann Arbor, MI, USA) p38 inhibitor BIRB-796 (75 nM, added 60 min before 6- and 10-h time points; Sigma, St. Louis, MO, USA), or opsonized *F. alocis* (MOI 10) for 6 and 10 h at 37°C in an incubator with 5% CO_2. After the pre-treatment, the volume in the tube was divided evenly between two tubes, with tube receiving TNFα (10 ng/ml) and the other nothing. All tubes were returned to the incubator for 4 or 12 h. After the TNFα stimulation period, cells were centrifuged, their supernatants collected, and the pellets tested for apoptosis. 1% protease and phosphatase inhibitors were added to the supernatants to protect them from degradation. IL-8 was measured in the supernatants using a commercially available kit (Invitrogen, Carlsbad, CA, USA). Cells were processed for Annexin V/7-AAD staining using the commercially available APC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA, USA). Samples were read on a BD FACSCelesta flow cytometer and analyzed using the FlowJo software (Ashland, OR, USA).

Statistical Analysis

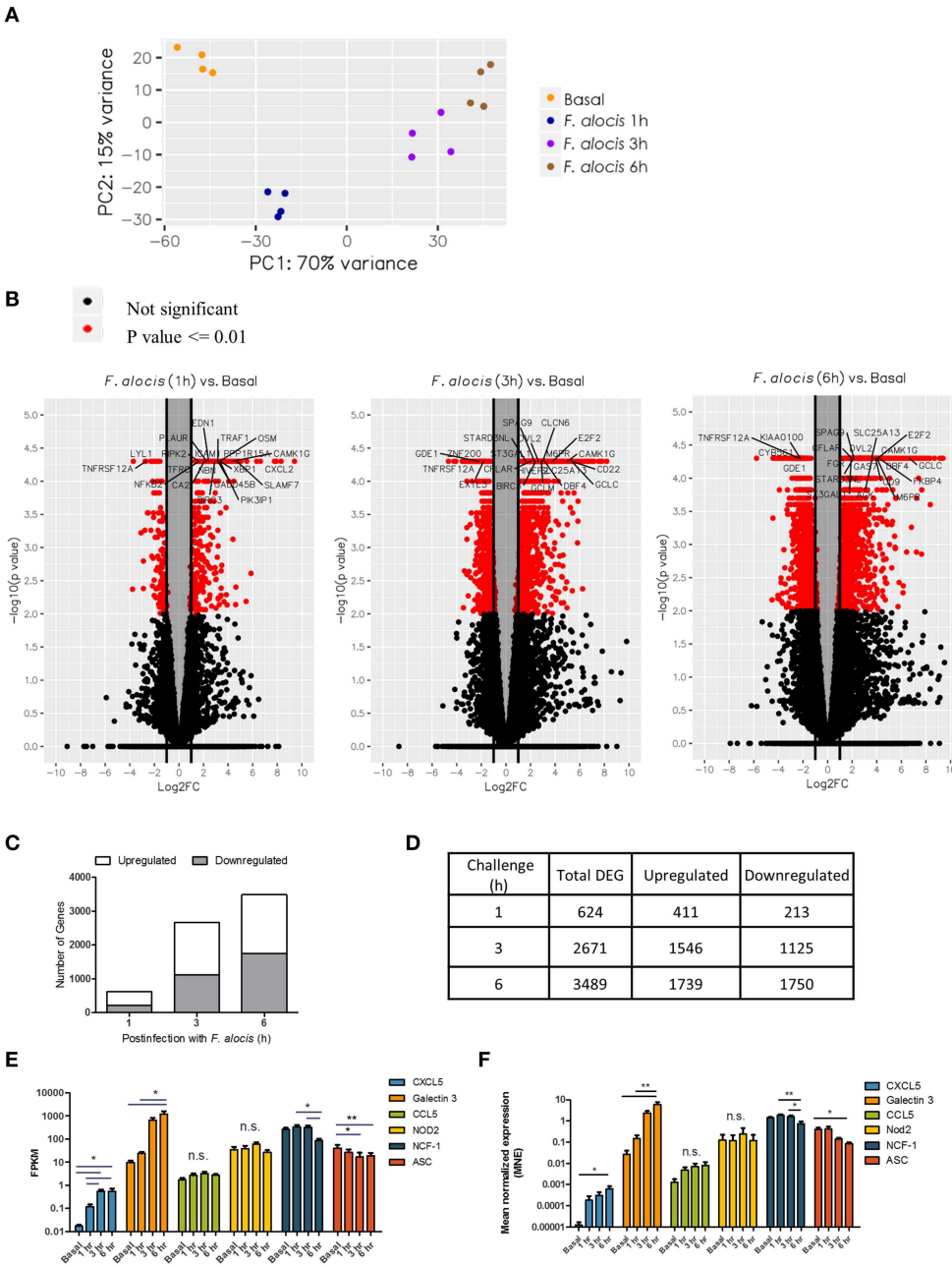
Unless otherwise noted, statistical differences among experimental conditions and time points were analyzed by a repeating measures two-way ANOVA, followed by Bonferroni post-tests using GraphPad Prism Software (Graphpad San Diego, CA, USA). Differences were considered significant at the level $P < 0.05$. When a two-way ANOVA was not applicable, a one-way ANOVA followed by the *post hoc* Tukey's multiple-comparison test was used.

RESULTS

F. alocis Induces Global Changes in Gene Expression

To assess changes in gene expression after *F. alocis* challenge, whole transcriptome by RNA-based next-generation sequencing (RNA-seq) was performed on human neutrophils from four individual healthy donors that were either left unstimulated or challenged with *F. alocis* for 1, 3, or 6 h. All time points for each donor were mapped onto a principal component analysis (PCA) plot to determine the variation in the dataset (**Figure 1A**). All four donors clustered together for each experimental condition, showing that donor variability is not a major contributor in our dataset. Contrastingly, the transcriptional profile of *F. alocis*-stimulated neutrophils clearly separated from basal conditions at each time point. Next, differential expression analysis was completed between each treatment condition using the Tuxedo Suite Program Cuffdiff2, where a p-value cutoff ≤ 0.01 and a log fold change $|\log_2 FC| \geq 1$ was utilized to compile a list of differentially expressed genes (DEG) for further analyses. Volcano plots for each time point showed that *F. alocis* challenge induced a time-dependent change in gene expression, as the number of red-colored dots, which represent the significant DEG, grew at each time point (**Figure 1B**). On the volcano plots, the most significant DEG for each time point was identified. Out of these highlighted DEGs, *CAMK1G* was upregulated and *TNFRSF12A* was downregulated at all three time points compared to unstimulated cells. The *CAMK1G* gene encodes a protein like calcium/calmodulin-dependent protein kinase; however, according to RefSeq, its exact function is unknown. *TNFRSF12A*, also known as Fn14, is a weak inducer of apoptosis that can activate NF-kB signaling pathways, promote oxidative stress, and is linked to high expression of matrix metalloprotease 9 (MMP-9) (42–45).

The number of DEGs was determined for each time point and plotted based on whether they were upregulated or downregulated compared to the basal control (**Figure 1C**). The biggest change in transcriptome occurred early in the time course, with 624 genes differentially expressed at 1 h and a steep



Challenge (h)	Total DEG	Upregulated	Downregulated
1	624	411	213
3	2671	1546	1125
6	3489	1739	1750

FIGURE 1 | Global changes in the transcriptome of *F. alocis*-challenged neutrophils. Illumina RNA sequencing was performed on human neutrophils from four individual healthy donors that were either left unstimulated (basal) or challenged with *F. alocis* for 1, 3, or 6 h. PCA analysis shows the variation between the four donors at each time point **(A)**. The Tuxedo Suite program Cuffdiff2 was used to acquire a list of differentially expressed genes (DEG) that were graphed in volcano plots **(B)**. Genes that had $p \leq 0.01$ and $\log_2$ FC$| \geq 1$ are colored in red, and genes with high fold changes are labeled. For each time point, the average number of upregulated and downregulated differentially expressed genes (DEG) was determined and plotted **(C,D)**. Six genes (two upregulated, two with no change, and two downregulated) were chosen to validate the RNAseq data by quantitative qPCR analysis. From the RNAseq data, the fragments per kilobase of transcript per million mapped reads (FPKM) for these six genes are plotted as mean ± SEM from the four donors **(E)**. From the qPCR analysis, the mean normalized mRNA expression (MNE) from five independent experiments are plotted in **(F)** as MNE ± SEM. One-way ANOVAs were performed on the expression levels from each gene to determine statistical significance between the basal condition and each time point. n.s.: non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

increase in the number of DEGs between 1 and 3 h (**Figure 1D**). At the 6-h time point, the number increased only marginally from 3 h. Throughout the time course, the number of genes induced was roughly the same as the number of repressed genes. While our DEG criteria are more stringent than other studies, these global changes in gene expression appear to be unique to *F. alocis*

as compared to other transcriptome studies between neutrophils and bacterial challenge (31–34). To validate the RNAseq data, two upregulated genes, two downregulated genes, and two genes with no change were selected for validation by quantitative PCR. **Figure 1E** shows the fragments per kilobase of transcript per million mapped reads (FPKM) expression values for all four donors from the RNAseq screen, while **Figure 1F** shows the mean normalized mRNA expression by qPCR. Overall, the qPCR results validate the RNAseq screen and provide confidence about the targets identified by the high throughput screening analysis.

F. alocis Affects Neutrophil Functional and Biochemical Processes

To reduce bias during the bioinformatic analysis, the DEG list was uploaded into two separate databases: Database for Annotation, Visualization and Integrated Discovery (DAVID) (46, 47) and MetaCore by Clarivate Analytics. In each database, we first identified the significant ($p < 0.01$) biological processes during challenge with *F. alocis*. From the DAVID analysis, 37, 74, and 86 processes were identified for the 1-, 3-, and 6-h time points, respectively, and categorized by cell function (Supplemental Table 1). Significant process categories in every time point include biological processes related to the inflammatory response, response to microbes, chemotaxis, signal transduction, gene expression and transcription factor regulation, cytokine-mediated responses and production, and apoptosis. However, as the time course progressed, there was a shift in the affected processes. While cytokine-related processes were most prominent at the earlier time points, biological processes related to phagosome maturation and metabolic processes became significant at the later time points. Moreover, processes related to protein folding only became significant at 6 h post-bacterial challenge.

Using MetaCore, 71 significant ($p < 0.01$) network processes were determined in our data set. Since the MetaCore software automatically categorizes the processes by cell function, we determined the frequency of each category (**Figure 2A**). Like the DAVID analysis, most processes were involved with inflammation, signal transduction, the immune response, and apoptosis. Cell function processes with a lower frequency include protein folding, cytoskeleton, transcription, chemotaxis, and autophagy. Next, we plotted the top 25 most significantly upregulated (**Figure 2B**) and downregulated processes (**Figure 2C**). Processes related to inflammation made up four out of the top five upregulated network processes, but the significance of the inflammatory processes decreased as the time course progressed. In fact, some of these inflammation processes from the upregulated list became significant in the downregulated processes during the later time points, as is the case with processes such as IL-6 signaling and neutrophil activation (Supplemental Table 2). This suggests that *F. alocis* may be dampening inflammatory processes between 1 and 3 h to prolong its survival or provide protection to bystander species. Together, this data shows that *F. alocis* challenge induces temporal changes in neutrophil functional mechanisms like cytokine production, chemotaxis, vesicular trafficking, and degranulation, as well as neutrophil biochemical mechanisms like the regulation of signaling pathways and metabolism. This coincides with previous data from our laboratory that shows that *F. alocis* affects neutrophil cytokine production, chemotaxis, vesicle trafficking, and degranulation functions (21, 22).

Based on previous work that demonstrated that *F. alocis* induces minimal intracellular and extracellular ROS production (21), we looked at whether the components of the NAPDH oxidase complex are affected during *F. alocis* challenge (**Table 2**). From the RNAseq data, the only statistically significant results show that the expression of CYBB (gp91phox subunit) increased in a time-dependent manner while the expression of NCF1 (p47phox subunit) decreased by 6 h of challenge, which was also validated by qPCR (**Figure 1F**). While the minimal ROS activation at the early time points cannot be attributed to modulation of gene expression by *F. alocis*, generation of ROS at later time points may be inhibited by the expression of a member of the galectin family of carbohydrate-binding proteins, galectin-3. The increased expression of galectin-3 has already been associated with inhibition of ROS production when human neutrophils were challenged with *Candida albicans* (48). In our dataset, both the FPKM expression as well as the qPCR validation show a time-dependent increase in galectin-3 mRNA expression (**Figures 1E,F**), and when tested by western blot, *F. alocis* induced a time dependent increase in galectin-3 protein expression (data not shown).

F. alocis Challenge Upregulates Cytokine Pathways and Downregulates Signaling Pathways

Next, we identified pathways relevant to challenge with *F. alocis*. Using DAVID, the DEG list was mapped onto predefined pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We limited our analysis to highly significant pathways with a $p < 0.01$, which resulted in 10, 26, and 33 pathways for the 1-, 3-, and 6-h time points, respectively (**Table 3**). The *F. alocis*-neutrophil transcriptome reinforced the pathogenic potential of *F. alocis* by the number of significant pathways linked to pathogens that subvert immune cells (*Salmonella*, *Legionella*, *Helicobacter pylori*, and Influenza A). Similarly, pathways for cancers, rheumatoid arthritis, and inflammatory bowel disease were significant for the *F. alocis*-challenged neutrophil transcriptome in both databases. Oral bacteria continue to be linked to systemic malignancies like those listed above (49), and although *F. alocis* has not been amongst the oral pathogens detected yet, these results hint that it could play a role in the pathogenesis of these diseases.

Two major bacterial recognition receptor signaling pathways, NOD-like receptor and Toll-like receptor signaling, were identified in our data set. These receptor pathways align with published data on *F. alocis*-induced cytokine production, where NOD1 is activated during challenge with heat-killed *F. alocis* to produce IL-6 in monocytes (50), and TLR2/6 activation of neutrophils leads to the production and release of cytokines and chemokines (22). Using Metacore, we divided the significant pathways into the top 20 upregulated (**Figure 3A**)

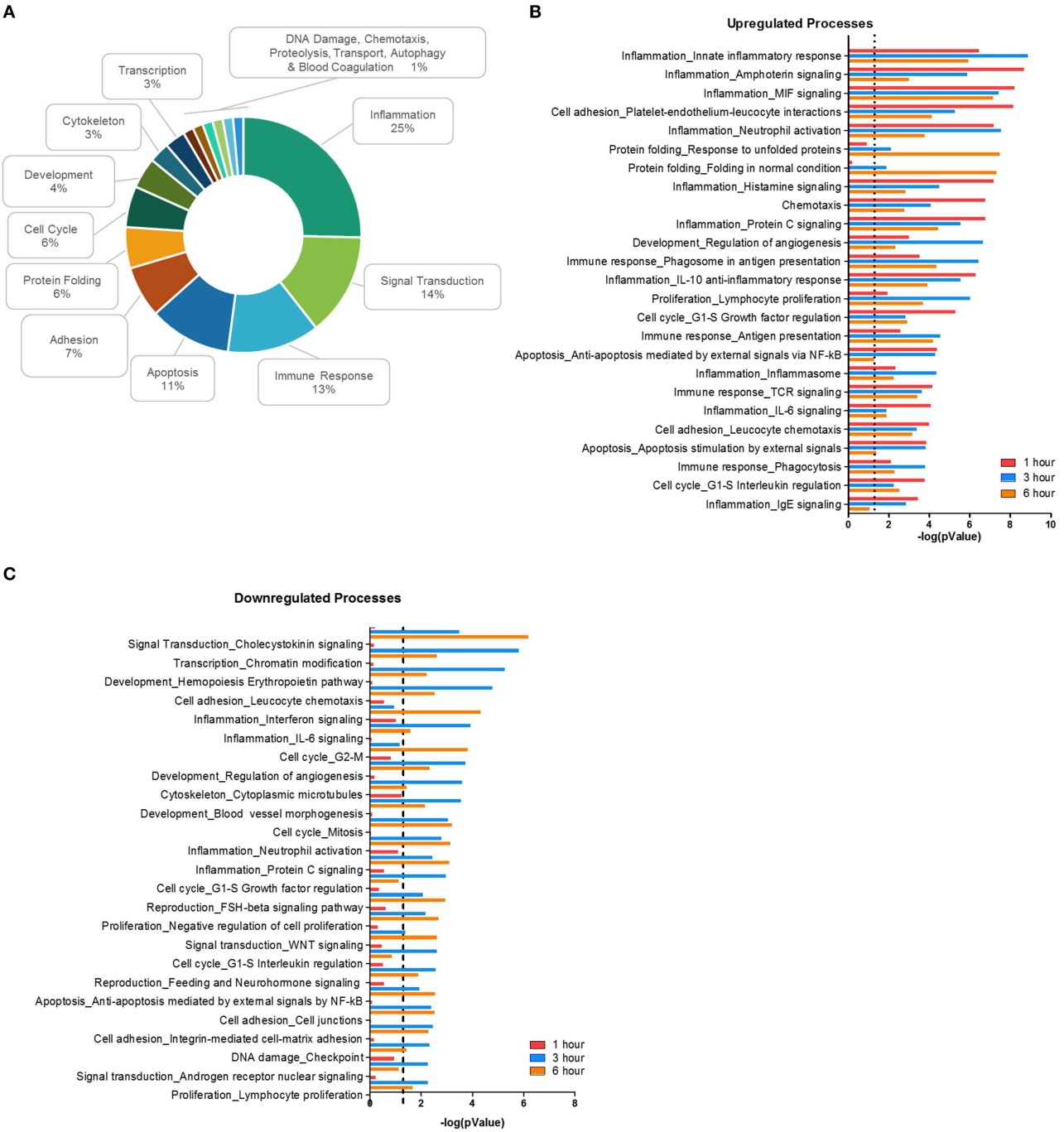


FIGURE 2 | Biological processes affected during *F. alocis* challenge. The list of differentially expressed genes (DEG) for each time point was input to the proprietary, manually curated MetaCore database and analyzed for biological processes that contained a significant number of DEG. MetaCore process networks are categorized by cell functions, so the frequency of each cell function was tallied for all the 71 significant ($p < 0.01$) processes. Percentages for each function are displayed as a donut graph **(A)**. The top 25 upregulated **(B)** or downregulated **(C)** network processes were then graphed according to the –log of the *p* value for each time point. The dotted line represents a *p* value of 0.01.

and downregulated (**Figure 3B**) plots. The list of upregulated pathways supports published data that shows initial contact with *F. alocis* results in the early transcription and production of cytokines (22). At 1 h, cytokine-related pathways such as cytokine-cytokine receptor interaction, TNF signaling pathway, and chemokine signaling pathway were also the most prominent pathways by KEGG analysis (**Table 3**). In both databases, the NFκB signaling pathway was significant and upregulated,

TABLE 2 | Fold change of Components of NADPH Oxidase Complex compared to basal.

Ensembl Gene	Gene Symbol|Description	1 h	3 h	6 h
ENSG00000165168	CYBB|cytochrome b-245, beta polypeptide	1.4	3.04*	3.09*
ENSG00000051523	CYBA|cytochrome b-245, alpha polypeptide	0.9	1.4	1.1
ENSG00000158517	NCF1|neutrophil cytosolic factor 1	1.3	1.2	0.32*
ENSG00000116701	NCF2|neutrophil cytosolic factor 2	1.0	1.5	1.4
ENSG00000100365	NCF4|neutrophil cytosolic factor 4, 40kDa	1.0	0.8	1.1
ENSG00000128340	RAC2|ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	0.9	1.4	1.3
ENSG00000116473	RAP1A|RAP1A, member of RAS oncogene family	1.1	0.8	0.9

Asterisk denotes $p < 0.05$ compared to basal unstimulated conditions.

suggesting this transcription factor is likely responsible for the cytokine and chemokine transcriptome response.

While many of the upregulated pathways were related to inflammation and cytokine responses, the downregulated list was largely comprised of signal transduction pathways (**Figure 3B**). The pathways were significantly affected at the later time points and include signal transduction by MAPK, GPCR, Rho GTPases, PI3K, PTEN, AKT, and PKA. Out of the list of 20 downregulated pathways, seven relate to MAPK signaling. To further support this analysis, under the signal transduction biological process category, positive regulation of ERK1 and ERK2 cascade and activation of MAPK activity are significant at 1 h, but inactivation of MAPK activity becomes significant at 3 and 6 h (**Table 3**). We focused on this pathway and determined if *F. alocis* is modulating MAPK signaling in human neutrophils.

F. alocis Challenge Does Not Affect fMLF-Stimulated MAPK Signaling

Since G-protein coupled receptor (GPCR) and MAPK were both hits in our dataset, western blots to evaluate ERK1/2 and p38 MAPK activation were performed on lysates from human neutrophils pretreated with media or media containing *F. alocis* for 1, 3, 6, and 10 h followed by stimulation with the bacterial peptide N-Formylmethionine-leucyl-phenylalanine (fMLF) (**Figure 4A**). Densitometry analysis of the western blots bands for phosphorylated and total ERK1/2 showed that stimulation with *F. alocis* alone has a time-dependent increase in phosphorylation of ERK1/2 (**Figure 4B**). This suggests there is a bimodal response in the activation of ERK1/2 since it was previously published that ERK phosphorylation peaks at 15 min and then decreases (20). In the case of p38 MAPK, the levels of phosphorylated p38 MAPK are also increased in the *F. alocis* pre-treated cells as compared to neutrophils cultured in media alone (**Figure 4C**). However, the levels remain steadily elevated throughout the time course. This pattern of phosphorylation for the MAPK is also observed, although at different magnitudes, when neutrophils are pre-treated with heat-killed *F. alocis* (**Figures 4D,E**) and the TLR2/6 agonist FSL1 (**Figures 4F,G**). Despite the increased basal levels of phosphorylated ERK1/2 and p38 MAPK in the viable and heat-killed *F. alocis* pretreated cells, when the neutrophils are stimulated with fMLF after pre-treatment with *F. alocis,* the phosphorylation of both ERK and p38 MAPK is comparable to that of cells cultured in media alone (**Figures 4B,C**). Contrastingly, cells pretreated with FSL1 showed increased phosphorylation of ERK1/2 when stimulated with fMLF, which became significant at 10 h compared to media-cultured cells stimulated with fMLF alone (**Figure 4F**). A similar trend was observed at the 10-h time point with phosphorylation of p38 MAPK, but the data did not reach statistical significance when compared to fMLF alone (**Figure 4G**).

F. alocis Challenge Dampens TNF-α-Stimulated MAPK Signaling

From the DAVID analysis, one of the KEGG pathways that was significantly modulated by *F. alocis* at each time point was the TNF signaling pathway (**Table 3**). Similarly, four of the upregulated pathways and one of the downregulated pathways from the MetaCore analysis involve TNF signaling (**Figures 3A,B**). Since it is well-documented that stimuli like LPS and TNFα can activate the p38 and MEK/ERK pathways in neutrophils (25, 51, 52) and high levels of TNFα are present in periodontitis active sites (53, 54), we tested the effect of *F. alocis* pre-treatment on TNFα-induced MAPK signaling cascade. Whole cell lysates from neutrophils pre-treated with media or *F. alocis* followed by stimulation with TNFα were immunoblotted for phosphorylated and total ERK1/2 and p38 MAPK (**Figure 5A**). Densitometry analysis of the ERK immunoblots showed that pre-treatment with *F. alocis* did not impact TNFα-driven phosphorylation of ERK1/2 (**Figure 5B**). However, the TNFα-driven phosphorylation of p38 MAPK was significantly dampened in neutrophils pre-treated with *F. alocis* for 6 and 10 h as compared to neutrophils cultured in media alone (**Figure 5C**). This effect is dependent on the bacteria being viable, because when the neutrophils were pre-treated with heat-killed *F. alocis* before stimulation with TNFα, there was no decrease in the p38 phosphorylation at 6 or 10 h (**Figure 5D**). Additionally, ligation of TLR2/6 is insufficient to elicit the phenotype observed (**Figure 5E**). This data shows that viable *F. alocis* modulates TNFα-induced activation of the MAPK signaling pathway by selectively interfering with the phosphorylation of p38 MAPK, but not ERK1/2.

In human neutrophils, activation of p38 MAPK by TNFα results in the downstream phosphorylation and activation of AKT (55). Therefore, since the TNFα-induced activation of p38 MAPK was affected when neutrophils were pre-treated with *F. alocis*, the activation of AKT should also be compromised. To test this hypothesis, the lysates from media and *F. alocis* pre-treated neutrophils stimulated with TNFα were immunoblotted for phosphorylated and total AKT (**Figure 6A**). Densitometry analysis of the Western blots demonstrated that TNFα-mediated phosphorylation of AKT was also dampened in *F. alocis*-treated

TABLE 3 | KEGG pathways significantly enriched for differentially expressed genes during challenge with *F. alocis*.

Time point	DEG count	Description	*P*-value
1 h	19	Cytokine-cytokine receptor interaction	5.40E-09
	13	TNF signaling pathway	2.50E-08
	10	Rheumatoid arthritis	3.50E-06
	9	NF-kappa B signaling pathway	2.70E-05
	8	Salmonella infection	1.50E-04
	6	Legionellosis	9.20E-04
	6	NOD-like receptor signaling pathway	1.10E-03
	9	Transcriptional mis-regulation in cancer	2.40E-03
	9	Chemokine signaling pathway	4.60E-03
	10	MAPK signaling pathway	9.10E-03
3 h	26	Rheumatoid arthritis	2.30E-08
	19	Legionellosis	1.50E-07
	29	Lysosome	4.10E-07
	26	TNF signaling pathway	1.40E-06
	18	NOD-like receptor signaling pathway	1.50E-06
	22	NF-kappa B signaling pathway	5.70E-06
	40	Cytokine-cytokine receptor interaction	4.00E-05
	17	Epithelial cell signaling in Helicobacter pylori infection	8.50E-05
	19	Salmonella infection	1.20E-04
	22	Toll-like receptor signaling pathway	1.30E-04
	30	Chemokine signaling pathway	6.40E-04
	20	Chagas disease (American trypanosomiasis)	8.00E-04
	13	Vibrio cholerae infection	9.00E-04
	28	Influenza A	1.00E-03
	23	Measles	1.30E-03
	34	Endocytosis	2.70E-03
	24	Phagosome	2.80E-03
	13	Apoptosis	4.40E-03
	20	Epstein-Barr virus infection	5.30E-03
	13	Inflammatory bowel disease (IBD)	5.70E-03
	17	Inflammatory mediator regulation of TRP channels	6.40E-03
	26	Herpes simplex infection	8.70E-03
	10	Sphingolipid metabolism	0.014
	12	Pancreatic cancer	0.017
	32	HTLV-I infection	0.018
	27	Viral carcinogenesis	0.019
6 h	22	Legionellosis	2.70E-07
	46	Chemokine signaling pathway	1.20E-06
	20	Apoptosis	1.80E-05
	30	Lysosome	1.20E-04
	32	Measles	1.20E-04
	37	Influenza A	4.40E-04
	24	Estrogen signaling pathway	9.10E-04
	22	Rheumatoid arthritis	1.00E-03
	40	Viral carcinogenesis	1.40E-03
	16	NOD-like receptor signaling pathway	1.60E-03
	45	Endocytosis	1.80E-03
	29	Insulin signaling pathway	2.50E-03
	24	TNF signaling pathway	2.70E-03
	26	Sphingolipid signaling pathway	2.90E-03
	8	Other glycan degradation	3.00E-03
	26	Epstein-Barr virus infection	3.60E-03
	17	Epithelial cell signaling in Helicobacter pylori infection	4.00E-03
	24	Toxoplasmosis	4.00E-03
	23	Chagas disease (American trypanosomiasis)	4.10E-03
	23	Toll-like receptor signaling pathway	5.20E-03
	13	Sphingolipid metabolism	6.80E-03
	16	Pancreatic cancer	7.30E-03
	33	Tuberculosis	8.20E-03
	21	Phosphatidylinositol signaling system	9.10E-03
	21	Inflammatory mediator regulation of TRP channels	9.10E-03
	14	Non-small cell lung cancer	0.011
	17	Pertussis	0.012
	19	Prostate cancer	0.013
	13	Vibrio cholerae infection	0.015
	25	Platelet activation	0.016
	16	Leishmaniasis	0.016
	27	Hepatitis B	0.018
	11	Bladder cancer	0.018

cells as compared to media-cultured neutrophils (**Figure 6B**). The reduced AKT phosphorylation followed the timing of the decreased p38 MAPK phosphorylation, with the phenotype reaching statistical significance only at 6 and 10 h. Like the p38 MAPK phenotype, the lowered AKT activation was dependent on interaction with viable *F. alocis* (**Figure 6C**) and was not mediated solely through ligation of the TLR2/6 receptor (**Figure 6D**). Also downstream of p38 MAPK phosphorylation is the activation of translation machinery such as the S6 ribosomal protein (25). Thus, the phosphorylation of S6 was tested in whole cell lysates from neutrophils pre-treated with *F. alocis* prior to TNFα stimulation (**Supplemental Figure 1**). Densitometry analysis showed that the activation of S6 in response to TNFα was significantly dampened in neutrophils pre-treated with *F. alocis* for 6 h in comparison to media treated controls. While this trend continued in the 10-h pre-treatment condition, it did not reach statistical significance. Together, these results demonstrate that *F. alocis* actively modulates the TNFα signaling pathway by dampening the activation of p38 MAPK and its downstream effectors, AKT and S6 ribosomal protein.

Functional Effects of *F. alocis'* Inhibition of TNFα-Mediated p38 Phosphorylation

TNFα stimulation can prime the ROS response of neutrophils, extend their lifespan, and induce cytokine production [reviewed

FIGURE 3 | Pathways affected during *F. alocis* challenge. Using the MetaCore database, we determined the top 20 upregulated **(A)** or downregulated **(B)** pathways in the transcriptome of *F. alocis*-challenged neutrophils. These pathways are graphed according to the $-\log_{10}$ of the *p*-value for each time point. The dotted line represents a *p*-value of 0.01.

in (56)]. To determine if the interference of TNFα signaling resulted in any phenotypic changes, these three TNFα-mediated functional responses were tested on cells cultured with *F. alocis* for 6 and 10 h prior to TNFα stimulation. The RNA seq screen showed that the mRNA levels of some NADPH oxidase components were affected; thus, before testing the

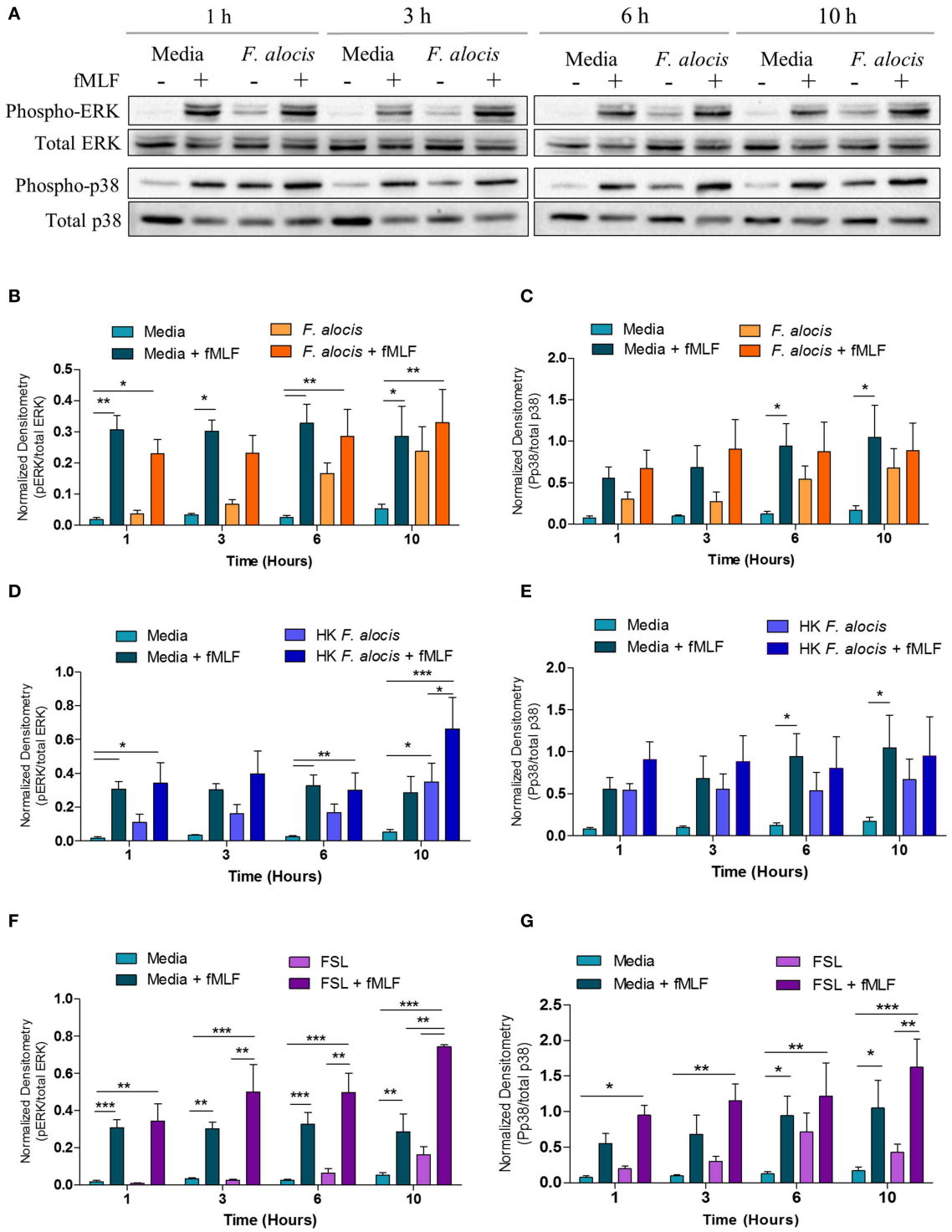


FIGURE 4 | *F. alocis* effect on fMLF-induced MAPK activation. To assess whether *F. alocis* is interfering with MAPK signaling, human neutrophils were cultured in media with or without *F. alocis* at an MOI of 10 for 1, 3, 6, or 10 h followed by stimulation with fMLF for 1 min. Western blots of whole cell lysates were probed for phosphorylated and total p38 and ERK1/2 **(A)** and quantified by densitometry **(B,C)**. Alternatively, neutrophils were cultured in media alone, media with heat-killed *F. alocis* **(D,E)** or the TLR2/6 agonist FSL **(F,G)** for 1, 3, 6, or 10 h before fMLF stimulation. Densitometries are plotted as the mean ± SEM from 6 **(B,C)** and 4 **(D–G)** independent experiments. Statistical differences among experimental conditions and time points were analyzed by a repeating measures two-way ANOVA, followed by Bonferroni post-tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

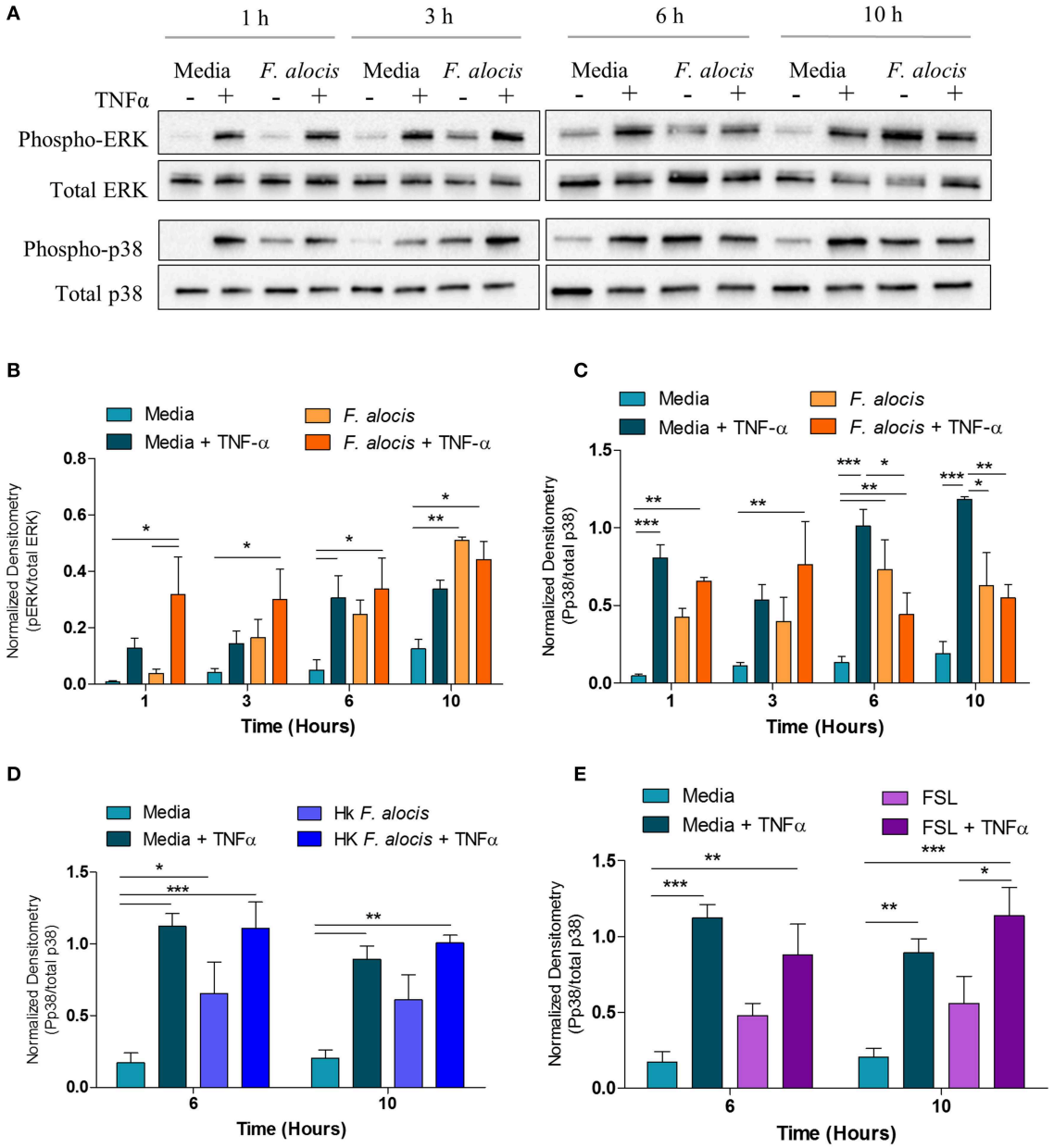


FIGURE 5 | *F. alocis* effect on TNFα-induced MAPK activation. Human neutrophils were cultured in media with or without *F. alocis* at an MOI of 10 for 1, 3, 6, or 10 h followed by stimulation with TNF-α for 15 min. Western blots with whole cell lysates were probed for phosphorylated and total p38 and ERK1/2 **(A)** and quantified by densitometry **(B,C)**. To determine if the decreased p38 phosphorylation at 6 and 10 h was specific to the viable bacteria or a consequence of TLR 2/6 ligation, neutrophils were cultured in media alone, media with heat-killed *F. alocis* **(D)** or the TLR2/6 agonist FSL **(E)** for 6 or 10 h before TNF-α stimulation. Western blots of whole cell lysates were probed for phosphorylated and total p38. Densitometries are plotted as the mean ± SEM from three independent experiments. Statistical differences among experimental conditions and time points were analyzed by a repeating measures two-way ANOVA, followed by Bonferroni post-tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ROS priming response, the protein expression of two of the subunits p47phox (NCF1) and p67phox (NCF2) was determined (**Supplemental Figure 2**). The RNAseq screen showed the gene expression of p67phox was unchanged when the cells were challenged with *F. alocis*, but the gene expression of p47phox was significantly decreased in *F. alocis*-treated cells (**Table 3**). At the protein level, there was no significant difference between media cultured neutrophils and those exposed to *F. alocis* at any time point tested for either p47phox or p67 (**Supplemental Figure 2**). Stimulation with TNFα also had no effect on either subunit's protein expression in media-cultured neutrophils or those exposed to *F. alocis,* demonstrating that any changes observed in the ROS priming response could not be due to differences in the availability of NADPH oxidase components. The basal

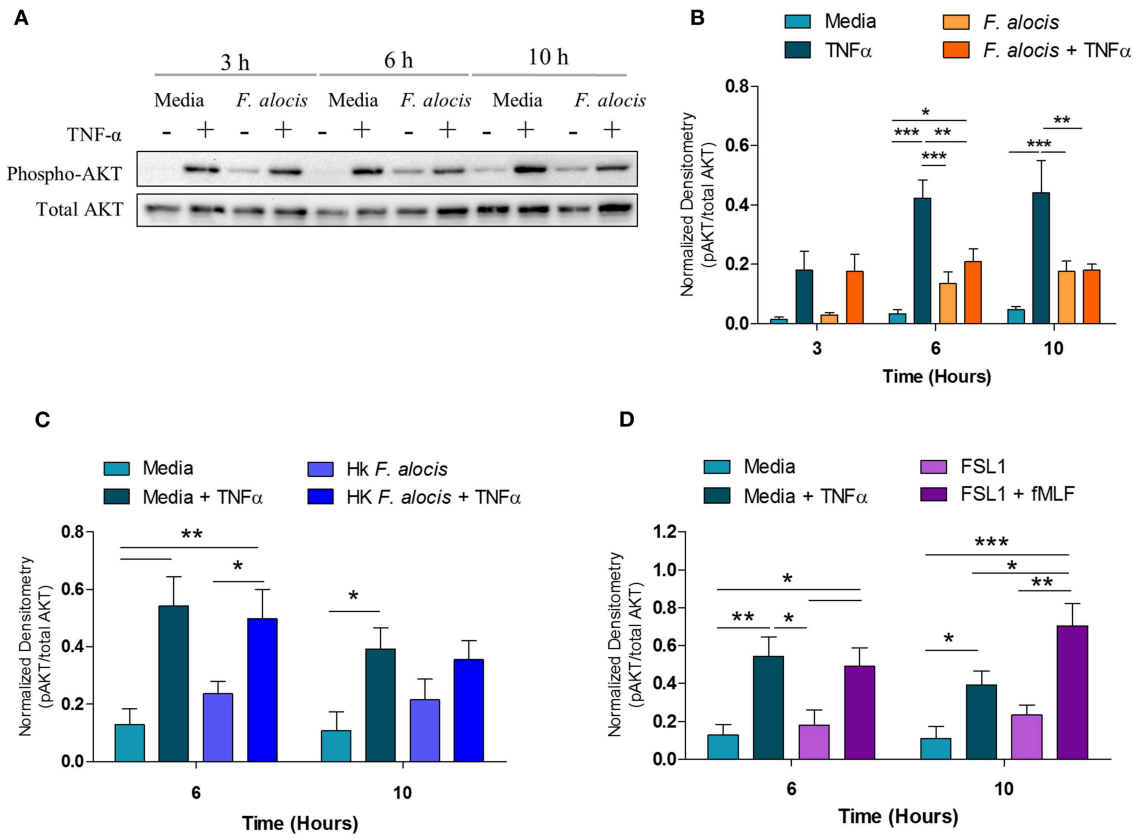


FIGURE 6 | *F. alocis* effect on TNFα-induced AKT activation. Whole cell lysates from human neutrophils pre-treated with media alone or *F. alocis* (MOI 10) for 3, 6, or 10 h followed by TNF-α stimulation were probed for phosphorylated and total AKT **(A)** and quantified by densitometry **(B)**. Similarly, neutrophils were cultured in media alone, media with heat-killed *F. alocis* **(C)** or the TLR2/6 agonist FSL **(D)** for 6 or 10 h before TNF-α stimulation. Densitometries are plotted as the mean ± SEM from 3 **(B)** and 4 **(C,D)** independent experiments. Statistical differences among experimental conditions and time points were analyzed by a repeating measures two-way ANOVA, followed by Bonferroni post-tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

extracellular superoxide production was similar in cells cultured in media or in media with p38 inhibitor BIRB-796 or *F. alocis* for 1, 6, and 10 h (**Figure 7A**). However, when BIRB-796 and *F. alocis* pre-treated cells were primed with TNFα followed by stimulation with fMLF, the superoxide production was comparable to that of neutrophils cultured in media prior to the TNFα priming. Thus, we conclude that inhibiting p38 activation through a chemical inhibitor or *F. alocis* does not affect the ability of TNFα to prime neutrophils' ROS response.

In human neutrophils, TNFα stimulation activates MAPK kinase kinase, TAK1 (also known as MAP3K7), which leads to the downstream phosphorylation of ERK1/2 to delay apoptosis and the phosphorylation of p38 to induce cytokine production [(25, 55), **Supplemental Figure 5**]. First, the effect of *F. alocis*-impaired p38 activation was tested on apoptosis. Neutrophils were cultured in media or media with a TAK1 inhibitor (5Z)-7-Oxozeanol (30 min), a p38 inhibitor BIRB-796 (60 min), or *F. alocis* for 6 h, followed by ± TNFα stimulation for 12 h (**Figure 7B**, **Supplemental Figure 3**). Based on Annexin V and 7-AAD staining, TNFα stimulation of cells cultured in media was able to decrease the number of apoptotic cells compared to cells left in media alone. When TAK1 was inhibited by (5Z)-7-Oxozeanol, neutrophils became apoptotic, especially when treated with TNFα. Neutrophils pre-treated with BIRB-796 behaved similarly to media-cultured neutrophils, where TNFα stimulation is pro-survival because inhibition of p38 does not affect the TNFα-ERK1/2 mediated delay in apoptosis. Interestingly, pre-treatment of neutrophils with *F. alocis* alone resulted in a decrease in apoptotic cells, which was not reduced further with TNFα stimulation. Apoptosis was also assessed in cells pre-treated with *F. alocis* for 10 h prior to the 12-h stimulation with TNFα, and results matched the 6-h pre-treatment (**Supplemental Figure 4**). Together, this data reinforces the finding that only ERK1/2 signaling is important in TNFα-induced neutrophil survival and that *F. alocis* is selectively inhibiting p38 MAPK.

TNFα stimulation can also induce the production of cytokines and chemokines such as interleukin (IL)-8. Thus, the release of IL-8 was tested in the supernatants of cells cultured with media, (5Z)-7-Oxozeanol, BIRB-796, or *F. alocis* for 6 h followed by +/- TNFα stimulation for 12 h (**Figure 7C**). As expected, TNFα stimulation of media-cultured neutrophils induced significant release of IL-8. Culturing the neutrophils with the TAK1 and p38 inhibitors alone did not induce IL-8 production; however,

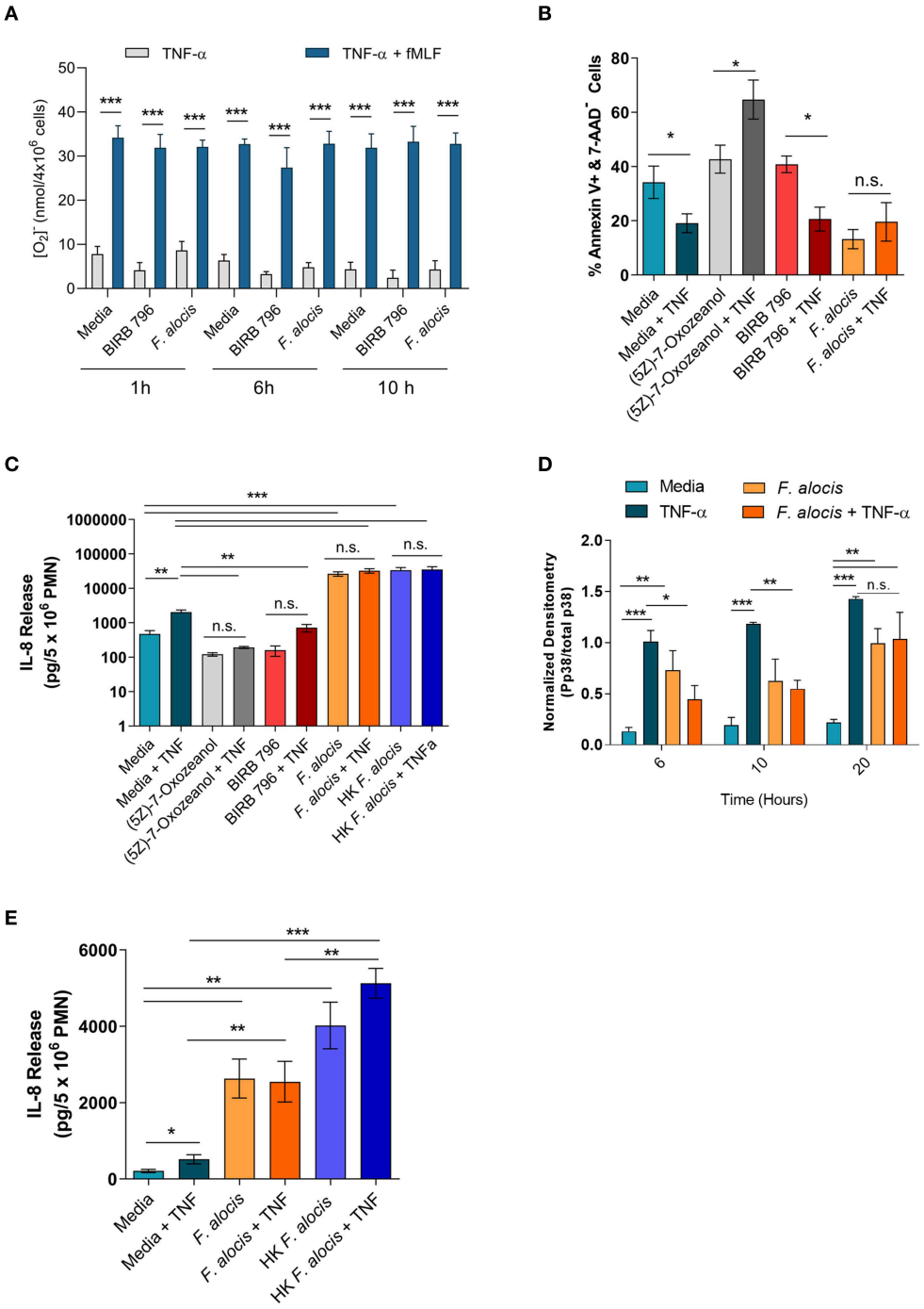


FIGURE 7 | *F. alocis* effect on TNFα-induced functional responses. The ability of TNFα to prime media, BIRB 796 or *F. alocis* pre-treated neutrophils was assessed as the production of superoxide release **(A)**. Cells were cultured in media, pre-treated with *F. alocis* for 1, 6, and 10 h, or the p38 inhibitor BIRB 796 (60 min) followed by TNFα alone or TNFα + fMLF. Results from three independent experiments are shown as the mean ± SEM superoxide production from basal TNFα stimulation or from cells primed with TNFα followed by fMLF stimulation. To test apoptosis, neutrophils were cultured in media or pre-treated with the TAK1 inhibitor (5Z)-7-Oxozeanol (30 min), BIRB-796 (60 min), or *F. alocis* for 6 h followed by a 12-h TNFα stimulation **(B)**. The apoptosis data are plotted as the mean ± SEM percent of early apoptotic cells (Q3: Annexin V+, 7-AAD-) from three independent experiments. IL-8 production was also tested in neutrophils described above as well as neutrophils pre-treated with heat-killed *F. alocis* for 6 h prior to a 12-h stimulation with TNFα **(C)**. The cytokine data are graphed as the mean ± SEM IL-8 release from five independent experiments. The duration of *F. alocis*' inhibitory effect on TNFα-mediated p38 phosphorylation was tested at 6, 10, and 20 h **(D)**. Normalized western blot densitometries are summarized from three independent experiments. Finally, IL-8 production was also tested in neutrophils cultured in media or pre-treated with live or heat-killed *F. alocis* for 6 h prior to a 4-h stimulation with TNFα **(E)**. Data is graphed as the mean ± SEM IL-8 release from four independent experiments. N.s, no significance, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TAK1 and p38 inhibition reduced the release of IL-8 by TNFα stimulation. Contrastingly, the *F. alocis* pre-treatment alone caused robust release of IL-8, which significantly surpassed the IL-8 release of TNFα-activated, media-cultured cells. Despite the potent IL-8 production by *F. alocis* alone, the further stimulation of *F. alocis* pre-treated cells with TNFα did not cause significant, additional release of IL-8. To rule out the possibility that *F. alocis* treatment alone exhausted the neutrophils' ability to produce IL-8, we also tested neutrophil IL-8 production after pre-treatment with heat-killed *F. alocis*, which show normal p38 activation in response to TNFα stimulation (**Figure 5D**). Similar to viable bacteria, the heat-killed bacteria-induced significant IL-8 production from neutrophils, and this production was not significantly enhanced with stimulation by TNFα (**Figure 7C**). This phenotype was also observed when neutrophils were pre-treated for 10 h with viable or heat-killed *F. alocis* prior to TNFα challenge for 12 h (**Supplemental Figure 4**), suggesting that despite a defect in TNFα-mediated p38 phosphorylation by the viable bacterium, alternative pathways are activated by *F. alocis* that result in maximal IL-8 production from neutrophils in the time points tested. This observation raised the possibility that the inhibition of p38 phosphorylation by *F. alocis* subsides during the 12-h TNFα stimulation period. Therefore, we tested the phosphorylation of p38 in neutrophils pre-treated with media or *F. alocis* for 6, 10 and 20 h prior to TNFα stimulation for 15 min. The inhibition of p38 phosphorylation by *F. alocis* had dissipated by 20 h, suggesting the MAPK dampening by *F. alocis* is a transient effect (**Figure 7D**). Finally, to determine if IL-8 production is affected during the period of infection where p38 phosphorylation is dampened, we shortened the TNFα stimulation to 4 h (**Figure 7E**). Despite the shorter stimulation period, TNFα still caused significant release of IL-8 from media cultured cells. Both viable and heat-killed *F. alocis* induced significant IL-8 production on their own, but their responses diverged after TNFα stimulation. While heat-killed *F. alocis* pre-treated cells produced a greater amount of IL-8 upon addition of TNFα, viable *F. alocis*-treated cells were incapable of generating more IL-8. Combined, this data demonstrates that viable *F. alocis* blocks TNFα-mediated p38 activation to reduce transiently the production of pro-inflammatory cytokines, but this interference has no effect on other TNFα induced effector functions like ROS priming or pro-survival response.

DISCUSSION

As executioners of the innate immune response, neutrophils are recruited to the gingival tissue to provide the host with protection against infection. However, in active periodontal disease sites, the interaction between neutrophils and the dysbiotic microbial community results in dysregulated inflammation, which is detrimental to the host. Composition analysis of the dysbiotic microbial community identified high concentrations of emerging periodontal pathogens such as *F. alocis*. We recently demonstrated that *F. alocis* survives within human neutrophils by inducing minimal ROS production and blocking granule recruitment to the bacteria-containing phagosome (21). Despite *F. alocis* causing significant changes in the mRNA expression of different neutrophil-derived cytokines and chemokines, lower levels of these inflammatory mediators are released when compared to the response elicited by other oral pathogens (22). Therefore, a systems biology-level approach was used to define global changes in human neutrophil transcriptome modulated by *F. alocis*. This unbiased approach provides insights into how this emerging oral pathogen might undermine the innate immune system and contribute to disease progression. Our results show that among the 71 significant biological processes modulated by *F. alocis,* the highest percent were related to inflammation, signal transduction, immune response, and apoptosis. Furthermore, the KEGG pathway analysis revealed that uptake of *F. alocis* significantly downregulated the expression of genes associated with signal transduction pathways, primarily the MAPK cascade and the TNFα signaling pathways. To the best of our knowledge, our results are the first to show that TNF-α-induced p38 MAPK activation is significantly impaired in *F. alocis*-challenged neutrophils.

Research studies from the last 20 years have corrected the misconception that neutrophils were unable to induce changes in gene expression because they are short-lived, differentially terminated cells with a densely condensed nucleus (57–60). Microarray-based approaches show that significant changes in neutrophil gene expression take place after 3–6 h following microbial uptake (59). Likewise, our results show a significant increase in neutrophil DEGs from 624 genes up to 2671 following 1 and 3 h of *F. alocis* challenge, respectively (**Figures 1C,D**). By 6 h post bacterial challenge, the number of DEGs continued to rise up to 3,489 with a similar number of upregulated (1,739) and downregulated (1,750) genes (**Figures 1C,D**). Interestingly, the transcriptome studies performed thus far on neutrophils following bacterial interactions reveal common as well as pathogen-specific transcriptional profiles, providing novel information about the potential pathogenic persona of the microorganism studied (60). For example, *Anaplasma phagocytophilum* induces minimal ROS production by neutrophils and a microarray study following 1.5 up to 24 h post-infection shows that the inability to mount the response is not due to modulation of the genes encoding for the different components of the NADPH oxidase (32). We recently showed that *F. alocis* is phagocytized by human neutrophils but induces minimal ROS production (21). In contrast to the transcriptional neutrophil profile elicited by *A. phagocytophilum*, our RNAseq and qPCR results indicate a significant downregulation of the gene that encodes for one of the cytosolic components of the NADPH oxidase, p47phox, and a significant time-dependent increase in the gene expression of galectin 3 (**Figures 1E,F** and **Table 3**). P47phox, together with p67phox and p40phox, form the triad cytoplasmic complex, in a 1:1:1 stoichiometric ratio, which is essential for NADPH oxidase activation (61). Although the minimal ROS induced by *F. alocis*-challenged neutrophils was monitored between 1 and 90 min and the significant decrease in p47phox gene expression was observed at 6 h post-infection, these pathogen-induced changes in the transcript levels could leave neutrophils defective in mounting an appropriate respiratory burst response. Furthermore, cytosolic

galectin-3 acts as a negative regulator of ROS production in both human and mouse neutrophils by modulating complement receptor 3 signaling pathway during *C. albicans* infections (48). Our results show a time-dependent increase in galectin-3 gene expression which could be one of the strategies used by *F. alocis* to inhibit ROS production. The mechanisms by which *F. alocis* modulates ROS production, both during early and late time points of infection, is an area under investigation in our laboratory.

To mount an efficacious antimicrobial response inside the neutrophil phagosome, the synergy between an optimal activation of the NADPH oxidase and the fusion of the different neutrophil granule subtypes with the bacteria-containing phagosome is essential (61). Microbial pathogens manipulate either one or both of these antimicrobial processes to evade neutrophil killing (62). The expression of CEACAM3-binding opacity (Opa) proteins on *Neisseria gonorrhoeae* renders the organism susceptible to neutrophil killing. The ability of *N. gonorrhoeae* to switch off the expression of Opa proteins, by phase-variation, prevents azurophilic granule fusion to the phagosome, thus promoting bacterial survival (63). During the phagocytic cup formation, effector proteins secreted by *Yersinia pseudotuberculosis* prevent fusion of specific granules to the forming phagosome in human neutrophils (64). Our transcriptome results show that *F. alocis* challenge significantly downregulated neutrophil processes and signaling pathways involved in the regulation of vesicle-mediated transport, neutrophil degranulation, and cellular pathways involved in transport (**Figures 2C**, **3B**, and Supplemental Table 1). This significant downregulation of vesicular trafficking and phagosome maturation transcripts is consistent with our previous results that *F. alocis* inhibits specific and azurophilic granule recruitment to the bacteria-containing phagosome (21). The mechanisms induced by *F. alocis* to modulate neutrophil vesicular trafficking to the phagosome is an area of active investigation in our laboratories.

Neutrophils isolated from periodontitis patients and from healthy controls were transcriptionally active following 3 h challenge with *Fusobacterium nucleatum,* which is found in high numbers in the subgingival plaque from periodontitis patients (33). In this microarray study, *F. nucleatum* induced significant upregulation of genes encoding pro-inflammatory cytokines and chemokines, and it has been shown that this organism induces the release of high levels of these inflammatory mediators from neutrophils (65). In our study we looked at the transcriptional response at 1, 3, and 6 h while the microarray study with *F. nucleatum* was performed only at 3 h. However, our results show that at 1 h post *F. alocis* challenge there is a significant increase in genes involved in pro-inflammatory cytokines such as IL-1β and IL-6, but those same signaling pathways were significantly downregulated by 3 and 6 h (**Figures 3A,B**). We recently showed that *F. alocis* induces an early increase in both gene expression and protein release of pro-inflammatory mediators such as IL-1β, TNFα, CXCL1, CXCL8, CCL1, CCL2, and CCL3, but the levels released by human neutrophils are significantly lower compared to the response elicited by *P. gingivalis* and *P. stomatis* (22). These results suggested that *F. alocis* might modulate the protein expression and/or release of these inflammatory mediators by neutrophils, and our current results confirm this hypothesis since *F. alocis* pre-treatment limited the release of IL-8 from TNFα stimulation.

An interesting observation from the RNAseq analysis was that *F. alocis* modulates MAPK signaling pathways. Activation of the different MAPK pathways plays a pivotal role in several inflammatory and antimicrobial functional responses both in macrophages and in neutrophils (29). Pathogenic organisms have evolved different strategies to modulate MAPK activation by releasing bacterial compounds into the host innate immune cell that cause kinase inactivation by proteolysis, post-translational modification at active enzymatic sites, as well as by induction of different phosphatases (28, 29). Either inactivation or sustained activation of the MAPK signaling pathways will lead to a dysregulated immune response. We showed that *F. alocis* challenge induces an early activation of p38 and ERK1/2 in human neutrophils that peaks between 15 and 30 min and decreases following 60 min of bacterial challenge (20). We expanded our initial observation, and the data from the present study revealed that *F. alocis* induces a second phase of MAPK activation in human neutrophils beyond 60 min of bacterial challenge. Besides modulation of MAPK signaling, RNAseq analysis showed that *F. alocis* also induced changes in gene expression associated with GPCR and TNF signaling pathways. In the inflamed gingival crevice environment, neutrophils infected with *F. alocis* will also be exposed to bacterial peptides, such as fMLF, as well as to inflammatory cytokines such as TNFα. Our results show a similar degree of fMLF-stimulated p38 and ERK1/2 phosphorylation in human neutrophils in the presence or absence of *F. alocis* infection. However, when TNFα was used as the second stimulus, *F. alocis*-challenged neutrophils showed a significant decrease in p38 MAPK activation. This modulation of TNFα-induced p38 phosphorylation was not seen when neutrophils were exposed to heat-killed *F. alocis* or to the TLR2/6 agonist, FSL-1, prior to the cytokine stimulation. Furthermore, the combination of *F. alocis* and TNFα had no impact on ERK1/2 activation induced by the pro-inflammatory cytokine. While many pathogenic organisms can modulate MAPK signaling in innate immune cells as a mechanism to increase bacterial virulence, this is the first time this observation has been shown for *F. alocis*.

In human neutrophils, TNFα stimulation results in activation of both p38 and ERK1/2, which are involved in the production of inflammatory cytokines and chemokines independently of NF-kB activation (55). However, blocking TNFα-induced activation of p38 but not ERK1/2 impaired both the transcription and translation of inflammatory cytokines by human neutrophils (25). Our results show that TNFα-induced activation of p38 MAPK is impaired in neutrophils infected with *F. alocis*, the extent to which this affects functional mechanisms was tested and is summarized in **Supplemental Figure 5**. This manipulation of MAPK signaling pathway by *F. alocis* limited the release of TNF-induced chemokine IL-8 by neutrophils. This phenotype has been described for other periodontal pathogens, which employ multiple mechanisms to manipulate IL-8 production and limit the influx of neutrophils (17). In human neutrophils, stimulation by TNFα also has a pro-survival response. It has been shown that activation of MEK and ERK1/2, which are

uncoupled in human neutrophils, participate in the prosurvival effect of TNFα (27). A previous study also showed that TNFα activated both p38 and ERK1/2 in human neutrophils, but that only activation of ERK1/2 was necessary for TNFα-mediated inhibition of caspase-3 activity and the pro-survival effect (66). In our study, *F. alocis* challenge did not affect TNFα-induced ERK1/2 activation, and when apoptosis was tested, the pro-survival effect of the cytokine was not impaired. In fact, stimulation with *F. alocis* alone had a pro-survival effect on neutrophils, which was also reflected in the RNAseq analysis where *F. alocis* up-regulated anti-apoptotic signaling pathways in neutrophils.

It has been shown that phagocytosis of pathogenic bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* induces changes in neutrophil gene expression involved in the acceleration of apoptosis whereas a different transcriptional profile, linked to delay neutrophil apoptosis, is induced following *A. phagocytophilum* and *Francisella tularensis* infection (32, 34, 60). A microarray study showed that *F. nucleatum* induces upregulation of anti-apoptotic genes in human neutrophils (33). Similarly, our RNAseq analysis identified several upregulated prosurvival and downregulated pro-apoptotic differentially-expressed genes in neutrophils after *F. alocis* challenge. Extending neutrophil life span delays cell turnover and prevents resolution of inflammation contributing to disease progression. The mechanisms utilized by *F. alocis* to delay neutrophil apoptosis is an area under current investigation in our laboratory.

In conclusion, our findings show that *F. alocis* induces significant changes in the human neutrophil transcriptome. In particular, biological processes involved with inflammation, signal transduction, vesicular trafficking, neutrophils activation, and apoptosis were significantly regulated. Furthermore, our results show that *F. alocis* modulated both the TNF and MAPK kinase signaling pathways. This resulted in decreased p38 MAPK activation by a secondary stimulus, i.e., TNFα, but not by fMLF. *F. alocis,* by selectively blocking p38 MAPK, but not ERK1/2, by the secondary stimulus TNF, will potentially maintain a delay of neutrophil apoptosis while dampening the release of inflammatory mediators.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in the GEO under accession number GSE137351.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the University of Louisville. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IM, AV, and MR performed the experiments, participated in data analysis, and interpretation. IM also performed the MetaCore software analysis, data interpretation, and was involved in drafting of the manuscript. ER performed the RNAseq bioinformatics analysis, the PCA, and volcano plots figures. XL participated in RNAseq data analysis and generating a list of differentially expressed genes and figures. SW performed the library preparations and sequencing run of the samples. RL contributed to study design and revision of the manuscript. SU performed the study design, data interpretation, drafting and critical revision of the manuscript, obtained funding, and study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00497/full#supplementary-material

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Citrullination Licenses Calpain to Decondense Nuclei in Neutrophil Extracellular Trap Formation

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Neutrophils respond to various stimuli by decondensing and releasing nuclear chromatin characterized by citrullinated histones as neutrophil extracellular traps (NETs). This achieves pathogen immobilization or initiation of thrombosis, yet the molecular mechanisms of NET formation remain elusive. Peptidyl arginine deiminase-4 (PAD4) achieves protein citrullination and has been intricately linked to NET formation. Here we show that citrullination represents a major regulator of proteolysis in the course of NET formation. Elevated cytosolic calcium levels trigger both peptidylarginine deiminase-4 (PAD4) and calpain activity in neutrophils resulting in nuclear decondensation typical of NETs. Interestingly, PAD4 relies on proteolysis by calpain to achieve efficient nuclear lamina breakdown and chromatin decondensation. Pharmacological or genetic inhibition of PAD4 and calpain strongly inhibit chromatin decondensation of human and murine neutrophils in response to calcium ionophores as well as the proteolysis of nuclear proteins like lamin B1 and high mobility group box protein 1 (HMGB1). Taken together, the concerted action of PAD4 and calpain induces nuclear decondensation in the course of calcium-mediated NET formation.

Keywords: neutrophil, citrullination, PAD4, calpain, cell death, nucleus, chromatin

INTRODUCTION

In recent years, a functional role of neutrophil extracellular traps (NETs) has been implicated in the setting of multiple diseases as diverse as autoimmunity, cancer, thrombosis, crystalopathies, and the response to large pathogens (1, 2). NETs serve an important role in the containment and remodeling of inflammatory foci. Chromatin decondensation is a crucial event in the formation of NETs (3). In prototypical apoptosis, nuclear chromatin condensation and caspase-dependent DNase activity result in characteristic DNA fragmentation at the internucleosomal level (4). On the other hand, NETs capable of pathogen immobilization and thrombus stabilization depend on large undegraded tangles of chromatin, which functionally depend on the framework of extracellular DNA (5, 6). The mechanism of chromatin decondensation in the setting of NET formation is incompletely understood. Proteolytic cleavage events by neutrophil serine proteases such as neutrophil elastase have been implicated in neutrophils treated with phorbol

esters acting in conjunction with reactive oxygen species (ROS) (7). Pharmacological inhibitors of peptidylarginine deiminases (PAD) and mice deficient in PAD4 (the only PAD enzyme with a nuclear localization signal) have been used in a wide variety of experiments to model deficient NET formation (8–10). PAD activity induces the modification of peptide-associated basic arginine residues to neutral citrulline. Citrullination thus disturbs polar protein interactions and the modification of the histone N-terminus is thought to diminish histone-DNA binding and account for chromatin decondensation. However, the role of citrullination by PADs as one of a multitude of potential posttranslational histone modifications remains enigmatic (11–14): In the setting of multiple posttranslational chromatin modifications *in vivo*, can loss of charge in arginine residues of histone proteins alone account for the massive decondensation observed in NET formation? Why should chromatin decondensation be sufficient to decondense an entire nucleus featuring a proteinaceous nuclear cytoskeleton and nuclear lamina? Currently, protein citrullination by PAD4 is not sufficiently integrated into previously defined signaling pathways of cellular demise and its function remains poorly defined. Its role in PMA- or pathogen-evoked NET formation has even been questioned recently (11, 15), while convincing results link PAD4-mediated NET formation to thrombus maturation (10), calling for a reevaluation of its role in NET formation.

Programmed cell death is typically associated with elevated protease activity. Apoptosis is characteristically driven by initiator and effector caspases (16). Caspase activity may be induced by extrinsic or intrinsic mechanisms. Additional protease cascades exist, which determine regulated necrotic cell death independently of caspase activity, often lacking characteristic morphological features of caspase-mediated apoptosis (17). Many years ago, members of the calpain family were identified as important calcium-dependent proteases in necrotic cell death (18). Calpains feature a peculiar mode of proteolytic cleavage. As opposed to tryptic-type serine proteases, which specifically cleave after arginine and lysine residues, calpain-mediated proteolysis is guided by the three-dimensional architecture of the substrate proteins and relies on the specific conformation of the protein (19): Large cytoskeletal proteins are hallmark substrates of calpain. Here, a regulated cleavage by calpain is achieved by controlled cleavage between domains, as observed for spectrin repeat domain proteins.

In this study, we observed that independent pathways lead to chromatin decondensation in neutrophils, typical of NETs. Moreover, these independent NET-forming pathways are characterized by differential proteolysis of nuclear membrane and chromatin proteins, and PAD4 intricately regulates substrate vulnerability to proteolysis (20). Citrullination by PAD4 is not necessary for elastase-mediated chromatin decondensation. However, PAD4 strongly enhances the capability of calpain to induce chromatin decondensation. Calpain alone did not induce chromatin decondensation in the absence of PAD4, whereas the concerted action of both was able to strongly decondense nuclear chromatin. We identified multiple proteins as relevant targets in this process including but not limited to high-mobility group box 1 protein (HMGB1), histone H3, lamins, and HP1 chromobox proteins.

RESULTS

Independent Pathways Induce Chromatin Decondensation in Neutrophils

To study the molecular mechanisms of NET formation, neutrophils were stimulated with prototypical instigators of NET formation, namely phorbol-myristate-acetate (PMA), known to result in protein kinase C activation, the assembly of the NAPDH oxidase complex and instigation of the oxidative burst. Additionally, neutrophils were stimulated with the bacteria-derived calcium ionophore ionomycin. Analyses by fluorescence microscopy revealed striking chromatin decondensation in response to both stimuli in healthy donor-derived neutrophils, whereas chromatin decondensation in response to PMA was drastically reduced in neutrophils derived from a patient with chronic granulomatous disease (CGD) (**Figures 1A,B**). However, ionomycin was perfectly capable of inducing chromatin decondensation even in the absence of the respiratory burst in CGD patient-derived neutrophils. Pharmacological inhibition of the NADPH oxidase complex by means of diphenyliodonium (DPI) revealed compatible findings (**Figure 1C**). DPI failed to inhibit ionomycin-induced chromatin externalization as detected using whole-well detection of DNA^{SYTOX}, whereas DPI was capable of inhibiting PMA-induced chromatin externalization. PMA induced a marked ROS generation in healthy neutrophils, whereas ionomycin did not (**Figure 1D**). As observed previously (21), these results point to a divergent necessity of NADPH-oxidase-derived ROS for independent pathways of chromatin decondensation in human neutrophils.

Recent publications have highlighted the role of peptidyl arginine deiminases and protein citrullination as a mechanistic feature of NET formation (9, 12, 13). Independent groups have shown citrullinated histone H3 as a hallmark feature of NETs (9, 22). In the prevailing view, citrullination of histone H3 causes a loss of charge of basic chromatin-bound proteins and thereby results in massive chromatin decondensation (12). We studied citrullination events in the course of both PMA- and ionomycin-induced NET formation *in vitro*. These experiments showed that, 120 minutes after stimulation, marked citrullination of histone H3 is restricted to calcium-ionophore treated neutrophils as opposed to untreated and PMA-treated cells under the specified experimental conditions (**Figure 1E**). Interestingly, citrullination is not restricted to histone H3 but rather affects a large number of proteins of various molecular sizes as assessed by specific immunodetection of modified citrulline residues. Processing by recombinant PAD4 of histone proteins and isolated nuclear protein fractions again showed low substrate specificity and rather broad protein citrullination by PAD4 (**Figure 1F**) (23). Taken together, neutrophil chromatin decondensation can be achieved via independent pathways (11). On the one hand, a need for NADPH-oxidase derived ROS is indicated. On the other hand, calcium ionophore-treated neutrophils are

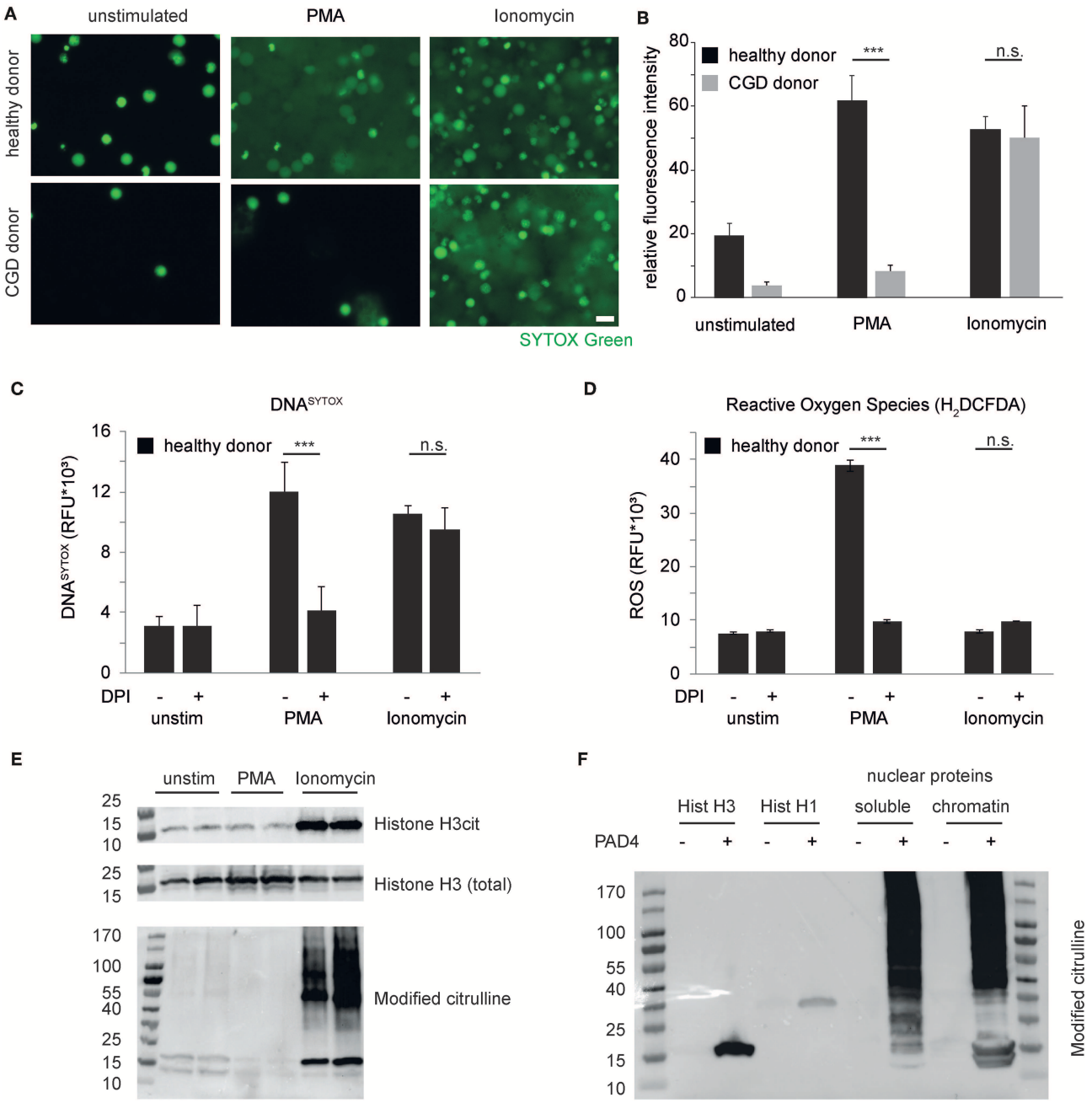

FIGURE 1 | Independent pathways induce chromatin decondensation in neutrophils. **(A)** Isolated neutrophils were stimulated in HBSS media with phorbol myristyl acetate (PMA, 50 nM) or ionomycin (5 μM). Imaging was performed 3 h after stimulation. The reproducibly observed response of peripheral blood neutrophils derived from healthy donors and a patient suffering from chronic granulomatous disease (CGD) is depicted. DNA of neutrophils is stained by SYTOX Green (in green), once the plasma membrane is disrupted and/or DNA has been expulsed. Decondensation of chromatin results in increased area and decreased intensity of SYTOX staining (scale bar = 20 μm). **(B)** SYTOX Green fluorescence intensity of microscopic images from equal numbers of cells as presented in **(A)** was quantified and represented as relative signal intensity (***$p < 0.001$ Student's *t*-test of technical replicates, $n > 4$ healthy donors, $n = 2$ independent experiments with CGD neutrophils). **(C,D)** Stimulation of healthy donor-derived human neutrophils in the presence or absence of Diphenyliodonium (DPI) and the previously mentioned stimuli and **(C)** whole-well DNA^{SYTOX} quantification as well as **(D)** whole well detection of reactive oxygen species (H2-DCFDA) was performed after 180 min in a Tecan M200 microplate reader. Results representative of 4 independent healthy donors. **(E)** Neutrophil granulocytes were stimulated with the indicated stimuli for 120 min before subsequent protein isolations. Citrullinated Histone H3, total histone H3 and citrulline-containing proteins were assessed subsequently. **(F)** Recombinant Histone H3 and Histone H1 as well as nuclear protein fractions of A549 cells were treated with recombinant PAD4. Protein citrullination was assessed by antibody based detection of modified citrulline residues after reaction with diacetyl monoxime/antipyrine. Unless otherwise indicated, results are representative of at least 3 independent experiments using independent healthy donors.

characterized by marked protein citrullination, which is not restricted to histones.

Differential Proteolytic Cleavage Events in NET Formation

Programmed cell death pathways are characterized by proteolysis of intracellular target proteins (24). As loss of nuclear integrity is a characterizing feature of NET formation, we studied proteolysis of nuclear proteins in healthy donor-derived neutrophils after PMA or ionomycin challenge. Interestingly, ionomycin induced a marked loss of the nuclear envelope protein Lamin B1 as well as of the nuclear chromatin-binding protein high mobility group box protein 1 (HMGB1) in the course of 2 hours, which was not yet observed after 120 minutes of PMA stimulation (**Figures 2A,B**). As citrullination is a key feature of ionophore-stimulated neutrophils, we asked whether this would impact

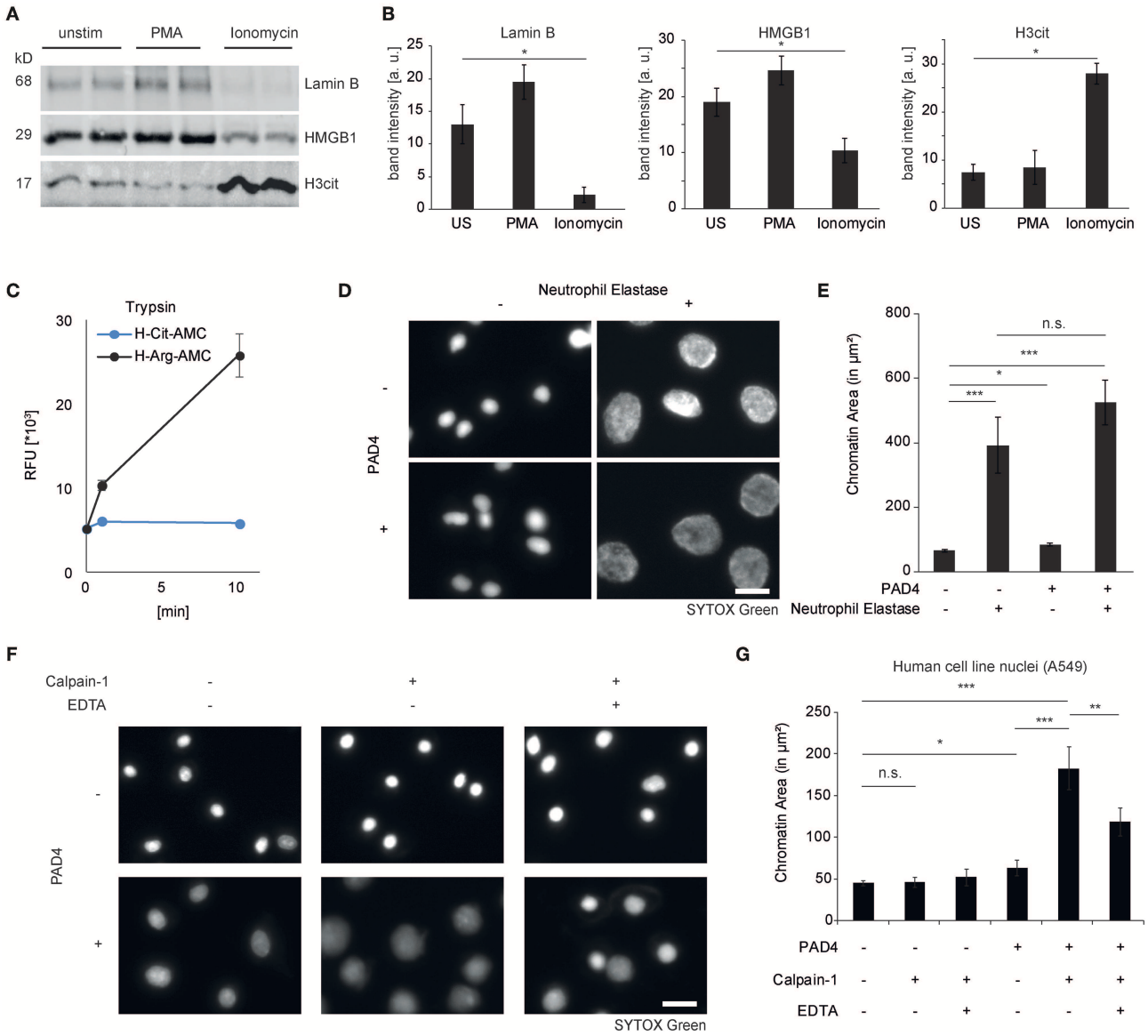

FIGURE 2 | Differential proteolytic cleavage events in NET formation. **(A)** Lamin B1, HMGB1, and citrullinated Histone H3 were assessed by western blotting in lysates from human neutrophils after stimulation for 120 min with PMA or ionomycin ($n = 3$ independent healthy donors). **(B)** Band intensity calculations of western blots as in **(A)** were performed using Image J-assisted density quantification of at least 4 lanes (**$p < 0.01$ ANOVA and *post-hoc* Tukey HSD test). **(C)** The fluorogenic substrates H-Cit-AMC (25 µM) and H-Arg-AMC (25 µM) were subjected to trypsin digestion and emitted fluorescence was measured. **(D)** Isolated A549 nuclei were subjected to neutrophil elastase (60nM) for 4 h after preincubation in the presence or absence of PAD4 (5 µM) for 90 min (scale bar = 20 µm). **(E)** Area of SYTOX Green-stained single nuclear chromatin was quantified (at least 100 nuclei in each of 3 independent experiments, depicted as mean + standard error, *$p < 0.05$, ***$p < 0.001$, ANOVA and *post-hoc* Tukey HSD test). **(F)** Isolated A549 nuclei were subjected to human calpain-1 for 4 h after preincubation in the presence or absence of PAD4 (5 µM) for 60 min (scale bar = 20 µm). **(G)** Nuclear chromatin area of SYTOX-Green-stained single nuclei was quantified as described in **(E)**.

regulated proteolysis. It is well-known that serine proteases of the trypsin type cleave proteins specifically after basic arginine and lysine residues and not after citrulline, as evidenced by specific fluorogenic substrates (**Figure 2C**). Furthermore, proteolysis by the serine protease neutrophil elastase had previously been shown to induce chromatin decondensation in isolated cell nuclei of different origin (7). However, biochemically, neutrophil elastase shows no arginine or lysine-guided specific proteolysis. It was, therefore, not surprising to observe that elastase was able to mediate chromatin decondensation of isolated nuclei independently of protein citrullination by PAD4 (**Figures 2D,E**). PAD4 alone was able to induce a minor chromatin decondensation in isolated nuclei but failed to reach the pronounced effect of proteolytic nuclear decondensation guided by neutrophil elastase (**Figures 2D,E**). Ionomycin has been known to promote the activity of calpain proteases (25). Calpain and peptidyl arginine deiminases share the feature that both need high calcium concentrations to fulfill their enzymatic activity. Supported by recent publications, we argued that calpain proteolysis might be enhanced by protein citrullination in nuclear decondensation assays (20, 26). We, therefore, stimulated isolated nuclei with calpain and PAD4 and a combination of both enzymes. We chose non-granulocytic cell lines in this approach to limit confounding effects of intrinsic PADs and granular proteases. Notably, isolated nuclei resisted calpain treatment and their morphology remained unaltered (**Figures 2F,G**). As observed before (27), recombinant PAD4 alone was able to induce a mild increase in nuclear area of 20%. However, nuclei enzymatically processed by PAD4 suddenly showed a striking susceptibility to calpain: In response to calpain, the nuclear area strongly expanded and displayed a reduced intensity as observed in NET formation (**Figures 2F,G**). This effect was partially reversed by EDTA. We next explored the effect of citrullination enhanced calpain-mediated nuclear decondensation in an independent preparation of mouse embryonic fibroblast nuclei. As observed in A549 cell nuclei, combined challenge with both enzymes induced a marked chromatin decondensation in MEF nuclei, whereas single treatment failed to strongly decondense the nuclei (**Figure S1**). Taken together, proteolysis of nuclear proteins differs in PMA- and ionomycin-induced chromatin decondensation. Citrullination selectively affects proteolytic events. Whereas, elastase can decondense nuclei irrespective of citrullination, PAD4 strongly enhances nuclear decondensation by calpain.

Breakdown of the Nuclear Envelope and Chromatin-Bound Proteins by the Concerted Action of PAD4 and Calpain-Mediated Proteolysis

We next explored the consequences of citrullination and calpain-mediated proteolysis on the protein level. Isolated nuclei were amenable to extensive histone H3 citrullination and subsequent chromatin decondensation when treated with recombinant PAD4 and human calpain (**Figure 3A**). Nuclear envelope disintegration was observed by immunocytochemistry. Untreated isolated MEF nuclei displayed surrounding lamin A+C positive nuclear lamina. After citrullination and calpain-mediated proteolysis, the nuclear lamina was lost (**Figure 3B**). Also HMGB1 was detected in the border area of the isolated nuclei (**Figure 3C**) (its chromatin association was possibly shifted during the isolation protocol). The concerted action of PAD4 and calpain removed the HMGB1 positive border surrounding the nucleus and induced a pronounced and significant nuclear decondensation (**Figure 3D**). These effects were also assessed by western blot analyses. The previously identified targets of proteolysis in NETs were specifically lost in MEF nuclei treated with both of these enzymes. Citrullination by PAD4 is known to delay migration in gels due to loss of charge leading to two separate bands in the presence of PAD4. Loss of A-type lamins by calpain (**Figures 3E,F**) was favored in the presence of PAD4. Additionally, the cleavage of histone H3 at its N-terminus as well as of HP1a was observed. Taken together, nuclear decondensation in response to PAD4 and calpain showed a marked citrullination of chromatin-bound histones by PAD4 and induced nuclear decondensation accompanied by a loss of lamins and chromatin-bound proteins, most likely mediated by proteolysis.

Concerted Action of PAD4 and Calpain Determines Nuclear Envelope Breakdown in Calcium Ionophore-Induced NET Formation

In order to further test our hypothesis of synergistic effects of PAD4-mediated citrullination and calpain-directed proteolysis in nuclear decondensation, we assessed the role of calpain and PAD4 in calcium-induced NET formation in human and murine neutrophils. Therefore, we treated healthy-donor-derived peripheral blood neutrophils with PMA and ionomycin as shown before. Pharmacological inhibition of calpain was achieved using PD150606. Inhibition of peptidyl arginine deiminases was achieved using the pan-PAD inhibitor Cl-amidine. Neither calpain inhibition nor Cl-amidine was able to inhibit NET formation induced by PMA, in which PAD activity was not as strongly observed. However, both calpain and PAD inhibition independently inhibited the nuclear decondensation observed in response to ionomycin as depicted by fluorescence microscopy (**Figure 4A**), nuclear area measurement (**Figure 4B**) and assessment of the cumulative distribution of the nuclear area (**Figures 4C,D**). This was further confirmed as ionomycin-induced neutrophil nuclear decondensation was significantly inhibited by two independent calpain inhibitors (PD150606 and the cysteinyl protease inhibitor E64c) and two PAD inhibitors (Cl-amidine, BB-Cl-amidine), respectively (**Figures 4E,F**). Pharmacological inhibition of PADs blocked ionomycin-inducible histone citrullination and chromatin decondensation in neutrophils. After pharmacological inhibition of calpains, citrullination was still evident in ionomycin-stimulated neutrophils. However, nuclear decondensation was blocked when calpain activity was inhibited (**Figures 4G,H**).

PAD4 Is Essential for ionophore-Induced Chromatin Decondensation and Nuclear Protein Degradation in Murine Neutrophils

In order to assess the direct contribution of PAD4 to nuclear proteolysis independently of pharmacological inhibitors, we

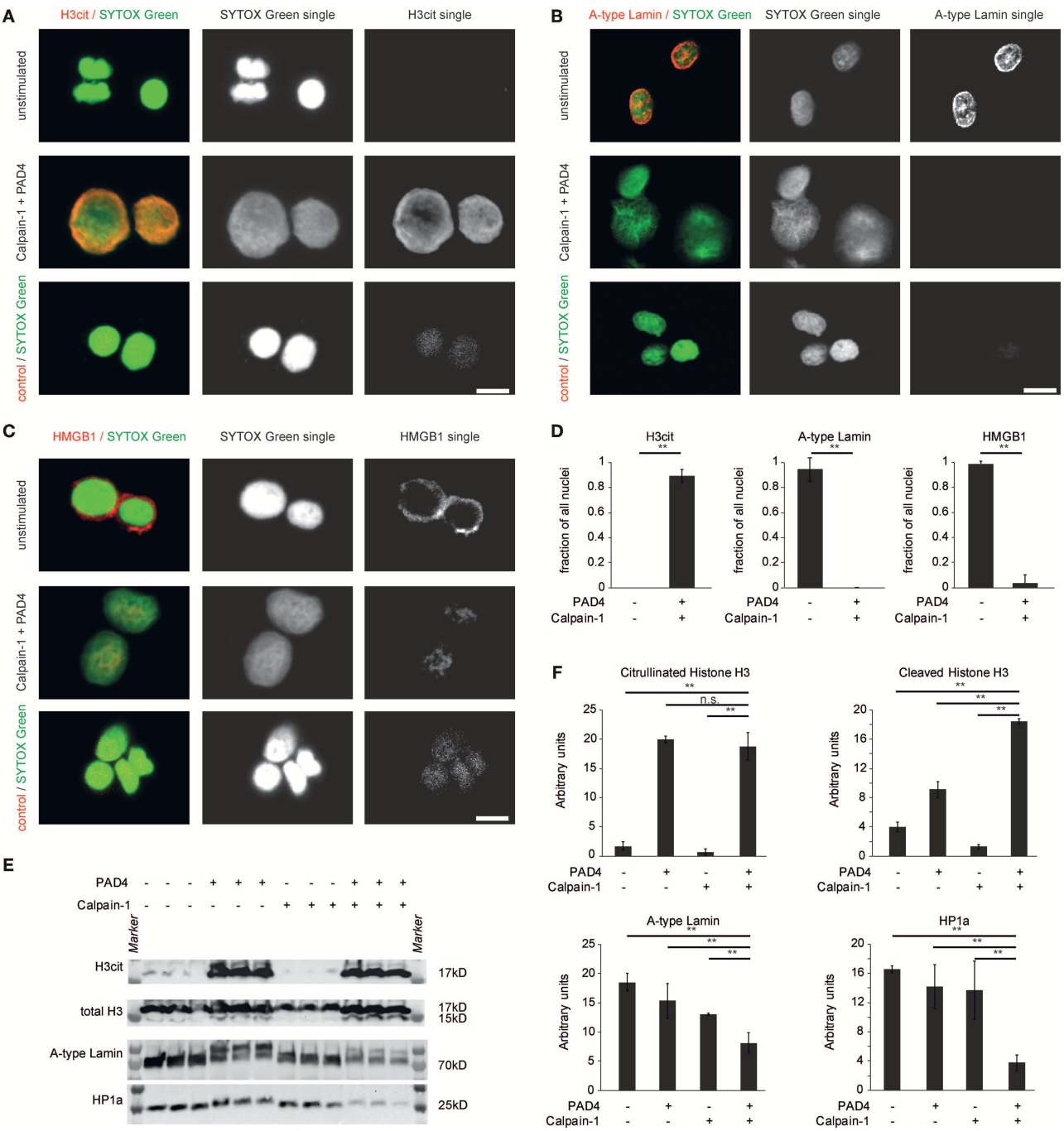

FIGURE 3 | Breakdown of the nuclear envelope and chromatin-bound proteins by the concerted action of PAD4 and calpain-mediated proteolysis. MEF nuclei were subjected to human calpain-1 after preincubation in the presence of PAD4 or left unstimulated and processed for immune cytochemistry of **(A)** citrullinated Histone H3, **(B)** Lamin A+C, and **(C)** HMGB1. DNA counterstaining was performed using SYTOX Green (scale bar = 20 μm) **(D)** H3cit^{+}, lamin A+C^{+} and HMGB1^{+}, respectively, were quantified as a fraction of all nuclei stained by SYTOX Green (**$p < 0.01$, student's *t*-test); data derived from 3 independent experiments). **(E)** Isolated A549 nuclei were subjected to the enzymatic activity of either calpain-1, PAD4 or the consecutive combination of both enzymes for 60 min each. Subsequently, proteins were isolated and processed for western blotting as indicated. **(F)** Band intensity calculations of western blots as in **(E)** were performed (**$p <$ 0.01, ANOVA and *post-hoc* Tukey HSD test). Three independent experiments were performed with similar results.

stimulated murine peritoneal neutrophils with ionomycin and assessed the integrity of the previously identified nuclear targets of proteolysis. Wildtype neutrophils displayed marked increases in histone H3 citrullination in response to ionomycin, which was absent in PAD4-deficient cells (**Figure 5A**). While ionomycin triggered a marked loss of both lamin B1

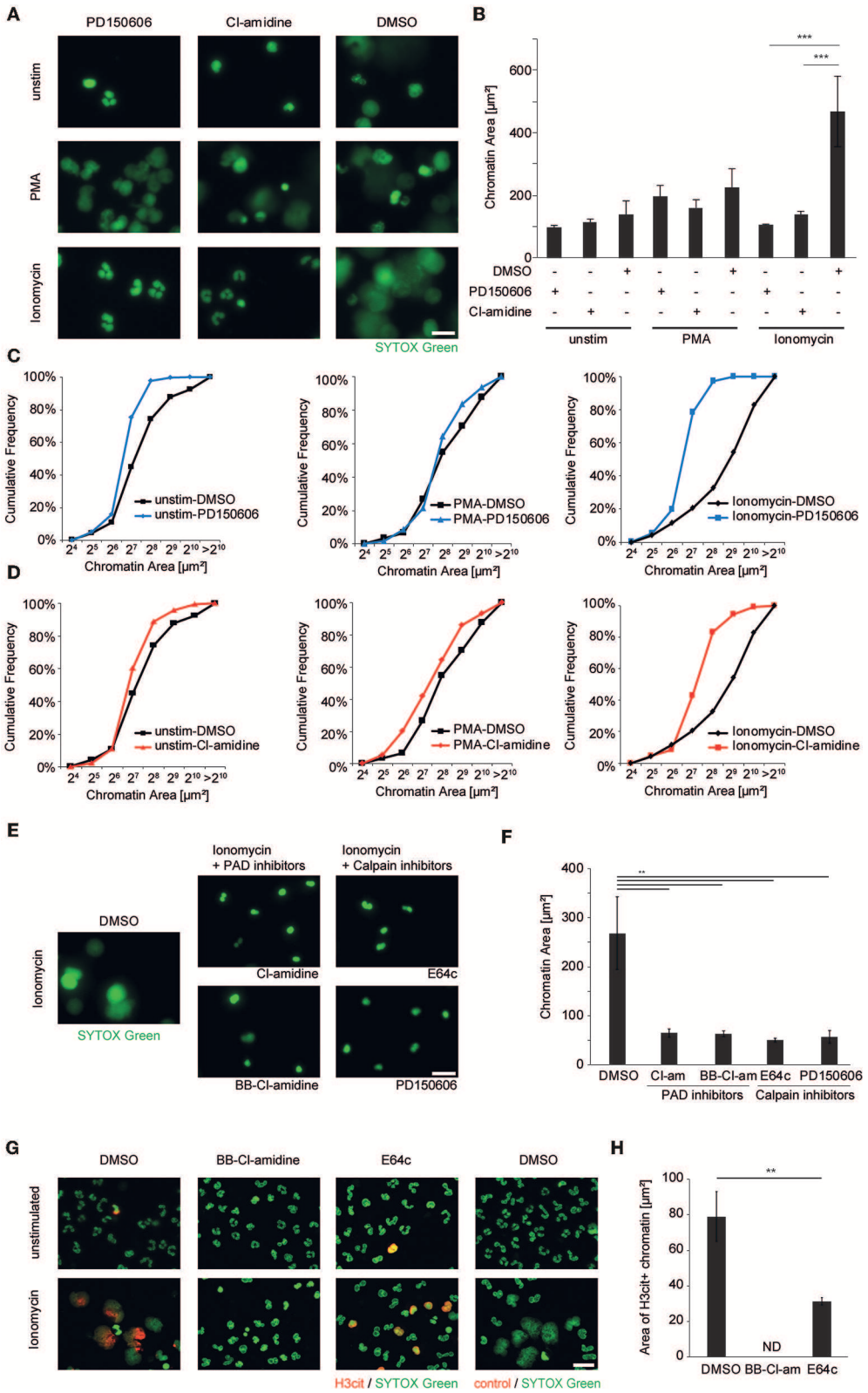

FIGURE 4 | Ionophore-induced chromatin decondensation of human neutrophil granulocytes requires both PAD and calpain activity. Human neutrophils from the peripheral blood of healthy donors were stimulated with PMA (50 nM), Ionomycin (5 µM) or left unstimulated after pre-treatment with DMSO control, the specific calpain inhibitor PD150606 (50 µM) or the PAD-inhibitor Cl-amidine (200 µM), respectively. **(A)** The morphology and spread area of the chromatin was analyzed after

(Continued)

FIGURE 4 | 2 h as assessed via fluorescence microscopy after SYTOX Green-mediated DNA staining. Representative images of at least four independent experiments are shown (scale bar = 20 μm). **(B)** Mean + SEM of the median chromatin area of at least 100 single cells of four independent experiments is depicted (***$p < 0.001$, ANOVA and *post-hoc* Tukey HSD test). **(C,D)** Histogram plots showing the distribution of single chromatin configurations as counted in **(B)** after PMA or ionomycin challenge with either PD150606 **(C)** or Cl-amidine **(D)** inhibitor treatment. **(E,F)** The effect of additional inhibitors of PAD enzymes and cysteinyl proteinases on ionomycin-induced chromatin decondensation was assessed using BB-Cl-amidine (20 μM), Cl-amidine (200 μM) and E64c (50 μM) and PD150606 (50 μM). Representative images are shown in **(E)** and chromatin area quantification in **(F)** (scale bar = 20 μm, **$p < 0.01$ in one-way ANOVA and *post-hoc* Tukey HSD tests, $n = 3$ independent experiments). **(G)** Granulocyte cultures stimulated as indicated were subjected to H3cit immunocytochemistry (scale bar = 20 μm) and **(H)** the area of H3cit immunopositive chromatin after ionomycin stimulation was calculated in each respective condition (**$p < 0.01$ student's *t*-test, $n = 3$ independent experiments; ND, not detected).

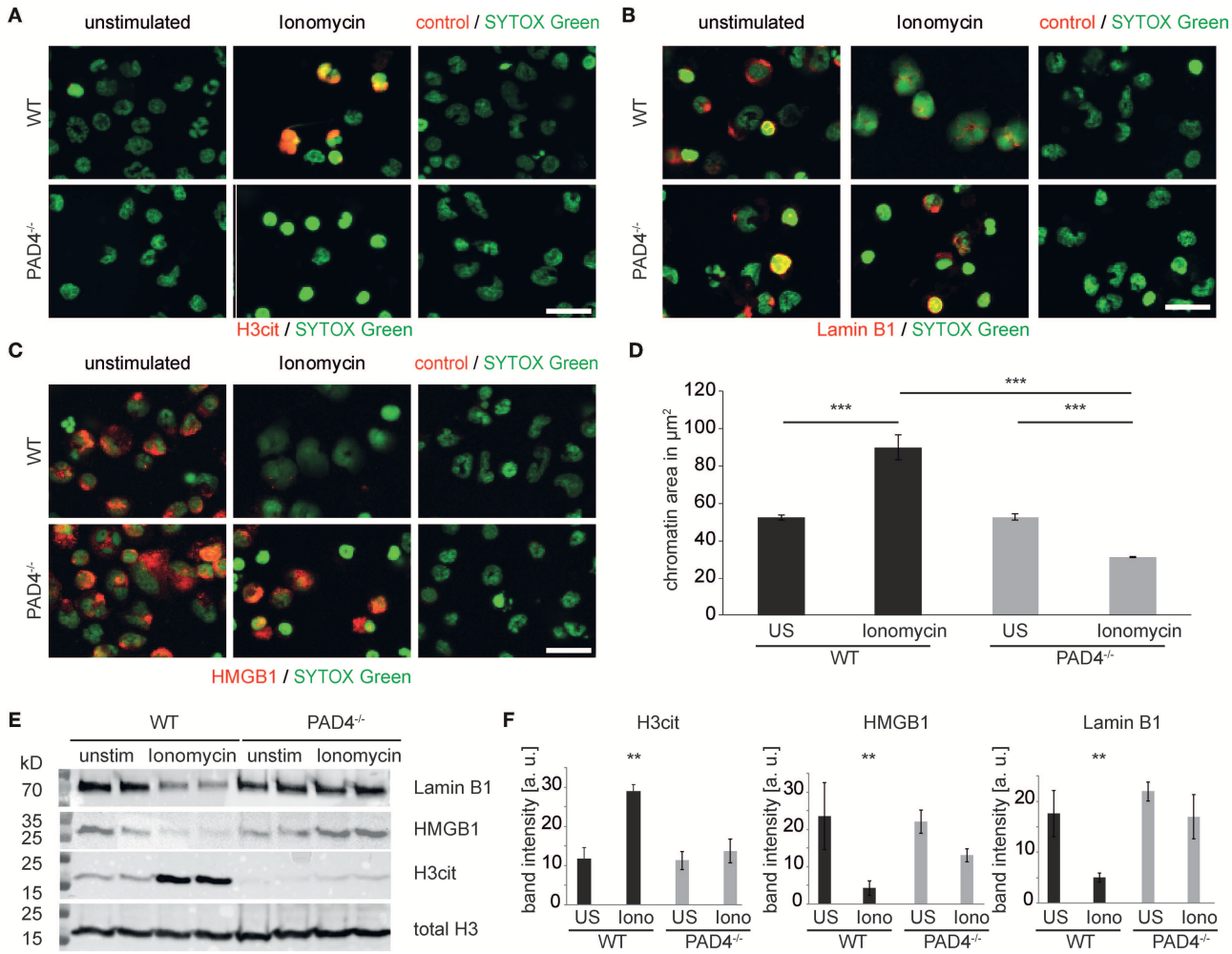

FIGURE 5 | PAD4 is essential for ionophore-induced chromatin decondensation and nuclear protein degradation in murine granulocytes. Murine peritoneal neutrophils were stimulated with ionomycin for 120 min or left unstimulated. **(A)** H3cit immunocytochemistry reveals PAD4 activity in WT cells and its absence in PAD4-deficient neutrophils ($n = 3$ independent experiments). **(B)** Identical cultures were subjected to Lamin-B1 and **(C)** HMGB1 immune cytochemistry. **(D)** The morphology and spread area of the chromatin was analyzed after 2 h as assessed via fluorescence microscopy after SYTOX Green-mediated DNA staining ($n = 4$ independent experiments with at least 100 nuclei per analysis, ***$p < 0.001$, ANOVA and *post-hoc* Tukey HSD test). **(E)** Western blots of total histone H3, citrullinated histone H3, Lamin B1, and HMGB1 of murine peritoneal neutrophil cultures were performed. One representative blot is presented. **(F)** Results of four independent western blots as in **(E)** were used for band intensity quantifications (all scale bars = 20 μm, **$p < 0.01$ ANOVA and *post-hoc* Tukey HSD).

(**Figure 5B**) and HMGB1 (**Figure 5C**), this did not occur in PAD4-deficient cells. Wild-type neutrophils responded to ionomycin with robust chromatin decondensation (**Figure 5D**), while PAD4-deficient neutrophils failed to decondense nuclear chromatin in response to ionomycin. Impressively, ionomycin-induced H3 citrullination was associated with Lamin B1 and HMGB1 loss in wild-type neutrophils. However, deficiency of PAD4 not only blocked H3 citrullination but furthermore significantly inhibited lamin B1 and HMGB1 degradation (**Figures 5E,F**).

Citrullination by PAD4 Facilitates Proteolysis of HMGB1 by Calpain

We further characterized calpain-mediated cleavage and citrullination *in vitro* using subcellular protein fractionation. Both soluble and chromatin-bound nuclear proteins were subjected to citrullination and calpain-mediated proteolysis. These analyses showed PAD-independent proteolysis of large spectrin repeat proteins e.g., Filamin and alpha-actinin as identified by mass spectrometry. However, at 24–27 kD, protein bands were apparent, that selectively disappeared after calpain cleavage and citrullination (**Figure S2**). HMGB1 was identified as a candidate protein: In soluble nuclear protein lysates of A549 cells, enhanced proteolysis of HMGB1 facilitated by citrullination was apparent (**Figures 6A,B,F**). Even more pronounced was the joint effect of calpain and PAD4 on proteolysis of HMGB1 in whole isolated nuclei (**Figures 6C,G**): Here, loss of HMGB1 was nearly complete, probably favored by protein conformation. HMGB1 is characterized by two L-shaped DNA-binding domains allowing the preferential binding of HMGB1 to kinked DNA (**Figures 6D,E**). Interestingly, calpain-mediated cleavage generated a cleaved form of HMGB1 that was detected in significantly increased amounts after nuclear protein citrullination (**Figures 6F,G**) using a polyclonal antibody directed against a C-terminal peptide of human HMGB1. In line with the concept of interdomain cleavage, the fragment was approximately half of the full-length HMGB1 (12-14kD). Recombinant HMGB1 was treated with PAD4 and Calpain-1 and the cleavage fragments were analyzed by mass-spectrometry (**Figure 6D**). Interestingly, HMGB1 showed evidence of citrullination specifically at the edges of the A- and B-box (R_{70}, R_{73}, R_{96}, R_{163}). Peptides spanning both A-box and B-box were detected in the cleaved band. This argues for an interdomain cleavage resulting in two fragments of similar size. We speculate that citrullination at the edges of the boxes facilitated calpain-mediated cleavage.

Taken together, we propose a central role for peptidyl arginine deiminase 4 in the regulation of calpain-mediated proteolysis of proteins of the nuclear lamina and core, as exemplified by lamins and HMGB1. The concerted action of calpain and PAD4 contributes to nuclear decondensation as observed in ionophore-induced NET formation.

DISCUSSION

In recent years substantial progress has been made in deciphering the functional role of NETs *in vivo*. Yet, seemingly contradictory findings regarding the molecular mechanisms of NET formation have contributed to confusion in a quickly expanding field of research (7, 9, 10, 12, 29). The landmark publications nicely described NET formation in response to phorbol-myristate acetate (PMA) and the necessity of the oxidative burst as a paradigm of NET formation (1, 3). PMA-induced NET formation further depends on the PI3K/Akt and the Ras/RAF/MEK/ERK pathway (30). Some of these features are also evident in response to microbial stimuli (31). On the other hand, NET formation has been described in response to calcium ionophores such as ionomycin (9, 12, 22, 32). Calcium-induced NET formation may occur in response to integrin-mediated NET formation (33, 34) or stimuli such as purins (35), pyroptotic membrane pores (36), bicarbonate-pCO2 alterations (29, 37) or calcium store-operated mechanisms (38). Calcium ionophore-induced NET formation is largely independent of NADPH oxidase, whereas mitochondrial ROS may play an important role (39, 40). Calcium ionophore-induced NET formation shows a striking dependency of peptidyl arginine deiminases as chromatin decondensation is blocked in the presence of both pan-PAD and selective inhibitors of PAD4 (41). Taken together, PMA and ionomycin induce independent pathways resulting in the extrusion of decondensed chromatin. As previously observed citrullination is rather restricted to calcium-dependent NET formation (32). Citrullination may also occur in neutrophils after PMA stimulation, once cell lysis has occurred, but does not seem to contribute to earlier chromatin decondensation (11). Furthermore, neutrophil chromatin decondensation in response to calcium ionophores is blocked in PAD4-deficient mice (10). In agreement with the concept of the existence of independent pathways of chromatin decondensation in neutrophils, Holmes et al. observed that decondensed chromatin, was still observable after ionomycin treatment of PAD4-deficient neutrophils, though strongly less abundant than in wild-type counterparts, most probably derived from citrullination-independent pathways (42).

PAD enzymes specifically citrullinate arginine residues. This property can strongly alter the susceptibility of proteins to proteolysis: Serine proteases with tryptic activity cleave behind basic amino acids such as arginine and lysine and citrullination inhibits trypsin-mediated cleavage after modified residues (43). The trypsin-related neutrophil serine protease Cathepsin G failed to induce chromatin decondensation in previous studies (7). It has been recently proposed, that citrullination actually increases serine protease activity by inhibiting serpin protease inhibitors (44). Many serpins make use of arginine baits in the P1 position to inhibit the target serine protease. Citrullination alters the bait and effectively blocks serpin inhibitory activity. Tilvawala et al. propose the complement system to have an important role in cell lysis. The experiments presented in our work were performed under serum-free conditions, making an important contribution of the complement system to our observations unlikely. Neutrophil elastase, previously implicated in PMA-induced NET formation, does not display arginine preference and is inhibited by serpins that do not contain an arginine at the P1 position (7). Proteolytic activity of neutrophil elastase, thus, appears unrelated to direct effects of PAD4, as shown above.

For many years, calpain has been implicated in cell death routines induced by high levels of intracellular calcium, especially in the setting of neurodegenerative diseases (45). Calpains are best known for limited interdomain cleavage of their many substrates which include cytoskeletal repeat domain proteins e.g., spectrins and mitochondrial factors, such as apoptosis inducing factor AIF, Bax, Bid or Bcl-2 (46–48). While it is known that nuclear proteins can be targets of calpain-induced proteolysis, little is known about the nuclear role of calpains (49). Inspired by the fact that peptidyl arginine deiminases and calpain share the requirement of high calcium concentrations

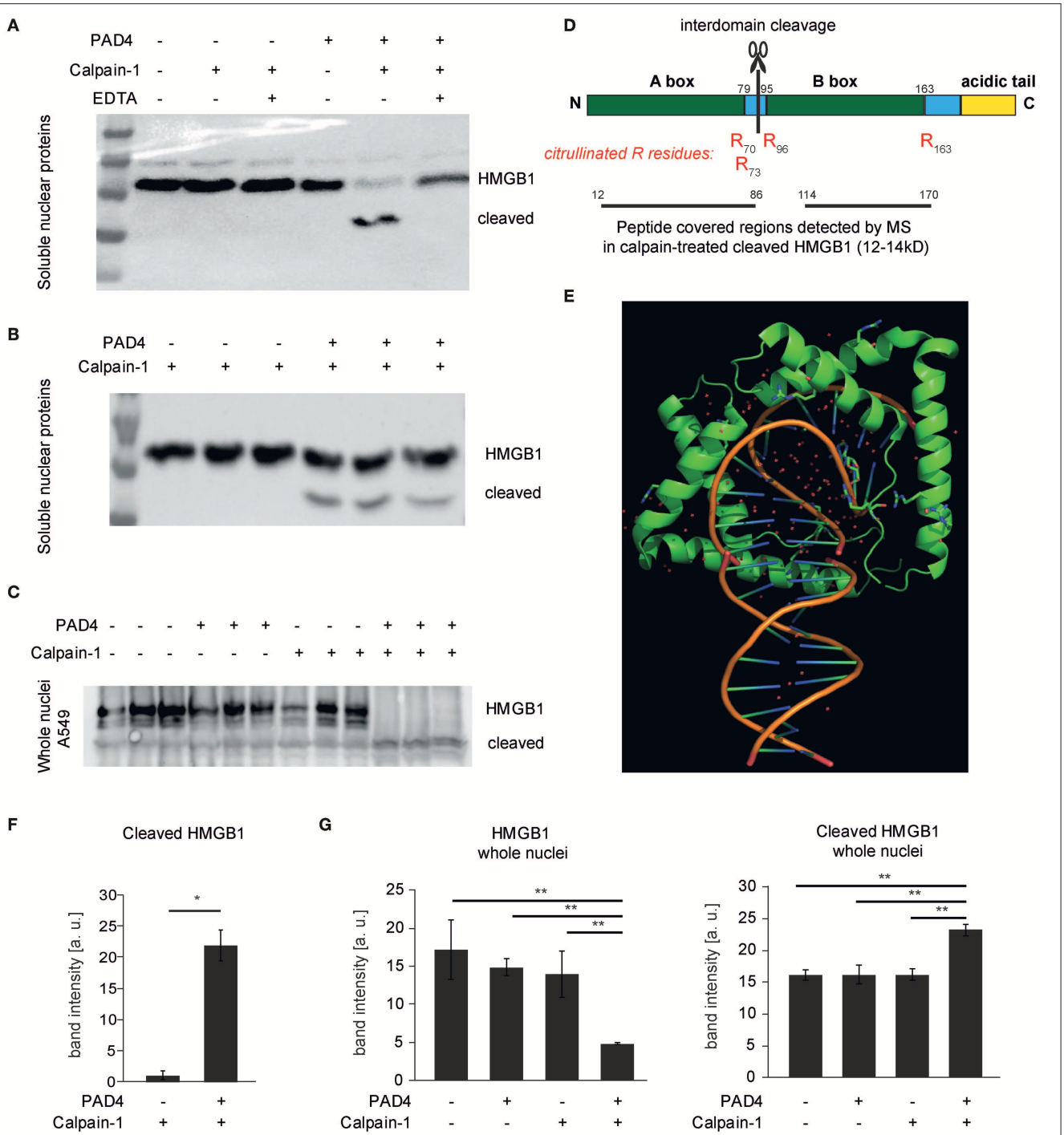

FIGURE 6 | Cleavage of HMGB1 by calpain and its selective dependence on PAD4. **(A,B,F)** Soluble nuclear protein lysates of A549 cells were subjected to PAD4-mediated protein citrullination and consecutive calpain-1-mediated proteolysis. HMGB1 was visualized by western blotting using a C-terminal primary anti-HMGB1 antibody (ab18256) (representative of 3 independent experiments). **(C,G)** Intact isolated A549 nuclei were subjected to enzymatic treatments as in **(B)** and subjected to western blotting. **(D)** The molecular organization of HMGB1 is depicted showing the two DNA-binding domains (Box A, Box B) and the acidic tail. Furthermore, the citrullinated arginine residues detected by mass spectrometry (MS) in cleaved HMGB1 (12–14 kD) are depicted in red (R70, R73, R96, R163). Regions covered by peptides identified in the cleaved HMGB1 fragment by MS are highlighted. **(E)** A structural representation of DNA-binding of two A-boxes of HMGB1 (*in green*) is depicted showing its binding to kinked DNA (based on PDB: 4QR9) (28). **(F)** shows respective relative band intensity quantifications of cleaved HMGB1 (student's *t*-test, $*p < 0.05$, representative of 3 independent experiments) **(G)** Intact isolated A549 nuclei were subjected to enzymatic treatments as indicated and subjected to western blotting. Relative band intensity quantifications are presented (ANOVA and *post-hoc* Tukey HSD, $**p < 0.01$, representative of 3 independent experiments).

and independent studies, which have shown facilitated calpain cleavage of citrullinated proteins (20, 26), we hypothesized that calpain and PAD4 synergize in ionophore-induced chromatin decondensation and NET formation. Activation of many membrane-bound receptors may lead to increased cytosolic calcium levels. Ionophore treatment bypasses specific receptor engagement, yet is a valid tool in assessing the downstream consequences of marked increases of cytosolic calcium levels.

Indeed, we observed, that calpain-1 alone did not induce chromatin decondensation. PAD4-treatment only modestly relaxed chromatin. However, incubation of citrullinated nuclei with calpain induces a marked and robust chromatin decondensation almost to the magnitude observed by elastase treatment. In an unbiased approach, we aimed to identify targets of calpain-1, targets of citrullination and proteins that were selectively cleaved by calpain-1 in the citrullinated state. These experiments revealed that large multi-repeat domain proteins in nuclear protein lysates (e.g., α-Actinin-4 and Filamin A as identified by mass spectrometry), once accessible, do not necessarily require citrullination in order to be efficiently processed by calpain-1. On the other hand, this approach revealed a band of 24–27 kD, which was selectively processed after citrullination of nuclear proteins (**Figure S2**). Western blot analyses of selected proteins identified HMGB1 as a protein of interest. Taken together, we observed a crucial synergy of calpain and PAD4, by which PAD4 licenses calpain to decondense chromatin.

How do calpain and PAD4 synergize in nuclear lysis and chromatin decondensation on a molecular level? To address this, we treated recombinant HMGB1 with calpain and PAD4. Citrullination of R residues was detected specifically at the edges of the DNA-binding A- and B-boxes. Tarcsa et al. described that citrullination favorably occurs in disordered regions of a protein (50). In line with this, citrullination at the edges of the domains and consecutive loss of polar interactions in this critical area might facilitate inter-domain cleavage by calpain. In line with substrate citrullination, citrullinated lamin C has been previously described (51). Thus, our experimental evidence points toward a synergy of calpain and PAD4 on the substrate level.

Additional modes of synergy of calpain and PAD4 might exist. Konig et al. detected calpastatin, the natural inhibitor of calpain among citrullinated proteins of human neutrophils (23). We speculate that citrullination might affect the interaction of calpain with its natural inhibitor. In the *in vitro* assays presented here, however, calpastatin was not present.

Our study integrates regulated proteolysis by calpain to the pathways of NET formation. Citrullination of chromatin and loss of charge of chromatin-associated proteins, as proposed by Wang et al. (12) may still be an important driving force in chromatin decondensation. However, we propose that efficient nuclear lysis requires additional proteolysis of the nuclear lamina and chromatin-bound proteins. These observations are also relevant to integrate seemingly contradictory observations regarding the studies of "vital" NET formation: Independent labs observed that NET formation *in vitro* is mostly associated to cell death but it was also discovered that under certain conditions nuclear expulsion occurs *in vivo* with remaining "viable" anuclear cytoplasts which still migrate and potentially phagocytize (52, 53). Calpain activity directly cleaves cytoskeletal components in a regulated fashion and is known for its essential functions both in the course of cell death and migration of viable cells (54–56). We speculate that disintegrating the cytoskeleton in a calpain- and PAD4-dependent manner on a specific location of the cytoskeleton can leave the rest of the cell body intact, while allowing targeted NET release possibly in response to receptor or adhesion molecule signaling (**Figure 7**). Future studies will need to provide further experimental evidence for this concept.

Taken together, we show that PAD4-mediated citrullination is a crucial prerequisite of nuclear decondensation in ionophore-induced NETosis coinciding and synergizing with specific calpain-directed cleavage of the nuclear lamina and chromatin-bound proteins. This finding links PAD4-mediated protein citrullination to calpain-directed proteolysis, considers calpain in the pathways of NET formation and thus proposes a novel mechanistic role of PAD4 in NET formation.

MATERIALS AND METHODS

Antibodies

Primary antibodies	Source	Dilution [ICC, WB]
ATP1A1	RRID:AB_2060983	WB 1:1000
Citrullinated Histone H3 (H3cit)	RRID:AB_304752	[1:200, 1:1000]
Neutrophil Elastase	RRID:AB_446409	[1:200, 1:1000]
GAPDH	RRID:AB_10622025	WB 1:1000
High Mobility Group Box Protein 1 (HMGB1)	RRID:AB_1139336	[1:200, 1:1000]
HP1 alpha	RRID:AB_10858495	[1:100, 1:1000]
Lamin A/C	RRID:AB_10860619	[1:500, 1:1000]
Lamin B1	RRID:AB_10107828	[1:200, 1:1000]
Modified citrulline	RRID:AB_10097769	According to recommended protocol
PARP	RRID:AB_777101	WB 1:1000
Vimentin	RRID:AB_10695459	WB 1:1000

Mice

$PAD4^{-/-}$ were kindly provided by K. Mowen, Scripps Institute, La Jolla, CA, USA and have been described previously (8). All mice studied were on the C57Bl/6 background. Mice aged 6–14 weeks were used for experimental procedures. All mice were kept under specific pathogen-free conditions at the animal facility of the University of Erlangen. Experimental procedures were approved by the local committees of Lower Franconia.

Cell Isolation Procedures

Human peripheral blood neutrophils were isolated from healthy donors and a patient suffering from CGD (hypomorphic gp91 mutation) after informed consent in agreement to local ethical regulations (Application No. 4489) and separated using PanColl (PanBiotech, Germany) density gradient centrifugation. Granulocytes were enriched from the erythrocyte pellet by

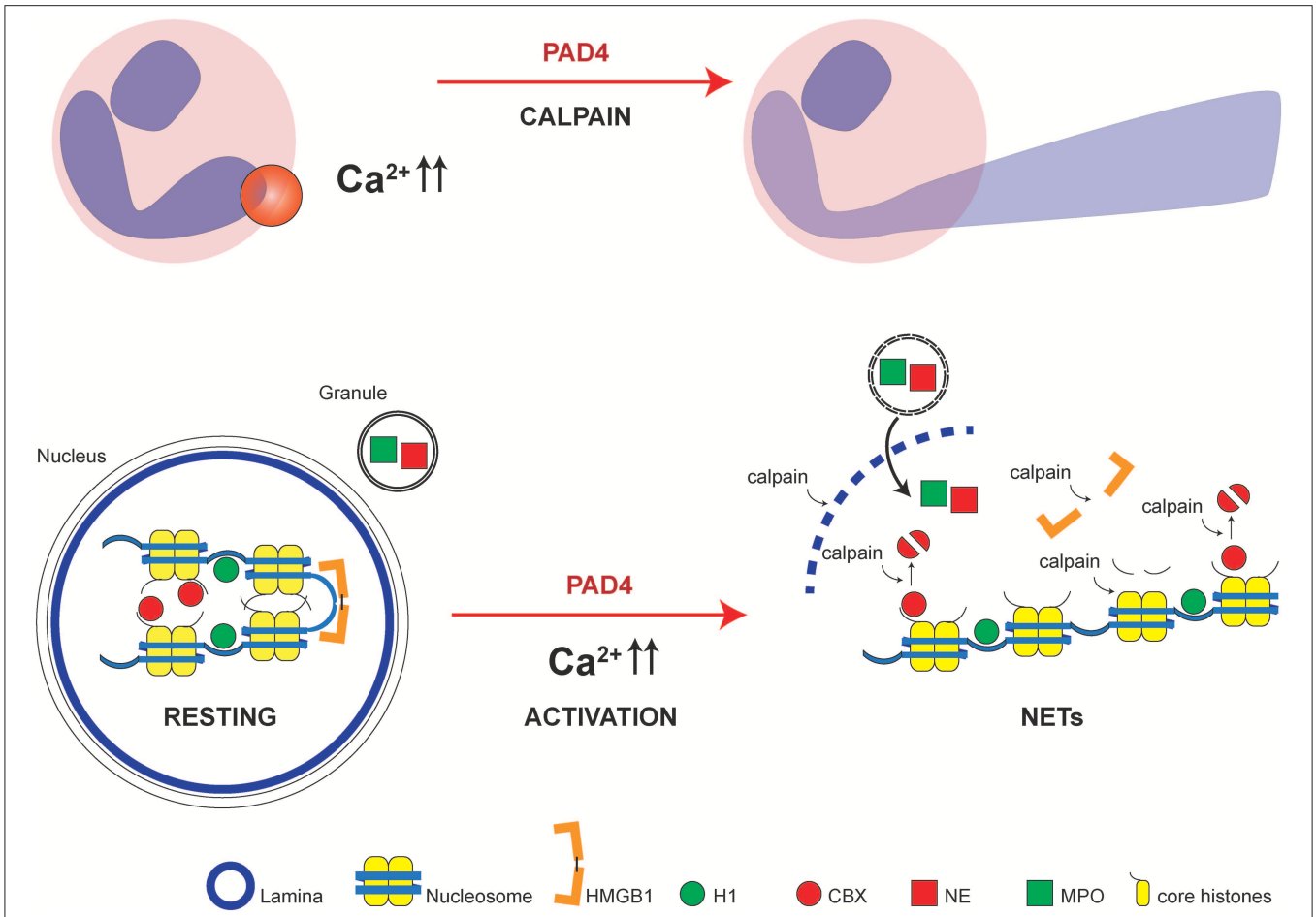

FIGURE 7 | Model of NET formation dependent on calpain and PAD4. Localized PAD and calpain activity allows local nuclear lamina breakdown and chromatin decondensation leading to disturbed polar protein interactions due to citrullination and consecutively increased accessibility and facilitated proteolytic cleavage by calpain of protein substrates. Cytosol-wide calcium increases in response to calcium ionophores induce wide-spread chromatin decondensation reminiscent of NET formation. High mobility group box protein 1 (HMGB1), histone H1 (H1), chromobox heterochromatin protein (CBX), neutrophil elastase (NE) and myeloperoxidase (MPO), and core histones with N-termini are highlighted.

consequent dextrane sedimentation (60 min, 1%, Carl Roth, Germany). The purity of neutrophil isolations was routinely above 90%. Murine neutrophils were isolated from the peritoneal cavity by peritoneal lavage of HBSS-EDTA solution and subsequent centrifugation on day 1 after thioglycolate instillation (3%) to the peritoneal cavity. Cells were counted and directly recultured in HBSS-based media (HBSS, 2 mM $CaCl_2$, 4 mM $NaHCO_3$) for further stimulation.

Neutrophil Stimulations and Treatment With Pharmacological Inhibitors

Neutrophil suspensions of human and murine origin were cultured in Hank's buffered saline solution containing 4 mM $NaHCO_3$ and 2 mM $CaCl_2$ and stimulated with the biochemical stimuli phorbol myristate acetate (Cayman Chem, 50 nM) and ionomycin (Cayman Chem, 5 μM) in the presence or absence of the peptidylarginine deiminase inhibitors Cl-amidine (Merck Millipore, 200 μM), BB-Cl-amidine (Cayman Chem, 20 μM), and the cysteinyl protease inhibitors E64c (Santa Cruz, 50 μM) and PD150606 (Santa Cruz, 50 μM). Pharmacological inhibitors were allowed to preincubate in the cell culture at 37°C/5%CO_2 for 1 h before stimulation. Neutrophil nuclear morphology was assessed directly after 120–180 min of cell culture via SYTOX Green-mediated chromatin staining and fluorescence microscopy.

Confocal Laser-Scanning Microscopic Analysis of Neutrophils

Neutrophil granulocytes were isolated, stimulated and consecutively processed for immune cytochemistry as indicated above. Stained glass slides (ibidi, BD Biosciences) were subjected to confocal laser-scanning microscopy (Leica inverted SP5) and Z-Stacks were acquired. Maximum intensity projections of Z-stacks (4.5, 0.5 μm slices) are depicted. Further image processing was achieved by Leica, Image J and Adobe Photoshop software.

Determination of Chromatin Decondensation

Size and area calculations of enzymatically treated nuclei and neutrophils were performed using fluorescent images of SYTOX Green-stained cell and nuclear cultures assisted by Adobe Photoshop measurement tools. Mean, median and standard deviation were calculated using Microsoft Excel. Mean of the median of single independent experiments and the standard error was calculated and presented. In additional approaches neutrophil nuclear morphology was determined from confocal laser-scanning microscopic pictures after immune cytochemistry.

Platereader-Based Quantification of Fluorescence

Plates containing polymorphonuclear granulocyte (PMN) cultures were analyzed under the conditions described above for 180 min in an infinite® 200 plate reader (TECAN, Germany). Excitation was performed at 488 nm and emission was detected at 523 nm. ROS were detected using H2-DCFDA and neutrophil chromatin was detected using SYTOX Green (ThermoFisher Scientific). Trypsin-mediated cleavage of H-Cit-AMC and H-Arg-AMC (25 μM, Bachem, Sigma Aldrich) was assessed in trypsin activity buffer (67 mM Sodium Phosphate Buffer, pH 7.6 at 25°C).

Immunocytochemistry

Immunofluorescence of cells stimulated in BD Culture Slides was performed as described below and recorded on either a confocal laser scanning-microscope or a standard fluorescence microscope (Leica, Germany) using overnight hybridization with primary Abs specific for the target proteins. Concentrations and antibody sources can be deduced from the table above. Detection was performed using directly labeled Alexa 488 or Alexa 555-conjugated target species antibodies (Abcam, 1:200–1:1000). Before examination, the nuclei were counterstained with either SYTOX Green or NucRed (Invitrogen Molecular Probes, Karlsruhe, Germany; BD, Heidelberg, Germany).

Nucleus Isolation Protocol

A549 cells and mouse embryonic fibroblasts were grown in T75 culture flasks in DMEM media, pelleted and washed in NB nucleus isolation buffer [10 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl2, 1 mM DTT, PMSF, phosphoSTOP (Roche)]. Cells were lysed in NB+0.2% Triton-X for 30 min with intermittent vortexing. Lysed cells were washed in NB buffer and subjected to a sucrose gradient (NB buffer + 30% sucrose, 10′ centrifugation at 1,300 rpm, 4°C) and washed. A sample of the isolated nuclei was counterstained with Hoechst and the yield was microscopically counted. Isolated nuclei were enzymatically processed as indicated below or transferred to storage buffer (50 mM Tris-HCl pH 7.5, 250 mM Sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) for cryoconservation and subsequent protein isolation procedures.

Enzymatic Treatment of Proteins and Nuclei

Isolated nuclei, recombinant histones and nuclear protein fractions were enzymatically treated with recombinant PAD4 (Cayman Chem, 5 μM) for 90 min in PAD activity buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT). Consecutive incubation in the presence of human calpain-1 (Sigma) was performed for 4–16 h without a change in buffer conditions. For blotting and detection of citrullination and cleavage, recombinant proteins (2 μg per lane) and nuclear protein lysates (15 μg per lane) were consecutively treated with PAD4 (112 ng/624 ng) and human calpain-1 (400 ng/1 μg). Nuclei were digested by neutrophil elastase (60 nM, Sigma) in 100 mM Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT after treatment with or without recombinant PAD4 (5 μM).

Protein Isolation Techniques

Subcellular protein fractions were isolated using a ready-made kit following the instructions by the manufacturer (ThermoFisher Scientific). Granulocyte proteins and proteins of enzymatically treated isolated nuclei were isolated after adding HALT protease inhibitor (ThermoFisher Scientific) directly to the culture well and subsequent prompt heat denaturation at 95°C for 5 min in LDS gel loading buffer. Protein quantification was performed using Bradford reagent (Carl Roth). For further analysis, Western blots were performed after SDS-PAGE using ready-made gels (Bio-Rad). Digital image acquisition was performed using a Bio-Rad ChemiDoc Imaging System (Bio-Rad). Images of molecular size markers and specific Western Blots were merged using the manufacturer's recommended software. Nitrocellulose membranes of western blots were reprobed after washing in Tris buffered saline, 0.1% Tween-20 (TBS/T) and stripping in Tris-HCl pH 6.8, SDS and β-mercaptoethanol.

Mass Spectrometric Protein Identification

Bands from Coomassie-stained gels were excised using a sterile scalpel. The proteins were identified following trypsin in-gel digestion by matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) MS via peptide mass fingerprinting using a Bruker Ultraflex TOF/TOF MALDI instrument (Bruker Daltonics, Bremen, Germany). The instrument was operated in the positive-ion reflectron mode using 2,5-dihydroxybenzoic acid and methylenediphosphonic acid as a matrix. Sum spectra consisting of 200–400 single spectra were acquired. For data processing and instrument control, the Compass (version 1.1) software package, consisting of FlexControl (version 2.4), FlexAnalysis (version 3.0), and BioTools (version 3.0), was used. The proteins were identified via a MASCOT peptide mass fingerprint search (Matrix Science) using the NCBInr database.

Statistical Analysis

Data were analyzed as indicated using the unpaired Student's *t*-test using Microsoft Excel (Microsoft, Redmond, WA) or

an analysis of variance (ANOVA) with *post hoc* Tukey HSD tests as indicated. When appropriate, correction for multiple testing was performed. Figure preparation was performed using Adobe Creative Suite CS5/CS6, PyMOL and the Microsoft Office Suite 2010.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission UK Erlangen. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Regierungsbezirk Unterfranken, Germany.

AUTHOR CONTRIBUTIONS

ML conceived the study and designed the experiments. SG, AL, CM, and ML performed the experiments. ML drafted the manuscript. PG, MB, MH, and NN-B provided valuable experimental tools and advice for this study, mass spectrometry was performed and evaluated by GL. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02481/full#supplementary-material

Figure S1 | PAD4 prepares nuclei for calpain-mediated proteolysis. **(A)** Isolated MEF nuclei were subjected to human calpain-1 (1 μg) for 16 h after preincubation in the presence or absence of PAD4 (5 μM) for 90 min. **(B)** Nuclear chromatin area of SYTOX-Green-stained single nuclei was quantified (at least 100 nuclei per one of 3 independent experiments, depicted as mean + SEM, scale bar = 20 μm), $^{***}p < 0.001$, ANOVA and *post-hoc* Tukey HSD test).

Figure S2 | Subcellular fractionation and protein isolation for subsequent enzyme treatment. **(A)** A549 cells were subjected to subcellular fractionation and protein isolation according to manufacturer's instructions using the respective Thermo Scientific Kit. **(B)** Purity of the fractionation was assessed using Western Blotting of compartment-specific proteins (GAPDH, ATP1A1, PARP, HISTH3, VIM). **(C)** Isolated A549 nuclear proteins were subjected to the enzymatic activity of either calpain-1, PAD4 or the consecutive combination of both enzymes (PAD4 90 min, calpain-1 90 min). Selective bands were analyzed using LC/MS. The proteins in the indicated bands in lanes 1, 3, 4, and 6 were identified as Filamin A (Mascot-Score 211-306) and alpha-Actinin 4 (Mascot-Score 131-163).

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Cytokine Production and NET Formation by Monosodium Urate-Activated Human Neutrophils Involves Early and Late Events, and Requires Upstream TAK1 and Syk

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Gout is a prevalent and incapacitating disease triggered by the deposition of monosodium urate (MSU) crystals in joints, which are also massively infiltrated by neutrophils. The interaction of the latter with MSU crystals triggers several responses, including the generation of inflammatory mediators and of neutrophil extracellular traps (NETs). Though some of the signaling events mobilized by MSU in neutrophils have been described (e.g., Src family kinases, Syk, PKC, PI3K), the picture remains fragmentary. Likewise, the impact of these signaling events on cellular responses is incompletely understood. In this study, we examined transcriptomic changes triggered by MSU in neutrophils and their impact on the corresponding proteins, as well as the role of various signaling pathways in prominent functional responses. We report for the first time that neutrophils can secrete the monocyte chemoattractant, CCL4, in response to MSU. Accordingly, we found that transcription factors NF-κB, CREB, and C/EBP are belatedly activated by MSU crystals, and at least the former is involved in chemokine generation. Moreover, we show that MAPKs and Akt are activated by MSU in neutrophils, that they are under the control of TAK1 and Syk, and that they participate in cytokine generation and NETosis. In the latter instance, we found the phenomenon to be independent of endogenous ROS, but under the control of PAD4. We finally provide evidence that endogenous factors contribute to the belated phosphorylation of kinases and transcription factors in response to MSU. Collectively, our findings unveil potentially important therapeutic targets for gouty arthritis.

Keywords: neutrophils, signaling, cytokines, NETs, transcription factors

INTRODUCTION

Gout is a prevalent disease (about 1 in 50 people will develop it over a lifetime) that is very painful and incapacitating (recurring gout attacks can cause permanent joint damage). One clear distinction between gout and other arthritides is that its causative agent is known. Deposition of insoluble monosodium urate (MSU) crystals in the joint triggers an acute inflammatory reaction

that is partially initiated and driven by neutrophils. Accordingly, the main mediators detected in the synovial fluid of gouty joints (i.e., IL-1β, IL-6, CXCL8, CCL3, TNFα), whether in humans (1) or in animal models (2), can all be secreted by neutrophils. More compellingly, neutrophil depletion suppresses the inflammatory response to MSU in canine joints (3, 4). Likewise, colchicine, an effective (but poorly tolerated) treatment for acute gout, potently inhibits numerous neutrophil functions (5). Together, these observations leave little doubt that neutrophils and their products represent important elements in the pathogenesis of gout.

Interactions between neutrophils and MSU crystals are known to elicit several responses. One of the first to be documented was the production of reactive oxygen species (ROS) and the concurrent release of anti-microbial peptides and proteolytic enzymes (6, 7). Neutrophils were also shown to synthesize and release the potent neutrophil chemoattractant, leukotriene B4, as well as other neutrophil chemotactic factors in response to MSU (8–11). Likewise, MSU-activated neutrophils can secrete cytokines and chemokines in response to MSU, namely IL-1β, IL-1ra, and CXCL8 (12–14). Neutrophils stimulated with MSU crystals also display a significantly delayed apoptosis (15, 16), which presumably contributes to their increased recruitment and persistence during active gouty inflammation. Finally, the ability of MSU to elicit the generation of neutrophil extracellular traps (NETs) was recently reported (17, 18).

Because of the numerous actions of MSU crystals toward neutrophils, several studies have focused on the underlying mechanisms; despite this however, our knowledge of the signaling pathways being mobilized remains fragmentary. It has been shown, for instance, that MSU rapidly triggers the phosphorylation of several neutrophil proteins on tyrosine residues, and that accordingly, tyrosine kinases such as Syk and members of the Src family are rapidly activated by the crystals in these cells (19, 20). Other kinases, namely conventional PKCs, were reported to be activated by MSU in neutrophils, and there is evidence that these PKCs can associate with Syk, resulting in its phosphorylation and interaction with PI3Ks (21, 22). Finally, studies involving pharmacological inhibitors have indicated that Src family kinases, Syk, and PI3Ks act as key signaling molecules for MSU-elicited degranulation, ROS production, generation of chemotactic activity, and NETosis in neutrophils (10, 17, 20, 21).

In view of the prevalence of gouty arthritis and of the neutrophil involvement in its pathogenesis, a better understanding of both MSU-elicited responses and of their molecular bases is clearly needed. In this regard, our previous work has provided several potential clues, insofar as we have shown the crucial involvement of TAK1, MAPKs, PI3K, and Syk in cytokine generation, delayed apoptosis, and NETosis in response to several physiological neutrophil stimuli (23–27). Under the same stimulatory conditions, we have also established that several transcription factors (e.g., NF-κB, C/EBP, CREB) drive cytokine production in neutrophils (23, 26, 28, 29). These observations raise the possibility, that some of the same kinases (in addition to Syk and PI3K) and transcription factors similarly control MSU-elicited responses. In this study, we examined the genomic and proteomic changes triggered by MSU in neutrophils, as well as the role of various signaling pathways in this and other functional responses. We now report for the first time that neutrophils can secrete CCL4 in response to MSU. Accordingly, we found that transcription factors NF-κB, CREB, and C/EBP are belatedly activated by MSU crystals, and at least the former is involved in cytokine generation. Moreover, we show that MAPKs are activated by MSU in neutrophils, that they are under the control of TAK1 and/or Syk, and that they participate in cytokine generation and NETosis.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against P-Akt (#4060), P-ERK (#9101), P-p38 (#9212), P-Src (#2101), P-Syk (#2711), P-C/EBPβ (#3084), P-CREB (#9191), P-RelA (#3031), IκBζ (#9244), and MAP3K8 (#4491) were all from NEB-Cell Signaling (Danvers, MA, USA); antibodies against IκB-α (sc-371) and β-actin (sc-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ficoll-Paque Plus was from GE Biosciences (Baie d'Urfé, Qc, Canada); endotoxin-free (< 2 pg/ml) RPMI 1640 was from Wisent (St-Bruno, Qc, Canada). MSU was from Cayman Chemical (Ann Arbor, MI, USA); recombinant human cytokines were from R&D Systems (Minneapolis, MN, USA); UltraPure LPS (from *E. coli* 0111:B4) was from InvivoGen (San Diego, CA, USA). Actinomycin D, cycloheximide, culture-grade dimethyl sulfoxide (DMSO), N-formyl-methionyl-phenylalanine (fMLP), and phenylmethanesulphonyl fluoride (PMSF) were from Sigma-Aldrich (St. Louis, MO, USA). Diisopropyl fluorophosphate (DFP) was from Bioshop Inc. (Burlington, Ont., Canada). The protease inhibitors, aprotinin, 4-(2-aminomethyl)benzenesulfonyl fluoride (AEBSF), leupeptin, and pepstatin A, were all from Roche (Laval, Qc, Canada). Kinase inhibitors and fluorescent probes were purchased through Cedarlane Labs (Missisauga, Canada). PlaNET Blue reagent was from Sunshine Antibodies (https://sunshineantibodies.com/planet-002.html). All other reagents were of the highest available grade, and all buffers and solutions were prepared using pyrogen-free clinical grade water.

Cell Isolation and Culture

Neutrophils were isolated from the peripheral blood of healthy donors, following a protocol that was approved by an institutional ethics committee (Comité d'éthique de la recherche du CIUSS de l'Estrie-CHUS). The entire procedure was carried out at room temperature and under endotoxin-free conditions, as described previously (30). Purified neutrophils were resuspended in RPMI 1640 supplemented with 5% autologous serum, at a final concentration of 5×10^6 cells/ml (unless otherwise stated). As determined by Wright staining and FACS analysis, the final neutrophil suspensions contained fewer than 0.1% monocytes or lymphocytes; neutrophil viability exceeded 98% after up to 4 h in culture, as determined by trypan blue exclusion and by Annexin V/propidium iodide FACS analysis.

Immunoblots

Samples were prepared, electrophoresed, transferred onto nitrocellulose, and processed for immunoblot analysis as previously described (26, 31).

RNA Extractions, Real-Time PCR Analyses, and Gene Microarray Analyses

Procedures and primers used are exactly as described (28). When samples were prepared for gene microarray analysis, total RNA from 5×10^7 neutrophils was isolated as described (28), purified using a Qiagen RNeasy MinElute cleanup kit, and processed for gene microarray analysis using the Affymetrix Human Gene 2.0 ST chip (Génome Québec, Montréal, QC, Canada).

ELISA Analyses

Neutrophils (3×10^6 cells/600 μl) were cultured in 24-well plates at 37°C under a 5% CO_2 atmosphere, in the presence or absence of stimuli and/or inhibitors, for the indicated times. Culture supernatants, as well as the corresponding cell pellets, were carefully collected, snap-frozen in liquid nitrogen, and stored at −80°C. Samples were analyzed in ELISA using commercially available capture and detection antibody pairs (R&D Systems, BD Biosciences).

NETosis Assays

The procedure used was exactly as described (27).

Data Analysis

All data are represented as the mean ± SEM. Statistical differences were analyzed by Student's *t* test for paired data using Prism 7 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Transcriptomic Changes Elicited by MSU in Neutrophils, and Its Consequences on Cognate Proteins

We first revisited the issue of the genes induced by MSU crystals in neutrophils, a response that hasn't been systematically investigated to date. The cells were initially cultured for 1 h with MSU, in an effort to detect immediate-early genes, and total RNA was processed for gene microarray analysis. Disappointingly, no transcript was induced by more than 1.8 fold; likewise, no transcript was reduced by more than 2 fold (data not shown). Thus, transcriptomic changes exerted by MSU at early stimulation times are modest at best. We repeated these experiments using neutrophils stimulated with MSU for 3 h, to determine whether gene expression changes are more pronounced at later times. As shown in **Supplementary Figure 1**, most genes examined exhibited changes in expression that were lesser than 3 fold. Despite this, several genes encoding inflammatory products were detected, whose expression was induced 3-fold or more (vs. unstimulated cells). These included IL-1-1α/β and CXCL8, as already reported (12–14), but also included transcripts that had not yet been observed to be induced in response to MSU, such as TNFα, CCL4, and Tpl2/MAP3K8 (**Supplementary Figure 2**). Other genes were similarly induced, whose products are however unknown (**Supplementary Figure 2**). When we validated these results by qPCR, we confirmed that the TNFα, IL-1β, CXCL8, CCL4, MAP3K8, and IκBζ genes were indeed strongly induced by MSU in human neutrophils (**Figure 1A**).

We next investigated whether the corresponding proteins were also upregulated in MSU-treated neutrophils. Cells were

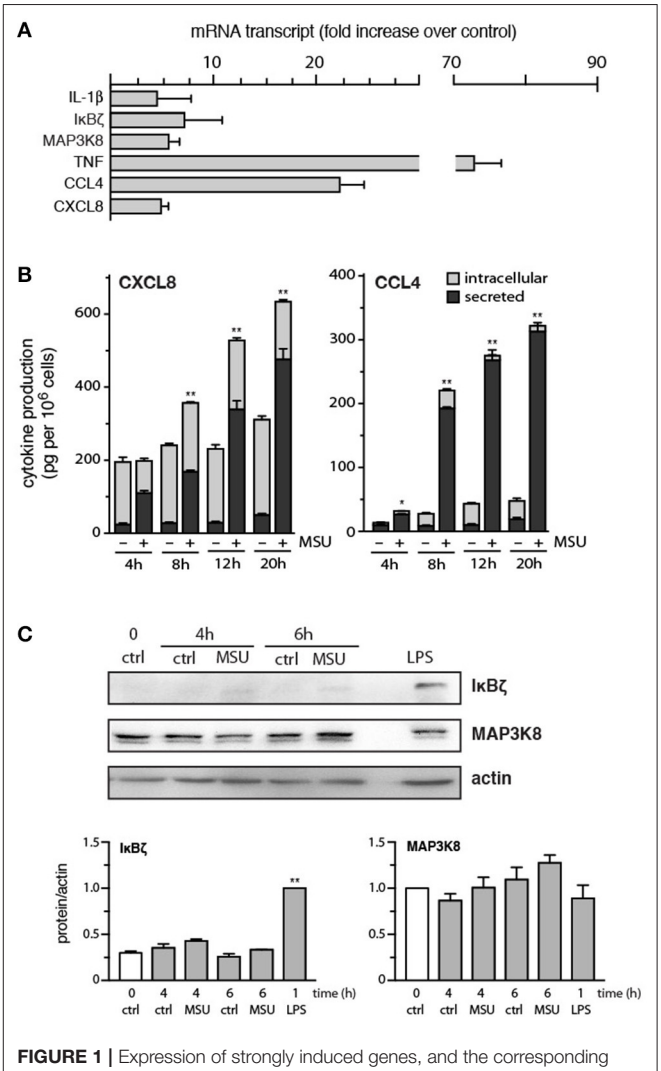

FIGURE 1 | Expression of strongly induced genes, and the corresponding proteins, in MSU-activated human neutrophils. **(A)** Cells were stimulated for 3 h with 1 mg/ml MSU, prior to RNA extraction, reverse transcription, and qPCR analysis. Values were normalized over RPL32 and are represented as fold increase relative to unstimulated cells. Mean ± s.e.m. from 3 independent experiments, each performed in duplicate. **(B)** Neutrophils were stimulated with 1 mg/ml MSU for the indicated times, prior to ELISA analysis of cell-associated chemokines and of chemokines in culture supernatants. Mean ± s.e.m. from 3 independent experiments, each performed in duplicate. $^*p < 0.05$ and $^{**}p < 0.01$ for total chemokine vs. the respective unstimulated controls. **(C)** Neutrophils were cultured in the absence ("ctrl") or presence of 1 mg/ml MSU or 1 μg/ml LPS for the indicated times, prior to immunoblot analysis of cellular IκBζ, MAP3K8, and β-actin (loading control). A representative experiment is shown, along with compiled data from at least 3 independent experiments. $^{**}p < 0.001$ vs. unstimulated control.

cultured for increasing lengths of time with the crystals, prior to ELISA or immunoblot analysis of the proteins of interest. As shown in **Figure 1B**, substantial amounts of CXCL8 and CCL4 were synthesized and secreted over time. Initially, most of the released CXCL8 came from preformed pools of the chemokine, whereas the later secretion of CXCL8 predominantly involved newly synthesized CXCL8 (**Figure 1B**). This is in contrast with the secretion of CCL4, which largely reflects the accumulation of newly-made chemokine (**Figure 1B**). By comparison, IL-1α/β or TNFα production was either undetected or at the detection limit at 20 h (data not shown). Finally, cellular levels of MAP3K8 were not significantly affected in MSU- or LPS-activated cells (**Figure 1C**). Cellular expression of IκBζ was also unchanged following MSU stimulation, though LPS did induce an accumulation of the protein, as expected (**Figure 1C**).

Signaling Cascades That Are Rapidly Elicited by MSU

Although some signaling intermediates are known to be activated by MSU in neutrophils, the picture remains incomplete; likewise, their eventual role in neutrophil functional responses needs be elucidated. When we monitored the kinetics of various signaling pathways in MSU-treated neutrophils, we confirmed that the Src and Syk pathways are quickly activated in these cells, with phosphorylated kinases slowly returning to near-baseline levels by 90 min in the case of Src, but still elevated in the case of Syk (**Figure 2A**). We additionally found that

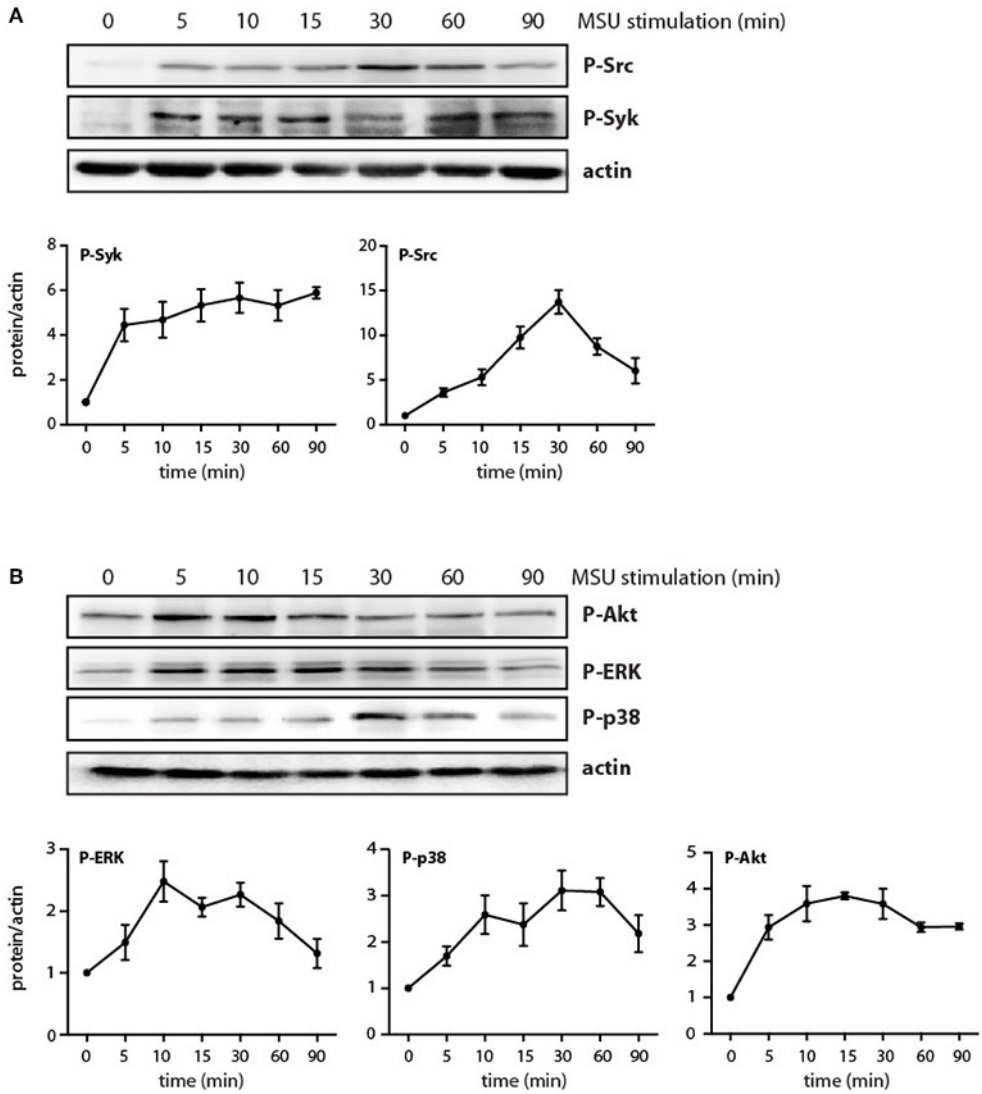

FIGURE 2 | Phosphorylation of signaling intermediates in MSU-stimulated neutrophils. Cells were stimulated with 1 mg/ml MSU for the indicated times, prior to immunoblot analysis of **(A)** cellular P-Src^{Y416} or P-Syk$^{Y525/526}$; **(B)** P-Akt^{S473}, P-ERK, or P-p38 MAPK; and β-actin (as a loading control). A representative experiment is shown in both panels, along with compiled data from at least 3 independent experiments.

MSU-stimulated neutrophils display a rapid activation of the PI3K/Akt, p38 MAPK, and ERK pathways (**Figure 2B**), with Akt showing sustained phosphorylation at 90 min, whereas p38 MAPK and P-ERK activation appeared to be more transient. By contrast, no changes were observed in cellular IκB-α levels; similarly, phosphorylated JNK was undetected; and little or no inducible phosphorylation of the transcriptional activators, RelA, C/EBPβ, and CREB, were observed under these conditions (data not shown). Thus, a discrete set of signaling pathways seem to be mobilized by MSU in neutrophils.

We have shown previously that the p38 MAPK, MEK/ERK, and PI3K/Akt cascades are controlled by the MAP3K, TAK1, in human neutrophils exposed to various physiological stimuli (24, 25, 32, 33). We therefore verified whether this is also the case in response to MSU crystals. As shown in **Figure 3A**, TAK1 inhibition mostly blocked the phosphorylation of all three kinases in response to MSU. We also reported that Syk and Src family tyrosine kinases can affect at least some neutrophil responses (26, 27) and our observation that MSU rapidly activates these kinases (**Figure 2A**) prompted us to examine whether they may also act upstream of MAPKs and Akt. As shown in **Figure 3B**, Syk inhibition profoundly hindered the phosphorylation of all three kinases, while Src inhibition only significantly affected that of p38 MAPK. Thus, both TAK1 and Syk act upstream of MAPKs and Akt, while Src family kinases contribute only to p38 MAPK activation.

Impact of Signaling Cascades on MSU-Elicited Cytokine Production, and Occurrence of Late Signaling Events

We next determined which signaling pathways contribute to MSU-induced cytokine production. To this end, neutrophils were pretreated with various inhibitors, prior to stimulation for 20 h. As shown in **Figure 4**, inhibition of TAK1, p38 MAPK, PI3K, and Syk impaired the generation of both CXCL8 and CCL4. In contrast, inhibition of the MEK/ERK or STK pathways had no significant effect on chemokine release (**Figure 4**). Blocking protein synthesis with cycloheximide, or transcription with actinomycin D, confirmed that MSU-elicited chemokine secretion largely depends on their *de novo* synthesis and gene expression, respectively (**Figure 4**). In addition, we found that pretreating neutrophils with the NF-κB blockers, MG-132 or 15-deoxy-PGJ2, profoundly ihibited chemokine production (**Figure 4**). This was quite unexpected, as both inhibitors target IκB-α degradation, which we had found not to occur following MSU exposure, at least over the first 60 min of stimulation (data not shown). This notwithstanding, we also observed that few mRNA transcripts accumulate in reponse to MSU in that time frame, requiring 3 h instead to be detected in abundance (**Supplementary Figures 1**, **2**). This prompted us to investigate whether transcription factors (and associated proteins) might be activated at later time points. As shown in **Figure 5A**, IκB-α degradation was evident by 2 h in MSU-treated neutrophils, and IκB-α levels had still

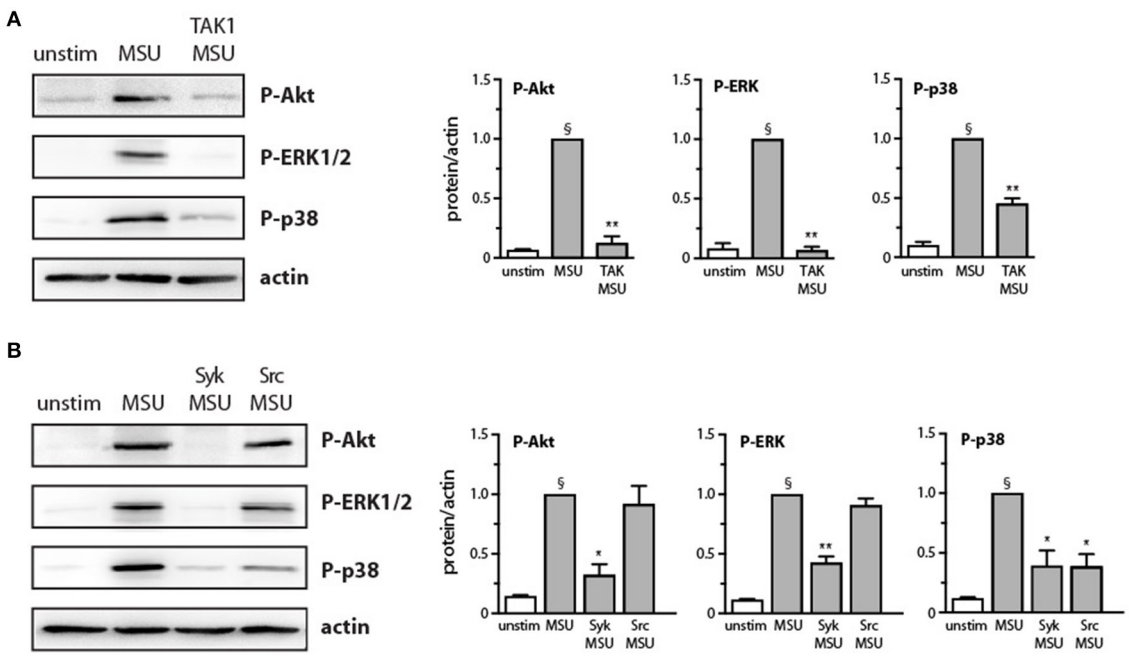

FIGURE 3 | Effect of Src, Syk, and TAK1 inhibition on Akt and MAP kinase activation in MSU-stimulated human neutrophils. Cells were pretreated for 10 min in the absence or presence of **(A)** a TAK1 inhibitor [1 μM 5(Z)-7-oxozeaenol] or **(B)** a Src inhibitor (10 μM SrcI1) or a Syk inhibitor (10 μM piceatannol), prior to stimulation for 15 min with 1 mg/ml MSU or diluent control ("unstim"). Whole-cell samples were processed for immunoblot analysis of P-Akt^{S473}, P-ERK, P-p38 MAPK, or β-actin (as a loading control). Representative experiments are shown, along with compiled data from at least 3 independent experiments. $^{§}p < 0.003$ vs. unstimulated controls; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MSU alone.

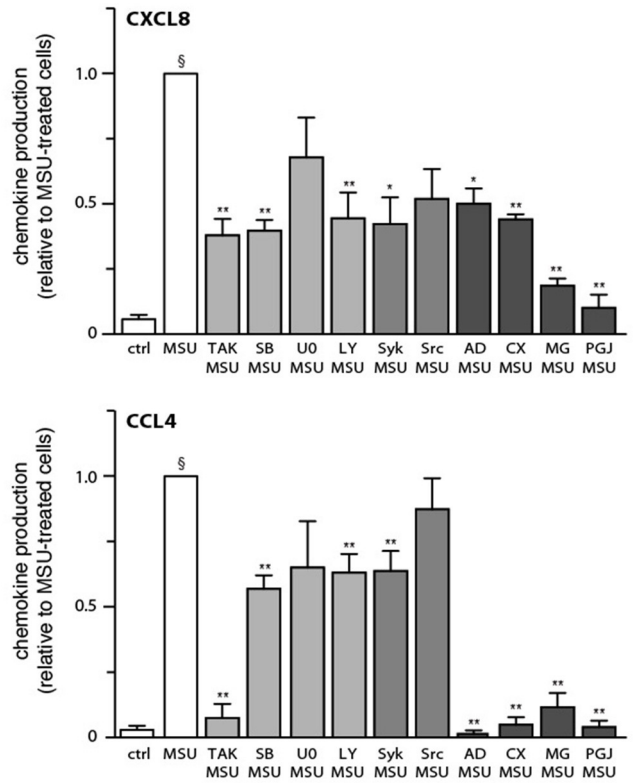

FIGURE 4 | Effect of various inhibitors on chemokine secretion in MSU-stimulated human neutrophils. Cells were pretreated for 10 min in the absence or presence of inhibitors of TAK1 [1 μM 5(Z)-7-oxozeaenol], p38 MAPK (1 μM SB202190), MEK (10 μM U0126), PI3K (10 μM LY294002), Syk (10 μM piceatannol), Src family kinases (10 μM SrcI1), transcription (5 μg/ml actinomycin D, "AD"), protein synthesis (20 μg/ml cycloheximide, "CX"), or NF-κB (1 μM MG-262 or 30 μM 15-deoxy-PGJ2). Neutrophils were then cultured in the absence ("ctrl") or presence of 1 mg/ml MSU for 20 h, prior to ELISA analysis of culture supernatants. Mean ± s.e.m. from 11 independent experiments, each performed in duplicate. Data is expressed as a ratio to MSU-stimulated cells, which amounted to 815 ± 60 pg/10^6 cells for CXCL8, and 375 ± 63 pg/10^6 cells for CCL4. $^{§}p < 0.0001$ vs. unstimulated controls; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. MSU alone.

not been replenished at 4 h of stimulation. An inducible phosphorylation of transcription factors RelA, C/EBPβ, and CREB was also found to follow a similar time course (**Figure 5A**). Thus, a belated induction of transcriptional events takes place in MSU-activated neutrophils, in keeping with the delay in gene expression.

We also found that in parallel to transcription factor phosphorylation, some kinases involved in cytokine production (e.g., p38 MAPK, Akt) were still phosphorylated at later time points (**Figure 5B**). However, they were decreasingly under the control of TAK1 or Syk (**Figure 5B**), compared to shorter stimulation times (**Figure 3**). By contrast, phosphorylated ERK remained firmly under the control of TAK1 but lost its dependence on upstream Syk (**Figure 5B**). The occurrence of phosphorylated transcription factors and kinases at late time points prompted us to investigate whether endogenously released factors might account for the phenomenon. To this end, neutrophils were stimulated for up to 2 h with MSU and the resulting culture supernatants were collected, depleted of MSU crystals, and used to stimulate fresh neutrophils for 10 min. As shown in **Figure 6**, supernatants from MSU-activated cells contain endogenous material that promotes transcription factor and kinase phosphorylation; this was especially evident in supernatants from cells that were stimulated for 2 h with MSU.

Signaling Cascades Involved in MSU-Elicited NETosis

Besides cytokine production, another major functional response of neutrophils is their ability to form NETs (34). This phenomenon was reported to occur in response to MSU crystals (17, 18, 35, 36). Using PlaNET reagents, which allow a specific, standardized assessment of NETosis (27), we confirmed these findings and could further establish that MSU represents the most potent NET inducer which we ever tested, even when compared to stalwarts like fMLP or PMA (**Figure 7A**). To determine which MSU-elicited signaling pathways influence NETosis, neutrophils were pretreated with various inhibitors, prior to being cultured with MSU. As shown in **Figure 7B**, inhibition of the TAK1, p38 MAPK, MEK, PI3K, and Syk pathways partially or totally prevented NET generation, whereas blocking Src family kinases had little or no effect on this response (**Figure 7B**). Because NETosis was initially thought to depend on endogenous ROS, and because MSU has long been known to promote the formation such molecules in neutrophils (37), we investigated whether blocking the NADPH oxidase would interfere with NET generation. As shown in **Figures 7B,C**, MSU-elicited NETosis was found to be ROS-independent, but it was largely prevented by inhibition of PAD4. Collectively, the above findings shed more light on the pathways and processes controlling NETosis in MSU-stimulated neutrophils.

DISCUSSION

Various aspects of the interaction between MSU crystals and inflammatory cells involved in gout pathogenesis have been studied in the last decades. Despite this, many gaps in our knowledge remain. In this study, we revisited the genomic changes triggered by MSU in neutrophils, their impact on the corresponding proteins, and the signaling pathways controlling MSU-elicited functional responses. This allowed us to uncover a new chemokine secreted in response to MSU; three trancription factors belatedly activated by the crystals; and signaling intermediates acting upstream of cytokine generation and NET formation.

Though some neutrophil genes were shown to be induced by MSU over the years, a systematic investigation of transcriptomic changes was (somewhat surprisingly) never undertaken. Herein, we found that unlike most neutrophil stimuli, which induce early gene expression within 30 min, MSU does not even modulate mRNA steady-state levels 2-fold over a 60-min stimulation.

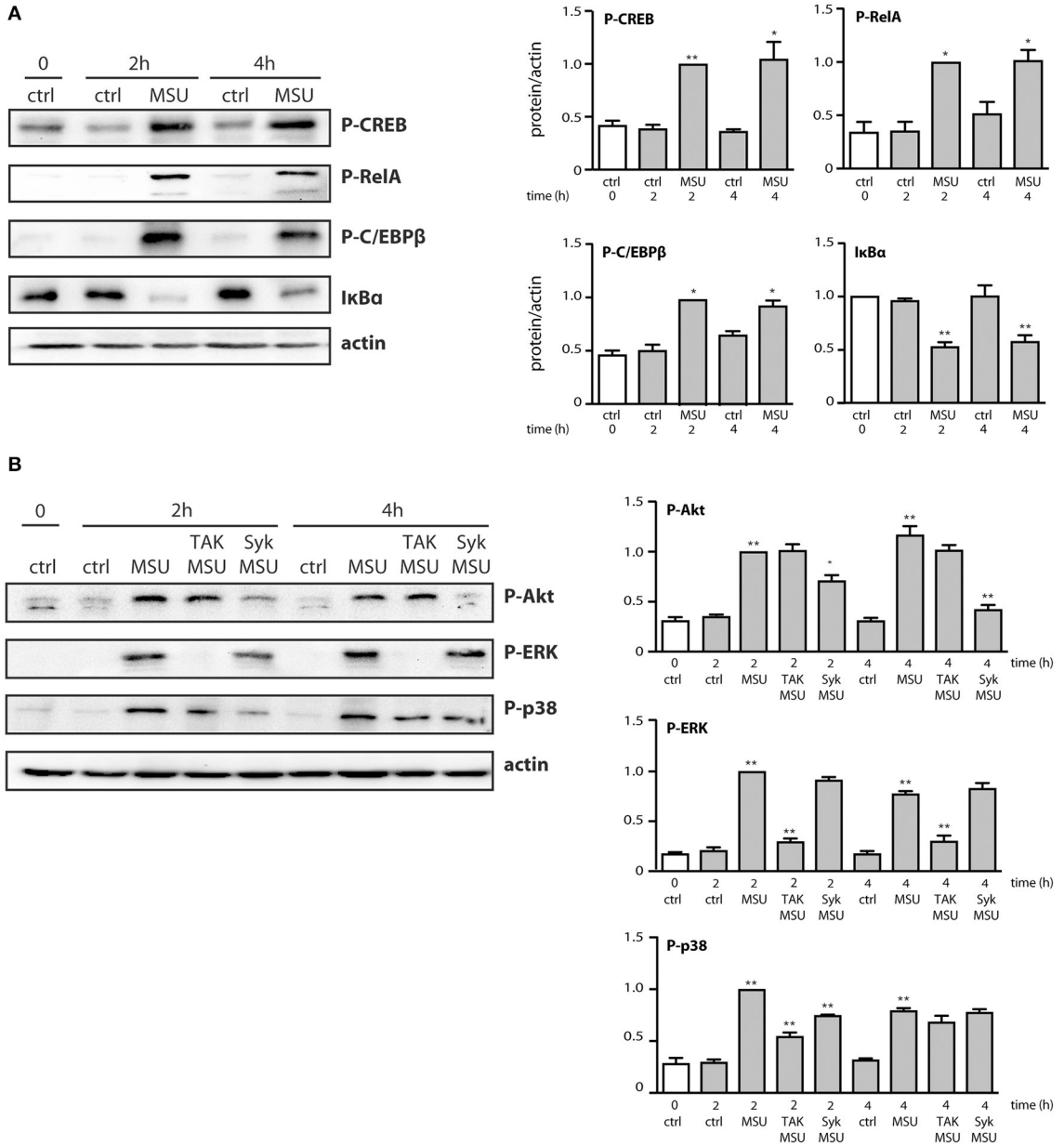

FIGURE 5 | Belated phosphorylation of transcription factors and associated proteins in MSU-stimulated human neutrophils. Cells were stimulated for the indicated times in the absence ("ctrl") or presence of 1 mg/ml MSU, prior to immunoblot analysis of cellular P-CREB^{S133}, P-RelA^{S536}, P-C/EBPβ^{T235}, IκB-α, and β-actin (as a loading control). **(A)** A representative experiment is shown, along with compiled data from at least 3 independent experiments. *$p < 0.05$ and **$p < 0.01$ vs. respective unstimulated controls. **(B)** Neutrophils were pretreated for 10 min in the absence or presence of a TAK1 inhibitor [1 μM 5(Z)-7-oxozeaenol] or a Syk inhibitor (10 μM piceatannol), prior to stimulation for the indicated times with 1 mg/ml MSU or diluent control ("ctrl"). Samples were then processed for immunoblot analysis using P-Akt, P-ERK, P-p38 MAPK, or β-actin (as a loading control). A representative experiment is shown, along with compiled data from 3 independent experiments. *$p < 0.05$ and **$p < 0.01$ vs. respective unstimulated controls.

After 3 h however, the expression of numerous transcripts was up- or down-regulated. Among those whose accumulation was induced 3-fold or more were previously reported transcripts such as IL-1α/β and CXCL8 (12–14), but also others that had never been observed before. Among the latter, some encode inflammatory mediators (e.g., CCL4, TNFα) or signaling machinery components (e.g., MAP3K8, IκBζ). Yet this still represents relatively few genes overall, especially when compared to classical neutrophil stimuli (such as LPS or TNFα) which, unlike MSU, strongly promote the expression of dozens of genes. Compounding the relative paucity of transcripts induced by MSU, is our observation that even fewer of the corresponding

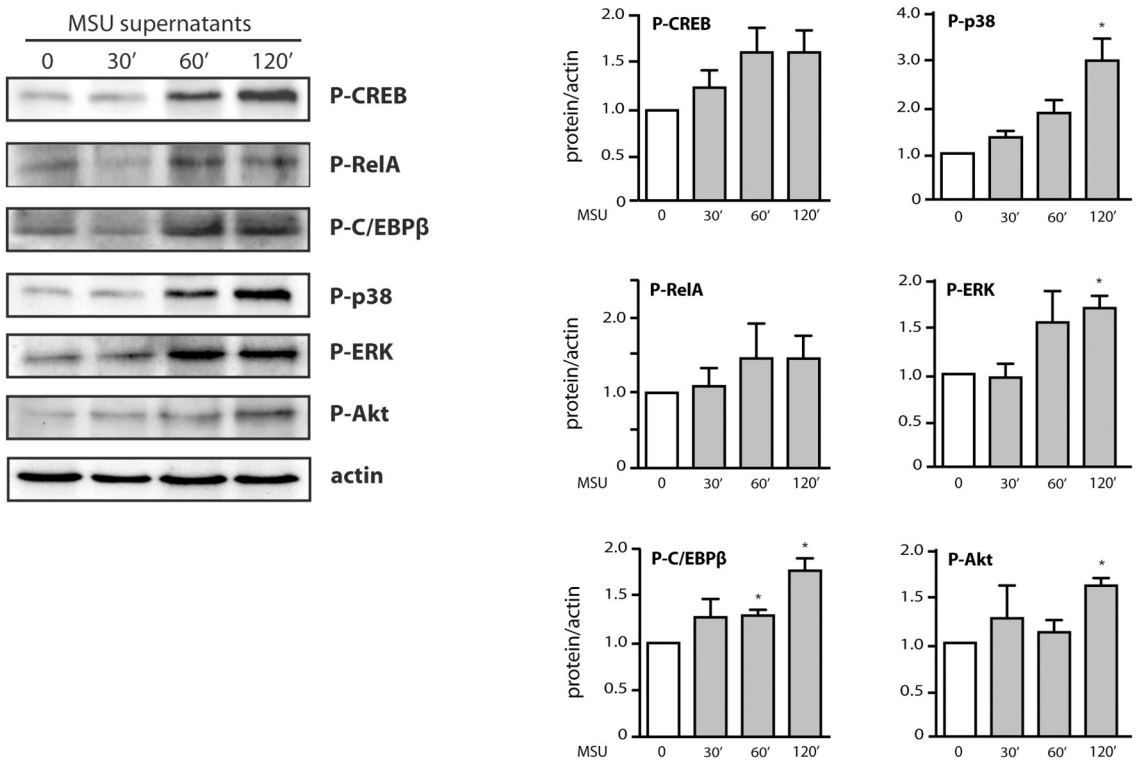

FIGURE 6 | Contribution of endogenous factors to the belated transcription factor and kinase activation observed in MSU-stimulated neutrophils. Cells were incubated for the indicated times in the presence of 1 mg/ml MSU; culture supernatants were collected, depleted of MSU crystals by centrifugation (15,000 g, 10 min), and incubated with fresh neutrophils for 10 min. Samples were then processed for immunoblot analysis of cellular P-CREB^{S133}, P-RelA^{S536}, P-C/EBPβ^{T235}, P-Akt^{S473}, P-ERK, P-p38 MAPK, and β-actin (as a loading control). A representative experiment is shown, along with compiled data from at least 3 independent experiments. *$p < 0.05$ vs. cells incubated with control supernatants.

proteins actually accumulate. A striking example is that of TNFα, whose gene was induced some 70-fold, yet without any detectable accumulation of intracellular cytokine. This raises the intriguing possibility, that MSU fails to fully mobilize the translational machinery of neutrophils; studies are in progress to elucidate this conundrum. Whatever the case may be, our data represents the first report that CCL4 can be secreted by MSU-treated neutrophils. This finding has potentially important biological implications, insofar as MSU-activated neutrophils can not only contribute to their own recruitment into inflamed joints by generating CXCL8, but can also attract monocytes through their ability to secrete CCL4. In keeping with this notion, both neutrophils and monocytes are recruited by MSU crystals.

The signaling events triggered by MSU crystals in neutrophils have been only partially elucidated to date. It has been shown, for instance, that Src family kinases, Syk, PKCs, and PI3Ks are activated upon MSU challenge (19–22). We confirmed herein that Syk and Src are rapidly phosphorylated in response to MSU; whereas this response was sustained for of P-Syk (for at least 90 min), it was transient in the case of P-Src. Importantly, we found that p38 MAPK, ERK, and Akt were also rapidly phosphorylated in MSU-stimulated cells, and that the phospho-proteins were still detected after 90 min. In the case of p38 MAPK, our data confirm and extend recent observations by Rousseau et al. (38), who however only detected weak p38 phosphorylation over a 5-min interval. By comparison, our data represents the first demonstration that ERK and Akt^{Ser473} can also be activated by MSU. Thus, the kinases activated by MSU are essentially the same as those mobilized by several physiological neutrophil agonists (23, 25, 26, 33, 39, 40). Morevoer, we found that the MSU-elicited phosphorylation of p38 MAPK, ERK, and Akt occurs downstream of TAK1 and Syk, much like it does in response to several classical neutrophil stimuli (24, 33). Thus, the undetectable synthesis of several proteins despite strongly induced corresponding genes in MSU-treated cells, cannot be attributed to a general defect in signaling. However, we observed that the extent to which Syk, Src, MAPKs, and Akt are phosphorylated is lower in response to MSU crystals, compared to classical stimuli such as LPS and TNFα. This notwithstanding, we showed that the Syk, TAK1, p38 MAPK, MEK/ERK, and PI3K/Akt pathways all contribute to chemokine generation and/or NETosis. Therefore, even a relatively weaker activation of these kinases by MSU is sufficient to entail functional consequences. On final note, it has been reported that the MSU-elicited synthesis and secretion of IL-8

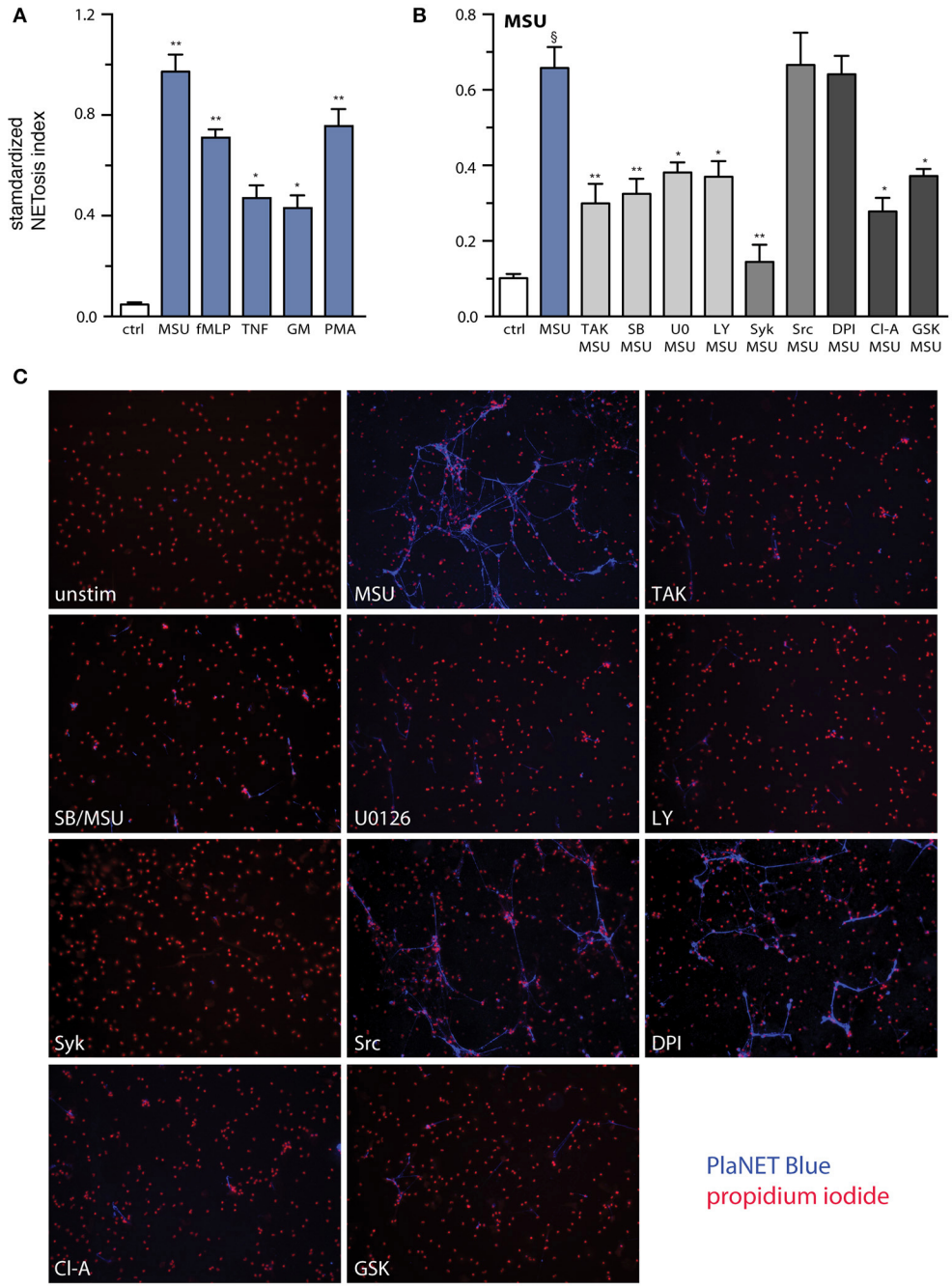

FIGURE 7 | Relative potency of MSU as a NET inducer, and signaling pathways controlling this response. **(A)** Neutrophils cultured on poly-L-lysine-coated coverslips were incubated for 4 h in the absence ("ctrl") or presence of 1 mg/ml MSU, 30 nM fMLP, 100 U/ml TNFα, 1 nM GM-CSF, or 50 nM PMA. NETosis was assessed using PlaNET Blue as described in *Methods*. Mean ± s.e.m. from 3 independent experiments. $^{*}p < 0.02$ and $^{**}p < 0.01$, relative to unstimulated cells. **(B)** Neutrophils cultured as described in **(A)** were pre-treated (15 min, 37°C) with the following inhibitors or their diluent (culture-grade DMSO): 1 μM (5Z)-7-oxozeaenol (TAK1 inhibitor); 1 μM SB202190 (p38 MAPK inhibitor); 10 μM U0126 (MEK inhibitor); 10 μM LY294002 (PI3K inhibitor); 10 μM piceatannol (Syk inhibitor); 10 μM SrcI1 (Src family kinase inhibitor); 10 μM DPI (a NADPH oxidase inhibitor); 10 μM chloraminidine ("Cl-A," a general PAD inhibitor); or 10 μM GSK484 (a PAD4 inhibitor). The cells were then further incubated for 4 h in the absence ("ctrl") or presence of 1 mg/ml MSU. NETosis was assessed using PlaNET Blue as described in *Methods*. Mean ± s.e.m. from at least 4 independent experiments. $^{§}p < 0.002$ vs. unstimulated control; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. stimulus alone. **(C)** Representative fields for each experimental condition shown in **(B)**, at 10X magnification.

in monocytes is dependent on the activity of Src kinases and of ERK1/2 (41, 42), whereas we found herein that Src inhibition had little impact on CXCL8 generation in neutrophils. This indicates that among the various signaling pathways mobilized by MSU, different combinations contribute to a given response depending on the cell type.

Another novel finding reported herein is that the NF-κB, C/EBP, and CREB transcription factors are activated in response to MSU crystals in neutrophils. This agrees well with the fact that both CXCL8 and CCL4, whose transcripts and proteins are also induced by MSU, feature cognate binding sites for these transcription factors in their proximal gene promoters, that are required for induction in human granulocytes (28, 29, 43). A singular characteristic of transcription factor activation by MSU, is that it was never detected at early time points (i.e., within 15 min), as is the case with other neutrophil stimuli, such as LPS, TNFα, or IL-18 (28, 29, 31, 39). Instead, phosphorylation of RelA, C/EBPβ, and CREB1, as well as IκBα degradation, were only observed at 120 min and beyond. This belated activation mirrors the delayed induction of chemokine genes occurring in response to MSU, which was detected at 3 h. This is again in contrast with stimuli such as LPS, TNFα, or IL-18, which typically promote chemokine gene induction within 30 min or less. Thus, whereas a similar set of transcription factors can be activated by cytokines, TLR ligands, and MSU in neutrophils, the latter stimulus does so belatedly, resulting in the late induction of target genes. This is not due to a slow ingestion of the crystals, as the process takes place within 15 min (44). On the contrary, the delayed mobilization of the transcriptional machinery, and even more so the sustained activation of MAP kinases and Akt at late time points, seem to reflect (at least in part) the production of endogenous mediators. Supportive evidence stems from our observation, that the addition of supernatants from neutrophils stimulated with MSU for 120 min consistently induced the phosphorylation of kinases (p38, ERK, Akt) and of at least some transcription factors. Studies are in progress to determine the nature of the endogenous factors involved.

Finally, MSU crystals proved to be the most powerful NET inducers that we ever tested. Whereas other investigators had already reported that this response requires the PI3K, RIPK, and MLKL pathways (17, 45), we showed herein that it also involves the TAK1, p38 MAPK, MEK/ERK, and Syk pathways. With regard to the cellular processes governing NET formation, we observed that MSU-elicited NETosis is independent of ROS generation, confirming recent reports (36, 46, 47). Conversely, our finding that MSU-induced NET formation depends on PAD4, is to our knowledge a first. Thus, MSU appears to function like most other physiological neutrophil agonists (e.g., TNFα, GM-CSF, fMLP, PAF, C5a, CXCL8) with respect to the involvement of endogenous ROS and PAD4 (27). Overall, our findings substantially extend our understanding of the mechanisms underlying NET generation, by showing that MSU crystals represent yet another class of physiological stimuli (in addition to growth factors, chemoattractants, and cytokines) (27) that employ common signaling components, as well as PAD4.

In summary, MSU crystals elicit a robust induction of a limited set of genes in neutrophils, including some that had not been reported to date (e.g., CCL4, TNFα, MAP3K8, IκBζ). However, only some of the corresponding proteins are similarly induced (e.g., CXCL8, CCL4). This involves several signaling pathways (e.g., Syk, TAK1, p38 MAPK, MEK/ERK, PI3K/Akt) and downstream effectors (transcription factors NF-κB, and possibly C/EBP and CREB as well). The same signaling pathways also participate in MSU-driven NET formation. Thus, our findings unveil several potentially important therapeutic targets for acute episodes of gouty arthritis, which feature a massive neutrophil influx. The fact that inhibitors for several of these molecular targets are already undergoing clinical trials (48–51) makes an eventual translation to the patient more than a remote possibility.

DATA AVAILABILITY STATEMENT

Datasets generated for this study are available upon request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique de la recherche du CIUSSS de l'Estrie—CHUS Project #2001-18, 01-16. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OT carried out the experiments for most aspects of the paper, compiled most the data, and wrote the first draft. TM compiled and analyzed the gene microarray data. VC completed experiments for **Figures 5B**, **6**. MI and SS-P carried out the initial experiments for this project. CD provided conceptual input. PM designed the research, mentored the other authors, and wrote the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02996/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Expression, Role, and Regulation of Neutrophil Fcγ Receptors

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Neutrophils are best known for their critical role in host defense, for which they utilize multiple innate immune mechanisms, including microbe-associated pattern recognition, phagocytosis, production of reactive oxygen species, and the release of potent proteases, mediators, antimicrobials, and neutrophil extracellular traps. Beyond their well-established contribution to innate immunity, neutrophils were more recently reported to interact with various other cell types, including cells from the adaptive immune system, thereby enabling neutrophils to tune the overall immune response of the host. Neutrophils express different receptors for IgG antibodies (Fcγ receptors), which facilitate the engulfment of IgG-opsonized microbes and trigger cell activation upon cross-linking of several receptors. Indeed, FcγRs (via IgG antibodies) confer neutrophils with a key feature of the adaptive immunity: an antigen-specific cell response. This review summarizes the expression and function of FcγRs on human neutrophils in health and disease and how they are affected by polymorphisms in the *FCGR* loci. Additionally, we will discuss the role of neutrophils in providing help to marginal zone B cells for the production of antibodies, which in turn may trigger neutrophil effector functions when engaging FcγRs.

Keywords: neutrophils, Fcγ receptors, IgG, immune complexes, B cells

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INTRODUCTION

Neutrophils are key players of the innate immune response. They are the most abundant leukocytes in the human blood (4.5–11 × 10^3/mm^3). Following a circadian rhythm, neutrophils are released from the bone marrow (1–3) and circulate in the blood for 4–6 days (4, 5). If they are not attracted to sites of inflammation, they will express markers of aged neutrophils, and preferentially home to the liver, spleen, or bone marrow, where they undergo apoptosis and are cleared by resident macrophages (6–8). This immunologically silent mechanism allows for maintaining a high number of functional neutrophils in the blood (55–70% of all blood leukocytes in the periphery), while guaranteeing a quick removal of deregulated or altered neutrophils. The tight control of neutrophil homeostasis is critical for the organism as many of their effector functions [i.e., production of reactive oxygen species (ROS), release of neutrophil extracellular traps (NETs), or granules containing potent proteases and lipophosphatases (9)] bare the potential to be deleterious for the host and damage surrounding tissues and organs.

Neutrophils express various receptors that enable them to respond almost instantaneously to diverse inflammatory stimuli and danger signals. Among these, receptors for the constant region of IgG immunoglobulins (FcγRs) stand out. They bestow on neutrophils the capacity to react in an antigen-specific way—hence to acquire a key feature of the adaptive immunity. FcγRs enable neutrophils to interact with and respond to monomeric or aggregated immunoglobulins,

antigen–antibody immune complexes, and opsonized (antibody-coated) particles, cells, or surfaces. Humans express six classical FcγRs: FcγRI/CD64, FcγRIIA/CD32A, FcγRIIB/CD32B, FcγRIIC/CD32C, FcγRIIIA/CD16A, and FcγRIIIB/CD16B (**Table 1**). All these FcγRs bind at least two of the four different human IgG subclasses with association constants (K_A) ranging from 8×10^7 down to 2×10^4 M^{-1} (10).

All FcγRs, except for FcγRIIB and FcγRIIIB, are classical activating receptors. Their activating signals are transduced by an immunoreceptor tyrosine-based activation motif (ITAM) that is either present in the cytoplasmic domain of the FcγR itself (FcγRIIA/FcγRIIC) or in an associated signaling subunit, notably the FcRγ chain. Upon FcγR aggregation by multimeric ligands, Src family kinases phosphorylate these motifs, allowing the activation of a signaling cascade, involving the spleen tyrosine kinase (SYK), phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC)-γ, Rho, and Rac, resulting in calcium mobilization, cell activation, cytokine/chemokine production, and cell migration (11–13). Counterbalancing these activating FcγRs, the inhibitory receptor FcγRIIB possesses an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracytoplasmic domain. Upon its co-engagement with an activating receptor, the phosphorylated ITIM recruits the inositol polyphosphate-5-phosphatase SHIP1 (14) that negatively regulates the signaling cascades initiated by ITAM-containing receptors (15–17). Moreover, several *FCGR* polymorphisms have been described in humans, adding to the complexity of this receptor family with overlapping functions and affinities for their ligands that collaborate, regulate, or compete with each other to tune cellular responses.

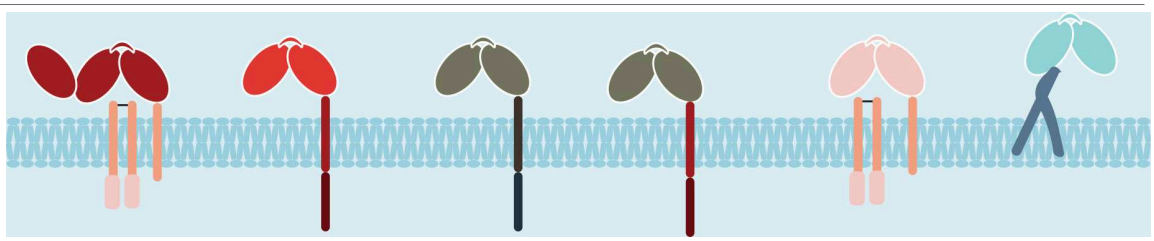
In this review, we will focus on IgG receptors (FcγRs) on neutrophils and their role and regulation in steady state and inflammatory conditions.

EXPRESSION AND ROLE OF FCγR ON NEUTROPHILS DURING HOMEOSTASIS

Blood neutrophils from healthy individuals express large amounts of a rather atypical FcγR, the FcγRIIIB. FcγRIIIB is a glycophosphatidylinositol (GPI)-anchored protein with no signaling capacity on its own. It was first described on neutrophils in 1982 with the means of a newly developed monoclonal antibody (mAb, 3G8) that also recognizes FcγRIIIA on monocytes and NK cells (18). Incubation of neutrophils with 3G8 could efficiently block binding of rabbit IgG-opsonized sheep erythrocytes and soluble rabbit IgG immune complexes (ICs), demonstrating that the newly identified receptor is an IgG Fc receptor (18). FcγRIIIB is one of the most abundant proteins on the surface of neutrophils, with each cell expressing between 100,000 and 200,000 copies (19). In resting neutrophils, the receptor is equally distributed over the cell membrane and is present in both low- and high-density detergent-resistant membranes (DRMs) (20). Additionally, intracellular storage compartments have been described that allow rapid FcγRIIIB mobilization to the cell surface upon receptor engagement (21, 22). Previously thought to have no signaling function, it is now generally accepted that FcγRIIIB can trigger neutrophil activation. Following multivalent cross-linking, FcγRIIIB accumulates in high-density DRMs (20) and elicits downstream signals, leading to Ca^{2+} mobilization, cell adhesion, and degranulation, but not to respiratory burst (23–27). The exact intracellular signaling cascade remains a matter of debate (20, 27–29), but seems to involve phosphorylation of the Src kinase Hck, mitogen-activated kinases (MAPKs) ERK (extracellular signal regulated kinase), and p38 and the tyrosine kinase Pyk2 (30–32). In this context, it is noteworthy that the 3G8 antibody, which is often used to block FcγRIIIB, can trigger

TABLE 1 | Classical FcγRs and their expression on neutrophils.

Structure



Name	FcγRI	FcγRIIA	FcγRIIB	FcγRIIC	FcγRIIIA	FcγRIIIB
CD	CD64	CD32A	CD32B	CD32C	CD16A	CD16B
Gene	*FCGR1A*	*FCGR2A*	*FCGR2B*	*FCGR2C*	*FCGR3A*	*FCGR3B*
Alleles	–	H_{131} R_{131}	I_{232} T_{232}	Q_{57} $stop_{57}$	V_{176} F_{176}	NA1 NA2 SH
Affinity	High	Low to medium	Low to medium	Low to medium	Low to medium	Low to medium
Expression on resting neutrophils	< 2,000 copies	30,000–60,000 copies	Low to none; increase when 2B4 promotor haplotype	Low to none	Low to none	100,000–200,000 copies
Neutrophil expression in inflammatory conditions	Up to 10-fold increased expression in presence of IFN-γ and G-CSF	Upregulated in presence TNF-α	Low to none; increase when 2B4 promotor haplotype	Low to none	Low to none	100,000–200,000 copies, subject to shedding

intracellular Ca-mobilization and neutrophil aggregation on its own. This cell activation requires co-engagement of another neutrophil FcγR, FcγRIIA *via* the Fc portion of the intact antibody (33). The main functions of neutrophil FcγRIIIB during homeostasis are the removal of spontaneously forming ICs from the vasculature, and the maintenance of the soluble FcγRIIIB (sFcγRIIIB) pool. FcγRIIIB-bound ICs are internalized through a mechanism used by GPI-anchored receptors and fluid-phase endocytosis (27), thereby clearing ICs without triggering further cell activation that could be deleterious to the host. sFcγRIIIB is present in serum of healthy individuals at concentrations of 5 nM (34). It is generated by proteolytic cleavage of surface FcγRIIIB on activated and apoptotic neutrophils (34, 35). Despite its relative abundance, the function of sFcγRIIIB remains elusive. sFcγRIIIB retains Fc-binding capacities and hence competes with membrane low-affinity receptors to dampen Fc-dependent immune reactions (36, 37). Due to the fact that FcγRIIIB binds to multimeric IgG1 and IgG3, but not or poorly IgG2 or IgG4 (10), the biological activity of these latter IgG subclasses should not be affected by sFcγRIIIB. Notably, both IgG2 and IgG4 also bind less well to other low-affinity FcγRs (with the exception of FcγRIIA-H131) (10). Adding to this possible immune-modulatory function, it also has been reported that sFcγRIIIB can bind to complement receptors CD11b/CD18 or CR3/Mac-1 and CD11c/CD18 or CR4 *via* lectin/carbohydrate interactions (38). These interactions can result in cytokine production by neutrophils and monocytes or may inhibit β2 integrin-dependent adhesion and subsequent transendothelial migration (38).

Neutrophils constitutively express a second low-affinity IgG receptor, the FcγRIIA. Albeit less abundant than FcγRIIIB, each neutrophil expresses between 30,000 and 60,000 copies of FcγRIIA (19). Interestingly, this receptor was described to have a lower affinity for IgG on resting than on primed or activated neutrophils (39), a feature that has been attributed to its interactions with integrins (40). Upon efficient cross-linking of FcγRIIA *in vitro*, neutrophils become activated, degranulate, and produce inflammatory mediators and ROS and trigger neutrophil extracellular trap (NET) formation (27, 41–44). More recent data, however, suggest that resting neutrophils rather poorly respond to FcγRIIA-induced activation. One possible explanation for these divergent observations may be found in the purification techniques used to isolate neutrophils. Indeed, density gradient centrifugation or dextran sedimentation used to be the standard techniques. Nowadays, neutrophils are mostly isolated by negative selection procedures that maintain the cells in isotonic buffer, but expose them to magnetic fields. Indeed, while comparative data between the procedures are sparse (45, 46), possible differences in neutrophil priming and purity need to be taken into account when interpreting the data.

In contrast to the abundant FcγRIIIB and FcγRIIA, neutrophils express less than 2,000 copies of the high-affinity FcγRI (19). Ligation of FcγRI on resting neutrophil with a specific antibody does not induce a significant degree of cell activation (47) and neutrophils show poor binding to monomeric IgG1 and phagocytosis of IgG-opsonized particles (48, 49). Neutrophils can also express FcγRIIB; however, its detection is variable among individuals ranging from low to undetectable (27, 50). Although it is well-established that co-engagement of FcγRIIB potently inhibits FcγR-driven cell activation (15–17), it is questionable if the low expression of FcγRIIB on neutrophils could oppose signals generated by the other FcγRs in this context. Finally, a very recent report suggests that neutrophils may also express modest amounts of FcγRIIIA (51), a receptor that was previously thought to be exclusively expressed by NK cells and monocytes (52). In this study, the authors report that FcγRIIIA engagement on neutrophils from FcγRIIIB-deficient and normal individuals efficiently triggers cell activation and mediates phagocytosis of IgG-opsonized beads that could not be blocked by anti-FcγRIIA $F(ab')_2$ fragments (51). Many different groups have studied FcγR expression on neutrophils before, but were incapable to affirm FcγRIIIA expression. This might be due to the fact that most antibodies used to study FcγRIII recognize both FcγRIIIA and FcγRIIIB, and that FcγRIIIB deficiency is rare. In a study from 1994, one can however appreciate some residual FcγRIII [3G8] staining on neutrophils from paroxysmal nocturnal hemoglobinuria patients [a disorder characterized by the deficiency of glycosyl phosphatidylinositol (GPI)-anchored proteins in blood cell membranes] and an FcγRIIIB-deficient patient, as well as on neutrophils treated with GPI-phospholipase C as compared to an isotype control (53). This residual binding, if specific, could support the hypothesis of a low expression of FcγRIIIA on neutrophils. The central piece of evidence for FcγRIIIA expression on neutrophils in the recent report is a single FcγRIIIB-deficient donor, whose genotype was confirmed by RT-PCR and not by sequencing of the FcγR locus. This allows speculation about a cryptic FcγRIIIB expression in this donor. Furthermore, if a low expression of FcγRIIIA can be confirmed in other FcγRIIIB-deficient donors, it will be necessary to clarify to what extent it can contribute to neutrophil effector functions *in vivo*. One point is certain, this study refuels the discussion about the capacity of FcγRIIIB to trigger cell activation and suggests that neutrophil activation observed following receptor cross-linking with anti-FcγRIII F(ab′)2 fragments or ICs can be rather attributed to FcγRIIIA than to the GPI-anchored receptor.

NEUTROPHIL FCγR IN AN INFLAMMATORY CONTEXT

Neutrophil FcγR expression can change dramatically in the context of an inflammation or infection. Notably, FcγRI is strongly upregulated in the presence of inflammatory cytokines such as interferon-γ (IFN-γ) or granulocyte colony-stimulating factor reaching up to 20,000 copies per cell (19, 48, 54, 55). This confers neutrophils the capacity to efficiently bind monomeric IgG (48), phagocytose IgG-opsonized bacteria (49), exert anti-fungal functions (56), and induce ROS production in response to FcγRI cross-linking (47). FcγRI upregulation also enables neutrophils to efficiently trigger antibody-dependent cytotoxicity (ADCC) (55). As a consequence, neutrophil FcγRI expression has been shown to reflect infection state and disease activity in numerous inflammatory conditions (57–61), and a low CD64 expression is a marker for sustained remission in Crohn's disease patients receiving infliximab (62). Consecutively, neutrophil

CD64 has been discussed as an interesting biomarker, especially in the case of sepsis (57). Sepsis diagnosis includes a blood culture that allows specific identification of the disease-causing bacteria and their antibiotic resistances, but takes up to 2 days to generate results. Precious time, during which patients with sepsis suspicion commonly receive broad-spectrum antibiotics to avoid deterioration of their condition. This common practice has it flaws, because antibiotics are not adapted in case of non-bacterial infections, and because some bacteria require specific antibiotics and are not targeted by broad-spectrum ones. Certainly, inappropriate use of antibiotics contributes to the increase of antibiotic resistance among different bacteria that has been classified as "one of the biggest threats to global health, food security, and development today" by the WHO. Neutrophil CD64 expression was proposed as a way to detect infection so that timely decisions and treatment refinement can be made. Neutrophil CD64 can be evaluated within 1–2 h, making it a rapid diagnostic and prognostic marker. Indeed, a study reported not only elevated CD64 expression in septic patients compared to healthy controls, but also a decrease in CD64 expression following treatment with an appropriate antibiotic compared to inefficient treatment (63). However, not all studies find in neutrophil CD64 a reliable marker for sepsis detection, and different studies report divergent sensitivity and specificity for sepsis detection by neutrophil CD64 (64–67). Two meta-analyses report a large heterogeneity in study design and results (68, 69). This may be due to the fact that this test can be run in any laboratory with a flow cytometer and that the results can be expressed either as percentage of neutrophils expressing CD64 or as mean fluorescent intensity of the whole neutrophil population. In the absence of a standardized assay, each laboratory needs to establish its own cutoff. Furthermore, confounding factors (previous use of antibiotics, delayed culture collection, etc.) may result in a negative result from the microbiological test that, as a consequence, poses problems with the classification of the patients. Given the low costs of the assay, neutrophil CD64 remains an interesting candidate to monitor in case of sepsis suspicion. Larger prospective studies are however required to conclude on its sensitivity and suitability as a biomarker, especially in light of new approaches for sepsis diagnosis and the evolution of our understanding of sepsis as a condition involving not only the bacterial infection and its resulting immune response, but also changes in coagulation, immunosuppression, and organ dysfunction (70).

IFN-γ treatment has little effect on neutrophil FcγRIIA expression but can induce FcγRIIB expression (albeit on a low level) and, depending on the experimental conditions, may induce FcγRIIIB down-modulation (19, 71). FcγRIIA expression on neutrophils may however be induced by TNF-α (72). As a consequence, primed neutrophils and neutrophils from individuals with inflammatory conditions show enhanced FcγR-dependent responses (73–75).

Finally, IgG ICs can also be at the onset of inflammation, allergic reactions, and autoimmunity (76, 77). This is notably the case, when the amounts of circulating ICs suddenly rise and exceed the body's capacity to silently remove them, when ICs form that are insoluble and "precipitate" onto endothelial cells, or when autoantibodies bind to large surfaces, i.e., cartilage, thereby opsonizing phagocytosis-resistant structures. All these conditions can be mimicked *in vitro* and helped to understand that soluble ICs require primed neutrophils to efficiently trigger external ROS production and degranulation, while insoluble ICs can activate unprimed neutrophils, leading to intracellular ROS production, degranulation, and sustained liberation of inflammatory mediators such as IL-8 and leukotriene B4 (LTB_4) that sustain neutrophil-driven inflammation (73, 78). An elegant study, using transgenic mice expressing either FcγRIIA or FcγRIIIB or both in the absence of endogenous activating FcγR, demonstrated that FcγRIIIB has a primordial role in the homeostatic removal of soluble ICs within the vasculature, whereas FcγRIIA engagement by soluble ICs in tissues generates NETs, a pro-inflammatory process linked to autoimmunity (27, 44). Engagement of either FcγR by deposited ICs leads to neutrophil accumulation (44). These data illustrate the specialized role of FcγRs in triggering neutrophil effector functions, despite their overlapping binding properties to IgG.

GENETIC VARIATIONS AFFECTING NEUTROPHIL FCγR EXPRESSION AND FUNCTIONS

A number of polymorphisms have been identified in the *FCGR* loci that affect their biological functions and may consequently impact the individual's susceptibility for diseases and their capacity to respond to therapies based on monoclonal antibodies. This is notably the case for polymorphisms that alter the affinity of FcγRs for IgG, thus directly affecting their capacity to clear immune complexes.

Until today, no polymorphism of FcγRI has been identified that modifies the affinity of the receptor for IgG or its associated functions. In contrast, the low-affinity IgG receptor locus on chromosome 1q23.3 coding for all *FCGR2/3* genes is home to multiple genetic variants, including single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs). These genetic variants display heterogeneity among ethnic groups (79). The best-characterized polymorphism of *FCGR2A* results in substitution of an arginine residue by a histidine at position 131 (rs1801274) that results in a receptor variant with an improved binding to IgG2 (and to a lesser extent to IgG1 and IgG3) (80). The *FCGR2A-R131* variant is therefore expected to show lower clearance of IgG immune complexes and is indeed associated with susceptibility to auto-immune disorders (81–85) and recurrent bacterial infections with encapsulated bacteria (86). The *FCGR2A*-H131 variant, on the other hand, predisposes individuals to Kawasaki disease and Myasthenia gravis (87, 88). More recently, a splice variant of *FCGR2A*, FcγRIIa(exon6*), has been described that retains a cryptic exon in the cytoplasmic tail of the receptor (89, 90). This results in a gain-of-function allele that increases neutrophil sensitivity to IgG stimulation and hence predisposes to anaphylactic reactions following IVIg infusions in patients with hypogammaglobulinemia (71). As described above, FcγRIIB is poorly expressed on neutrophils (50). A specific haplotype in the promoter region, termed 2B.4,

was shown to augment FcγRIIB expression on myeloid cells, including neutrophils (91). This promoter variant enables a more efficient binding of the transcription factors GATA4 and Yin-Yang1, resulting in a higher promoter activity and hence higher FcγRIIB expression (92). Surprisingly, this gain-of-function promoter haplotype was found to be associated with systemic lupus erythematous (SLE) (91). A possible explanation might reside in an FcγRIIB-dependent inhibition of IC-phagocytosis, but experimental data to support this hypothesis are still missing. Additionally, a polymorphism of *FCGR2B* (rs1050501) results in the replacement of a threonine by an isoleucine in the transmembrane domain (I232T). The presence of threonine in that position entails a failure of FcγRIIB to enter lipid rafts and, as a consequence, reduces its ability to inhibit activatory receptors (93). For *FCGR3B*, three different allotypes have been described, resulting from five non-synonymous polymorphisms that all affect the neutrophil antigen (NA) located in the membrane-distal Ig-like domain. These variants are termed NA1 (R_{36} N_{65} A_{78} D_{82} V_{106}), NA2 (S_{36} S_{65} A_{78} N_{82} I_{106}), and SH (S_{36} S_{65} D_{78} N_{82} I_{106}) (94). They do not result in detectable differences in affinity for hIgG subclasses (10). The NA1 allotype was nevertheless reported to increase phagocytosis of IgG-opsonized particles (95) and is associated with a reduced responsiveness to IVIG therapy in Kawasaki disease (96). The SH allotype is the rarest allele and less well-characterized. Recently, it has been reported to be associated to increased *FCGR3B* copy numbers (79) that could account for the higher FcγRIIIB expression levels described earlier (97).

A rather large number of studies have associated a single FcγR polymorphism with the induction or severity of antibody-related diseases, or the efficacy of antibody-based therapies. It is however important to bear in mind that all low-affinity IgG receptors are encoded in a single locus on chromosome 1 (1q23). Indeed, a high degree of linkage disequilibrium has been reported for the *FCGR2/3* locus (79, 98, 99) that are strongly linked to ethnic background (79). Association studies should therefore take into account the entire locus and not investigate an isolated gene (100). Adding to the complexity of the 1q23 locus, gene copy number variations (CNVs) have been described for *FCGR3A*, *FCGR3B*, and *FCGR2C* that directly impact the expression level of the receptors (97). These CNV can include deletions of parts of the locus, giving rise to *FCGR2A/2C* chimeric genes, reducing the expression and function (ROS induction) of the resulting receptor (101). CNVs of *FCGR3B* have been associated to a number of autoimmune disorders, including SLE, rheumatoid arthritis, and systemic auto-immunity (102–106). Indeed, fewer than two copies of *FCGR3B* have been associated to SLE susceptibility (107, 108), which has been confirmed in meta-analysis (109, 110).

Lastly, 0.03–0.1% of the population show a deficiency of *FCGR3B* (and the *FCGR2C* gene) (101, 107). While most studies did not find an association of the *FCGR3B*^{null} genotype with a disease phenotype, one report suggested an association with SLE (102, 111, 112). This apparent contradiction with the finding that low copy numbers increase SLE susceptibility could be due to the low frequency of this genotype in the population, resulting in an insufficient power for calculation. On the other hand, FcγRIIIB-deficient individuals were included at the estimated frequency in a selective cohort of healthy donors with a long list of exclusion criteria, suggesting that FcγRIIIB deficiency remains undetected in most carriers (113). This suggests that *FCGR3B* deficiency does not directly cause disease, but possibly aggravates disease pathogenesis when ICs are accumulating.

REGULATION OF NEUTROPHIL FCγRS

The best-described pathway of inhibiting activating FcγRs is, without doubt, through co-engagement of the inhibitory FcγRIIB by the same immune complexes (114, 115). However, as mentioned earlier, human neutrophils express little to no FcγRIIB (50), and it appears therefore mandatory that neutrophils rely on other mechanisms to regulate their activation by FcγRs.

Recently, several groups have reported that the glycosylation state of the IgG antibodies significantly modifies their affinity for FcγRs (116–118). Indeed, all human IgG contain a single N-linked glycan positioned at asparagine 297 in the antibody Fc portion, which is critical for their interaction with FcγRs. Several studies have illustrated how the composition of the Fc glycan influences IgG effector functions (119, 120) (**Figure 1A**). As an example, IgG Fc glycans lacking fucose display a greatly enhanced affinity to the FcγRs, FcγRIIIA, and FcγRIIIB, compared to fucosylated IgG. Besides their well-established improvement of NK cell-dependent ADCC *in vivo* (121–123), afucosylated IgG used to opsonize target cells also activate neutrophils more efficiently than wild-type IgG, inducing pro-inflammatory cytokines and phagocytosis of target cells, but no ROS production or antibody-dependent cellular cytotoxicity activity (124). Addition of an afucosylated anti-CD20 mAb (obinutuzumab) to blood samples from RA and SLE patients resulted in a superior B cell deletion than wt anti-CD20 mAb, concomitantly with neutrophil and NK cell activation (125), suggesting that both cells cooperate to eliminate target cells *in vitro*. Opposing these results, another study reported FcγRIIIB-dependent inhibition of neutrophil ADCC or trogocytosis of solid cancer cells coated with either anti-HER2 mAb (trastuzumab) or anti-EGFR mAb (cetuximab). Notably, copy numbers of FcγRIIIB could be linked to the inhibitory effect and blocking FcγRIIIB with $F(ab')_2$ 3G8 improved target cell killing (126). Further studies are required to determine whether these discrepancies depend on the target cell (solid tumor vs. hematological) and how neutrophil FcγRIIIB (and FcγRIIIA) (51) contribute to cancer elimination *in vivo*. Terminal sialylation of the Fc glycan, instead, decreases the affinity for activating FcγRs (while maintaining the affinity for inhibitory FcγRIIB), and sialylated IgG has reduced capacity to initiate ADCC *in vivo* (118, 127). This might explain why antibodies against autoantigens can often be detected months to years before the first sign of an autoimmune inflammation (128–130). De-sialysation of these antibodies might therefore be a hallmark of autoimmune disease progression (131, 132). In this context, it is noteworthy that a recent report evidenced the potential of *in vivo* glycan engineering of antibodies to

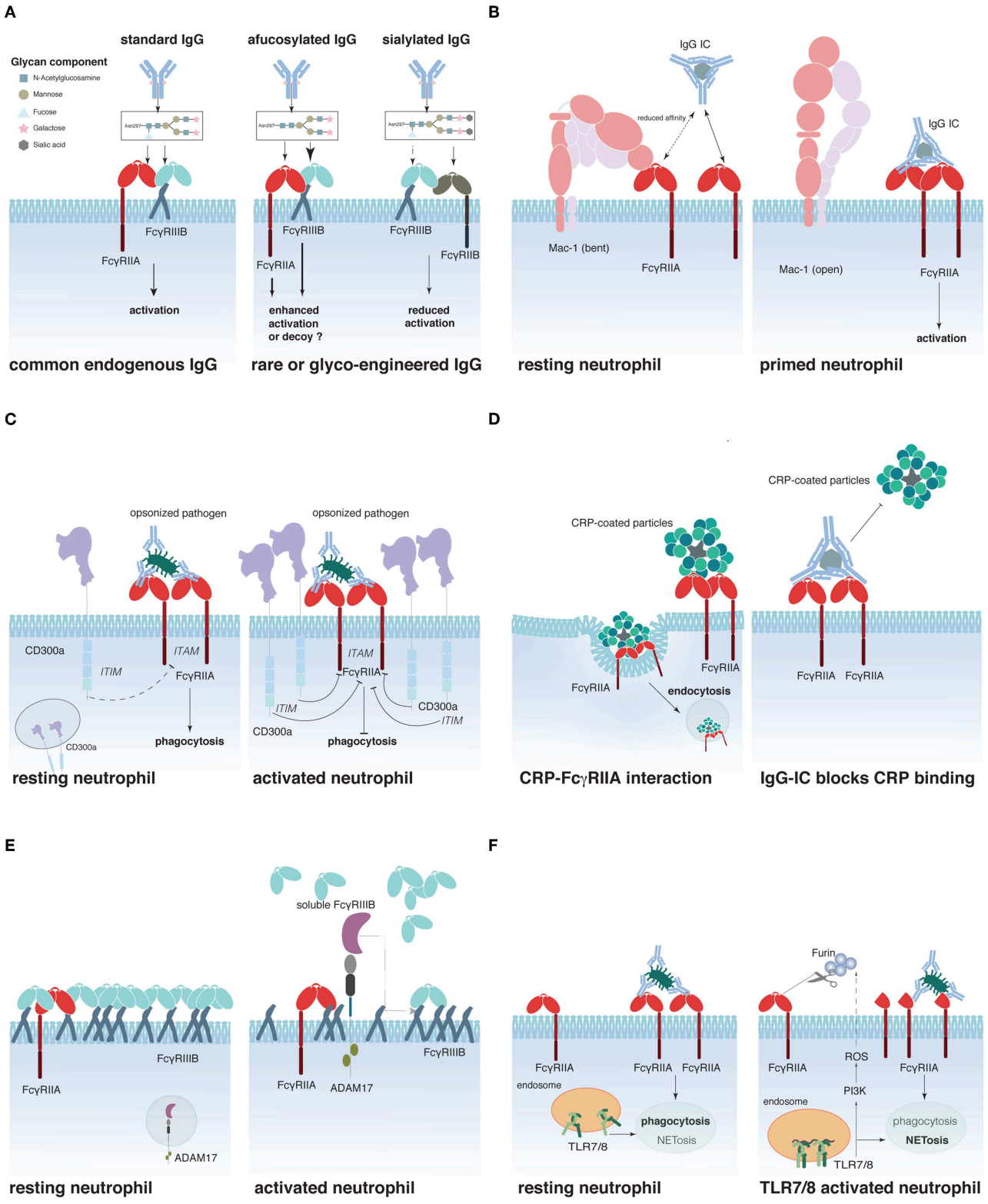


FIGURE 1 | Schematic representation of proposed mechanisms that regulate FcγR activity on neutrophils. **(A)** IgG glycosylation determines its affinity for FcγRs, **(B)** Mac-1 regulates FcγRIIA affinity for IgG, **(C)** CD300a-mediated FcγRIIA inhibition, **(D)** C-reactive protein (CRP)-driven FcγRIIA down-regulation, **(E)** FcγRIIIB shedding by ADAM17, and **(F)** Interplay between Toll-like receptors (TLR)7/8 and FcγRIIA.

modulate of IgG effector functions. Indeed, application of soluble glycosyl-transferases could reduce autoantibody-induced inflammation in models of arthritis and nephrotoxic nephritis (133). However, it remains to be determined whether these very promising findings are exclusively due to IgG glycan modulation or the result of a more complex alteration of multiple glycan structures.

Sialic acid residues have recently been reported to contribute to another mechanism of FcγR regulation (**Figure 1B**). Prompted by the finding that CD18-deficient neutrophils show an enhanced recruitment to IgG-coated endothelium (134) and that an SNP in the CD18 integrin Mac-1 (rs1143679) is a risk factor for SLE (135), Saggu et al. investigated the interaction between FcγRIIA and Mac-1 on the cell surface. The authors convincingly show that the extracellular portion of Mac-1 in its inactive bend configuration interacts with sialylated FcγRIIA on resting neutrophils and thereby lowers the affinity of the receptor for IgG (40). Interestingly, once the interaction between FcγRIIA and IgG is sufficiently strong to overcome this increased activation threshold, Mac-1 assists FcγRIIA to induce cell spreading (136). This study provides a mechanistic explanation for the observation made earlier that FcγRIIA appears to have a lower affinity for IgG on resting neutrophils than on pre-activated ones.

Other molecules have been suggested to interact and modulate FcγRIIA activity. These include the ITIM-containing CD300a that can be rapidly mobilized from an intracellular pool to the surface of peripheral blood neutrophils following stimulation. Co-engagement of CD300a and FcγRIIA reduced FcγRIIA-dependent activation in an *in vitro* system (137) (**Figure 1C**). Furthermore, two plasma proteins produced in the liver during the acute phase of inflammations were described to interact with FcγRIIA (and FcγRIIIB), the C-reactive protein (CRP) and the serum amyloid P (SAP) component (138, 139). CRP preferentially interacts with the FcγRIIA-R131-allele and can act like an opsonin, triggering the uptake of CRP-coated particles (140) (**Figure 1D**). Interestingly, IgG ICs can reduce CRP binding to FcγRIIA, whereas the reverse is not true (141). SAP was similarly described to act as an opsonin that could enhance phagocytosis *via* FcγRs (139). Additionally, it was described to reduce neutrophil adhesion by binding to FcγRIIA (142). Whether SAP binding can regulate IgG-dependent FcγRIIA activation remains however to be determined.

Another possibility to modulate FcγR-induced cell activation is to regulate receptor availability on the cell surface. This can be achieved by receptor internalization (8, 90, 93), translocation from intracellular storage compartments to the cell surface (22), or shedding of the extracellular portion of the FcγR. This latter phenomenon is best documented for FcγRIIIB, which is rapidly and efficiently cleaved from the cell surface following neutrophil stimulation (143), and during neutrophil apoptosis (34, 35). The main protease responsible for this ectodomain shedding appears to be ADAM17 (A Desintegrin and Metalloprotease-17) (144) that is rapidly activated following multiple cell stimulating pathways, such as FcγR/CR-dependent phagocytosis or stimulation with fMLP or PMA (145) (**Figure 1E**). ADAM17 activation appears to require activation of caspase 8 and mitochondrial ROS production (146). Supporting an important role for the regulation of cell activation by ADAM17, ADAM17 deficiency in humans has been associated with severe inflammatory disorders of the skin and the gut, resulting in recurrent sepsis and poor survival (147, 148). Similar to the ectodomain shedding of FcγRIIIB, reduction of surface FcγRIIA on Langerhans cells and activated neutrophils has been described (39, 149). These initial observations have recently gained attention by the demonstration that TLR-induced activation resulted in the cleavage of extracellular FcγRIIA on the neutrophil surface, thus removing the N-terminal portion of the receptor (150) (**Figure 1F**). This cleavage has functional consequences for the neutrophils. It reduces their phagocytic activity, while augmenting their propensity to produce NETs, thereby supporting the concept that phagocytosis and NETosis could be neutrophil effector functions that oppose each other, as had been suggested by the finding that NETosis was reduced when microbes where small enough to be phagocytized (151). Similarly, neutrophils from SLE patients and especially their low-density granulocytes that were reported to spontaneously release NETs (152) seem to express less "full-length" FcγRIIA than neutrophils from healthy donors (150). This might explain why neutrophils from SLE patients fail to efficiently clear circulating ICs and are NET-prone (153). The cleavage of the N-terminal portion of FcγRIIA involves a PI3K-dependent production of ROS and seems to be mediated by the serin-protease furin (150); the exact mechanism of its action, however, as well as the fate and role of the N-terminal fragment of FcγRIIA following cleavage remains to be discovered. Similarly, questions on the stability, function, and possibly altered affinity of the shortened membrane-bound FcγRIIA justify further research in this area.

Finally, it has been suggested that FcγRIIIB could represent an efficient modulator of FcγRIIA activity in neutrophils. Indeed, the weakly signaling FcγRIIIB predominantes FcγRIIA expression on resting neutrophils. Furthermore, CD16B extends out further from the cell surface membrane (154, 155), implying that FcγRIIIB is likely to capture circulating immune complexes, thus competing with and preventing FcγRIIA–IgG interactions. The picture is very different with regard to activated neutrophils that, through FcγRIIIB ectodomain shedding, grant access to cell-activating FcγRIIA (154).

CROSS-TALK OF NEUTROPHILS AND B CELLS

Collectively, these studies underline the critical involvement of IgGs in the modulation of neutrophil activity. Indeed, IgGs through FcγRs render neutrophils capable to act to threats to the host in an antigen-specific manner, but are also the trigger for tissue damage if autoantigens are being targeted. Interestingly, there is accumulating evidence that neutrophils are not mere effector cells of the immune system, but actively shape and modulate immune responses through interactions with other cells and the release of mediators. In the context of

IgG-dependent immunity, their recently described interactions with B cells are of particular interest.

Data from patients receiving G-CSF suggested that neutrophils can communicate with B cells through production of BAFF (B cell-activating factor) (156), a molecule known to sustain B cell survival and responsiveness (157, 158). Similarly, APRIL (A Proliferation Inducing Ligand) was suggested to be an important survival proliferation factor for human B cells, which additionally drives class-switching reactions (159–161). Neutrophils constitutively secrete APRIL (162), but circulating neutrophils fail to directly activate B cells. Upon infection, neutrophil-derived APRIL is retained by heparan sulfate proteoglycans in mucosal tissues, thereby creating a niche for local plasma cell survival and sustained antibody production (163). Similarly, it has been reported that diffuse large B cell lymphoma secretes chemokines to recruit APRIL-producing blood neutrophils to the tumor (162) and that high APRIL concentrations in tumors are correlated with decreased patient survival rates (164). In SLE, neutrophils interact with B cells in many different ways. SLE neutrophils were reported to show increased expression of BAFF, APRIL, and IFN-α that fuels B cell development and autoantibody production in the bone marrow. In the circulation, SLE neutrophils secrete increased amounts of IL-6 upon IFN-α stimulation that supports survival and maturation of B cells and plasma blasts (165). Concomitantly, they are also NET-prone and release LL37–DNA complexes that trigger polyclonal B cell activation *via* TLR9, giving rise to more NET-specific autoantibodies (166).

Neutrophils can also be found in multiple locations of the spleen, including the perifollicular zone, around the marginal zone (MZ) and the red pulp (167–170). Their exact role in each of these compartments is not fully understood. In the spleen, a specialized subset of neutrophils has been described that has the capacity to provide B cell support. These "B cell helper neutrophils" (N_{BH}) are located around the MZ of healthy human donors and express high levels of the B cell-stimulating cytokines BAFF, APRIL, and IL-21 in response to microbial stimuli and thereby provide help to MZ B cells to trigger antibody production against T cell-independent antigens (169). In patients with severe congenital neutropenia (SCN), $CD27^{+}IgD^{low}$ circulating MZ B cells and levels of IgM, IgG, and IgA antibodies against T cell-independent antigens were less abundant than in healthy subjects (169). Contradicting this report, no N_{BH} could be identified in spleen samples from organ transplant donors (171); also, similar numbers of CD27+IgD+ memory B cells were reported in patients suffering from chronic idiopathic neutropenia and healthy subjects (172). In addition to BAFF and APRIL, Pentraxin3 (PTX3) has been proposed to be a neutrophil-derived factor that supports B cell functions (173). PTX3 is stored in secondary granules of neutrophils and released upon stimulation with Toll-like receptor agonists (174). PTX3 binds to MZ B cells and enhances the secretion of class-switched IgG in the presence of BAFF (173). The capacity of neutrophils to secrete B cell-stimulating factors enables them to directly interact with the adaptive immune system and shape antibody responses, which, in turn, can trigger potent neutrophil effector functions. This underlines the complexity of our immune system and the multiple layers of regulation that are at play to efficiently protect us from external threats.

CONCLUDING REMARKS

Collectively, the discussed literature exemplifies how our understanding of neutrophils has evolved from their early descriptions as simple first-line defense cells, equipped with powerful weapons to defend the host but unable to differentiate between different threats, to portrayals of cells capable of tailoring their responses according to their environment. We now appreciate that neutrophils interact with various other cell types, integrate complex stimuli, and cooperate with other players of the immune system to fine-tune their responses. The role and regulation of FcγR on neutrophils is not an exception to this rule. Early reports frequently suggested that IgG ICs had very strong neutrophil-activating capacities, but more recently, a much more detailed picture has been drawn, taking into account the size, solubility, composition, and location of ICs. Furthermore, IgG enables neutrophils to function as antigen-specific cells; at the same time, accumulating evidence suggest that neutrophils in turn tune the activity of (at least some) B cells to regulate antibody production. Much remains to be discovered and hopefully new techniques will allow us to unravel previously unappreciated functions of neutrophils and revise others. We are looking forward to hearing more about these exciting cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Downregulation of HLA Class I Renders Inflammatory Neutrophils More Susceptible to NK Cell-Induced Apoptosis

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Neutrophils are potent effector cells and contain a battery of harmful substances and degrading enzymes. A silent neutrophil death, i.e., apoptosis, is therefore of importance to avoid damage to the surrounding tissue and to enable termination of the acute inflammatory process. There is a pile of evidence supporting the role for pro-inflammatory cytokines in extending the life-span of neutrophils, but relatively few studies have been devoted to mechanisms actively driving apoptosis induction in neutrophils. We have previously demonstrated that natural killer (NK) cells can promote apoptosis in healthy neutrophils. In this study, we set out to investigate how neutrophil sensitivity to NK cell-mediated cytotoxicity is regulated under inflammatory conditions. Using *in vitro*-activated neutrophils and a human skin chamber model that allowed collection of *in vivo*-transmigrated neutrophils, we performed a comprehensive characterization of neutrophil expression of ligands to NK cell receptors. These studies revealed a dramatic downregulation of HLA class I molecules in inflammatory neutrophils, which was associated with an enhanced susceptibility to NK cell cytotoxicity. Collectively, our data shed light on the complex regulation of interactions between NK cells and neutrophils during an inflammatory response and provide further support for a role of NK cells in the resolution phase of inflammation.

Keywords: neutrophil, NK cell, HLA class I, immunoregulation, neutrophil apoptosis

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INTRODUCTION

Neutrophils are innate immune cells that play a key role in the defense against invading microbes. In circulation, neutrophils are in a resting state, but in response to proinflammatory signals they become alerted, extravasate and migrate toward the site of damage or infection. Neutrophils contain numerous intracellular granules containing pre-formed proteins needed to execute their functions. Thus, when neutrophils are stimulated, they rapidly mobilize specific types of granules to equip the cell with new surface structures and to release soluble factors to the extracellular space (1). The transmigration process *per se* is dependent on degranulation, which enables attachment to endothelial cells, and subsequently chemotaxis-driven migration (2). However, the rearrangement

of surface structures does not only involve granule mobilization, but also cleavage or internalization of surface structures; CD62L, involved in attachment to endothelial cells, is for example shed from the neutrophil surface already in circulation (3).

Neutrophils have a rich arsenal of toxic substances and degrading enzymes stored in their granules, which are used to eradicate an ingested prey. The toxic content makes neutrophils a potential danger to the surrounding tissue and it is thus of importance that neutrophils are removed from the inflammatory site after they have fulfilled their tasks. A tightly controlled death process where the cell is degraded from the inside while the surface membrane remains intact, is therefore of importance for termination of the inflammatory process (4). Neutrophil apoptosis can be induced by interaction with other immune cells inducing pro-apoptotic signaling *via* death receptors on the neutrophil surface (5); e.g., we have previously reported that interaction between NK cells and neutrophils can promote neutrophil apoptosis (6).

Natural killer (NK) cells are cytotoxic cells that can kill aberrant cells without prior sensitization (7). Besides their undisputed role in the defense against viral infections and certain malignancies, a growing body of evidence points to a role for NK cells in immune regulation, both as an early source of cytokines but also by selective killing of immune cells (6, 8–13). The result of an encounter between an NK cell and a target cell is determined by the balance between signals originating from inhibitory and activating receptors expressed on the NK cell surface; thus, the presence of cognate ligands to NK cell receptors (NKRs) on the potential target cell determines the outcome of an NK cell—target cell interaction. When the inhibitory signaling is decreased, or the activating signaling increased, the NK cell cytotoxic machinery may be activated, resulting in release of cytotoxic granules into the immunological synapse. Among the major activating receptors are the natural-killer group 2, member D (NKG2D), recognizing MICA and MICB and different ULBPs; DNAX accessory molecule-1 (DNAM-1), recognizing polio virus receptor (PVR) and Nectin-2; 2B4 recognizing CD48; and the group of natural cytotoxicity receptors (NCRs; NKp30, NKp44, and NKp46), which in part constitute orphan receptors (14, 15), while NKp30 recognizes B7-H6 and BAG-6 (16, 17). The main inhibitory receptors are the inhibitory killer immunoglobulin-like receptors (iKIRs) and NKG2A/CD94 that bind to specific human leukocyte antigen (HLA) class I molecules on target cells. HLA-C binds to the KIR2DL receptors, while certain HLA-B and HLA-A molecules contain a Bw4 motif that is recognized by KIR3DL1. The NKG2A/CD94 heterodimer binds to the non-classical HLA-E molecule, which selectively presents the leader peptides of classical HLA molecules and thus reflects the overall expression of HLA class I in a cell. In addition, the inhibitory receptor LILRB1 recognizes HLA class I molecules. Moreover, HLA class I molecules may also bind to activating NK cell receptors, where the NKG2C/CD94 heterodimer binds to HLA-E, and activating KIRs recognize certain motifs on classical HLA class I molecules. The HLA class I molecules can be up- or downregulated from the cell surface in response to cellular signaling, and free soluble HLA class I molecules has been described in serum, either secreted from cells or shed from the cell surfaces due to proteolytic cleavage (18–20).

As mentioned above, NK cells have been ascribed immunomodulatory functions (21, 22) and previous work from our group has demonstrated that NK cells induce apoptosis in healthy neutrophils in an NCR- and Fas-dependent manner (6). Moreover, in a human *in vivo* blister model of sterile inflammation, NK cell entry into blisters coincided with the appearance of apoptotic neutrophils, suggesting a role for NK cells in terminating an inflammatory response (6). With this background, we hypothesized that modulation of neutrophil surface expression of NKR ligands upon neutrophil activation may lead to altered sensitivity to NK cell-induced apoptosis. In this study, we demonstrate that inflammatory neutrophils, both *in vitro*-stimulated blood neutrophils and *in vivo*-transmigrated neutrophils collected from skin chamber exudates, are more susceptible to NK cell-mediated apoptosis, and that the enhanced sensitivity is associated with a pronounced downregulation of neutrophil HLA class I expression.

RESULTS

Surface Expression of HLA Class I Molecules Is Decreased Upon Neutrophil Activation

NK cell cytotoxicity is triggered when the signals from activating receptor-ligand interactions overcome the inhibitory signals from inhibitory receptors. Thus, in a first set of experiments we investigated to what extent activated neutrophils modulate their expression of ligands to NKRs. In *in vitro* experiments, we combined GM-CSF with the TLR7/8 agonist CL097 (23, 24) to activate freshly isolated neutrophils and screened the neutrophil expression of ligands to activating and inhibiting NKRs using flow cytometry. As demonstrated in **Figure 1A**, neutrophils stimulated with CL097 and GM-CSF for 20 min at 37°C displayed strong up-regulation of CD11b and CD66, both classical markers for neutrophil activation, as well as loss of CD62L. In parallel, we observed a significant decrease of classical HLA class I molecules after 20 min stimulation at 37°C (HLA-ABC; **Figure 1A**). These results were confirmed with two additional clones recognizing HLA-ABC (G46-2.6 and A6-136; data not shown). Notably, a significant downregulation of HLA class I was seen also without addition of stimuli (**Figure 1A**). Furthermore, we observed decreased expression of the DNAM-1 ligand PVR, and increased expression of MICA/B and ULBP-3, both ligands to NKG2D, on the neutrophil surface after 20 min of stimulation with CL097/GM-CSF (**Figure 1B**), while no robust changes were observed regarding expression levels of Nectin-2, ULBP-1, ULBP-2/5/6, B7-H6, or NKp46-ligand, all ligands to activating NKRs (**Supplemental Figure 1**). Surface expression of CD48, a ligand to 2B4, could not be detected on neither resting nor activated neutrophils (data not shown). Extended GM-CSF and TLR stimulation further decreased neutrophil surface expression of classical HLA class I molecules, while expression levels of ligands

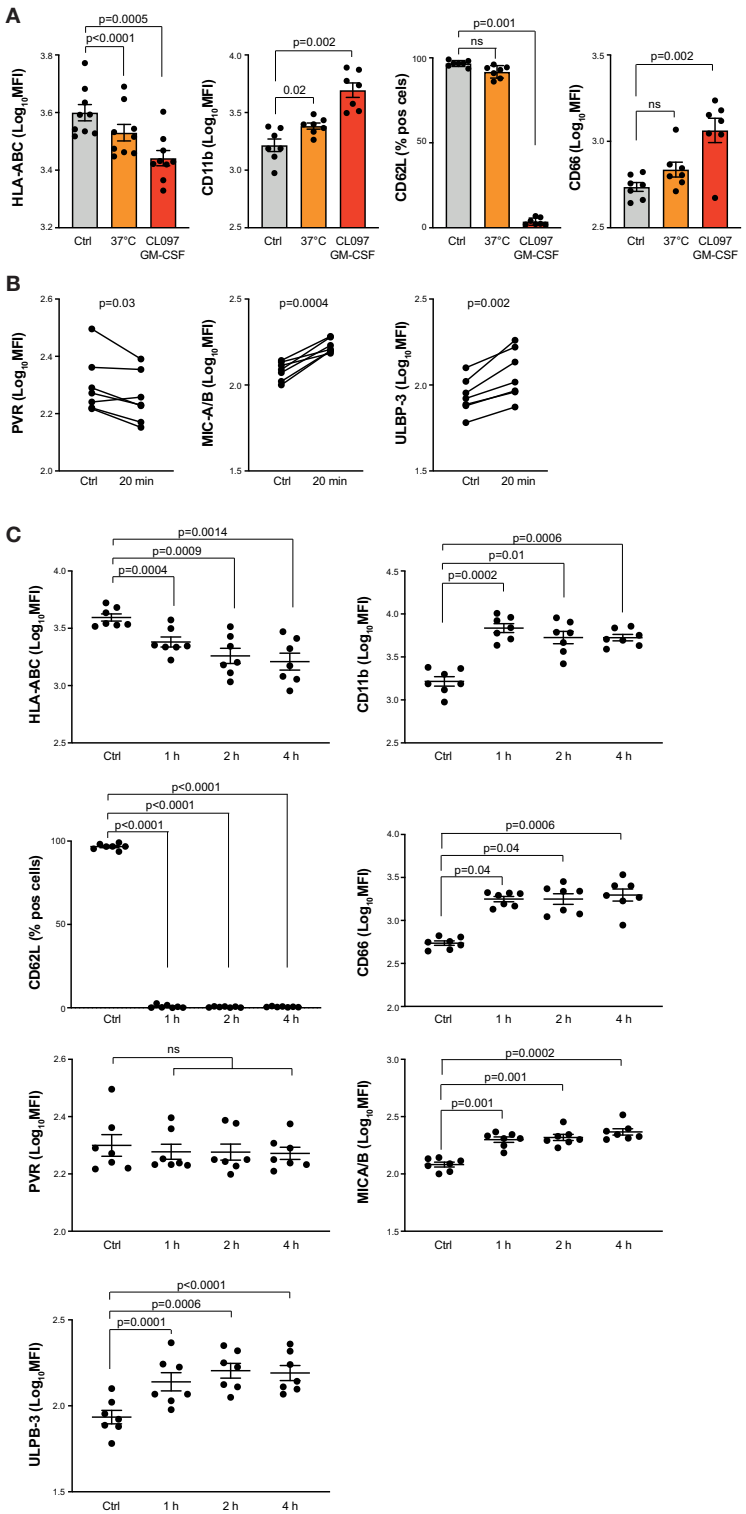

FIGURE 1 | NKR ligand expression on *in vitro*-activated neutrophils. **(A)** Surface expression of HLA-ABC, CD11b, CD66 or CD62L as indicated, on resting neutrophils kept on ice (ctrl) or *in vitro*-activated neutrophils stimulated for 20 min at 37°C alone or in presence of the TLR-agonist CL097 and GM-CSF (one-way ANOVA followed by Dunnett's multiple comparisons test). **(B)** Surface expression of indicated ligands to NKRs on resting neutrophils (ctrl) or *in vitro*-activated neutrophils stimulated for 20 min at 37°C with CL097/GM-CSF (paired *t*-test). **(C)** Expression of NKR ligands and neutrophil degranulation markers on resting neutrophils (ctrl) or *in vitro*-activated neutrophils stimulated for 1, 2, or 4 h at 37°C with CL097/GM-CSF (one-way ANOVA followed by Dunnett's multiple comparisons test). Error bars represent SEM.

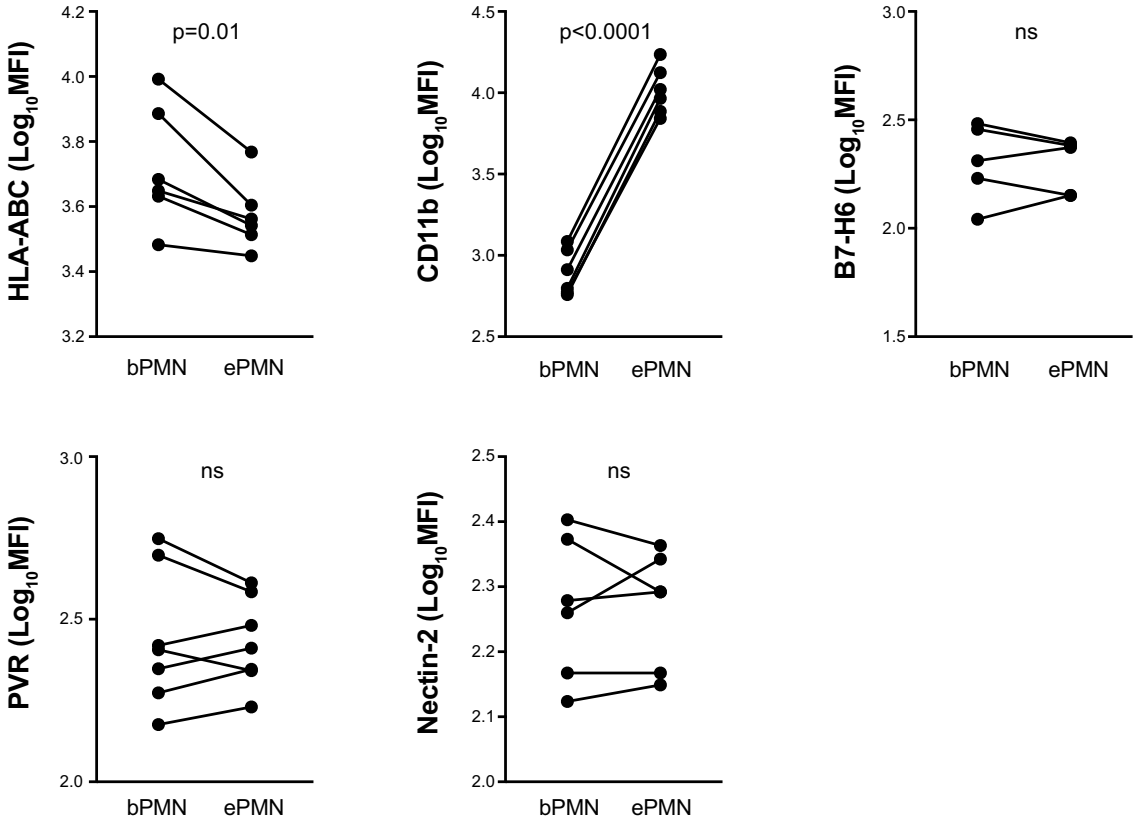

FIGURE 2 | Surface expression of ligands to inhibitory and activating NKRs on transmigrated neutrophils. Surface staining of indicated ligands to NKRs on transmigrated neutrophils (ePMN), collected from exudates in the skin chamber model, and autologous blood neutrophils (bPMN). Paired *t*-test, error bars represent SEM.

to activating NKRs displayed varying patterns (**Figure 1C**, **Supplemental Figure 1**).

To investigate whether our observations in neutrophils activated *in vitro* were consistent with expression patterns on activated, extravasated neutrophils *in vivo*, we made use of a human *in vivo* model where transmigrated neutrophils are harvested from a serum-filled skin chamber placed on exposed dermis (25). Transmigrated neutrophils were highly activated as reflected by an increased CD11b expression and loss of CD62L compared to resting autologous neutrophils harvested from blood (**Figure 2** and data not shown). Similar to the *in vitro*-activated neutrophils, transmigrated neutrophils displayed significantly decreased surface expression of classical HLA class I molecules. Consistent with our data from *in vitro*-stimulated neutrophils, transmigrated neutrophils did not display altered surface expression of Nectin-2 and B7-H6. However, in contrast to *in vitro*-stimulated neutrophils, transmigrated neutrophils did not display decreased expression of PVR.

Downmodulation of NK Cell-Regulatory HLA Molecules on Activated Neutrophils

HLA class I expression on hematopoietic cells is dominated by HLA-A and -B, which are approximately 15 times more abundant than HLA-C (26), which in turn is more abundant than the non-classical HLA class I molecule, HLA-E. Given the important role for HLA-C and HLA-E in regulation of NK cell effector functions (27), we next investigated to what extent these HLA molecules were also downregulated in response to neutrophil stimulation. As shown in **Figures 3A,B**, we observed a strong downregulation of both HLA-C and HLA-E already after 20 min of stimulation. The decreased HLA-C expression was observed also after longer neutrophil stimulation (**Figures 3C,D**; **Supplemental Figure 2**). Consistently, we observed a similar picture on transmigrated neutrophils *in vivo* (**Figures 3E,F**).

In an attempt to understand the mechanism of HLA downmodulation, we investigated whether HLA was internalized upon neutrophil activation. Neutrophils were stained with a FITC-conjugated anti-HLA-ABC antibody prior to stimulation. After 20 min CL097/GM-CSF stimulation, an anti-FITC mAb was added, quenching the extracellular FITC signal. However, as shown in **Figure 4**, the fluorescence detected from activated neutrophils was quenched to a similar extent as resting neutrophils, suggesting that internalization of HLA is not the main explanation to the HLA downregulation observed on activated neutrophils. Activated neutrophils release granules containing various proteolytic enzymes, such as serine proteases, e.g., elastase, proteinase 3, cathepsin G, and different matrix

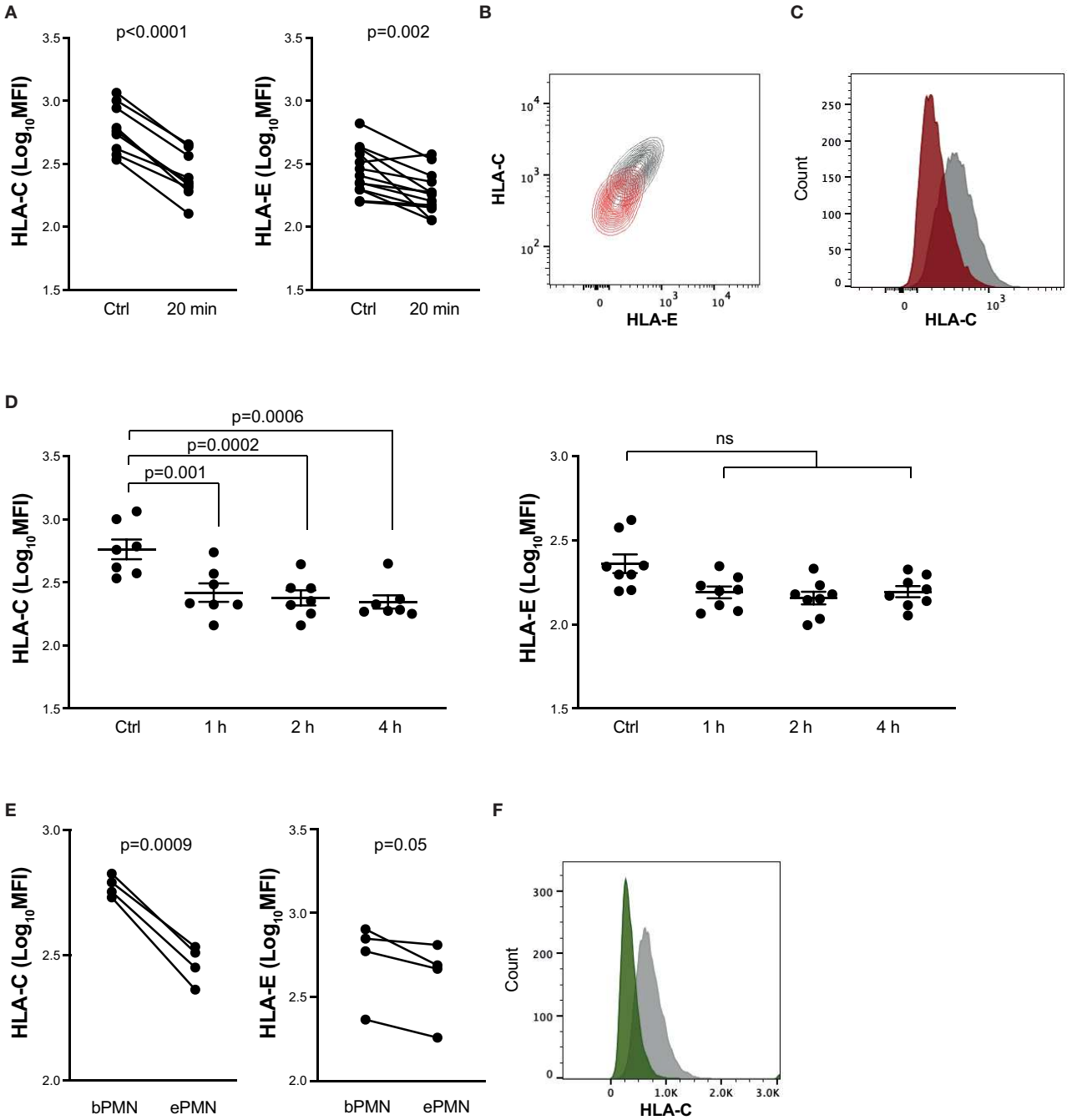

FIGURE 3 | Downmodulation of HLA-C and HLA-E on activated neutrophils. **(A–D)** Neutrophil staining of indicated HLA class I cell surface structures (HLA-C or HLA-E) binding to inhibitory NKRs. Resting neutrophils were kept on ice (ctrl) or stimulated for 20 min with the TLR-agonist CL097 and GM-CSF at 37°C (**A**; paired *t*-test) or for 1, 2, or 4 h under the same conditions (**D**; one-way ANOVA followed by Dunnett's multiple comparisons test). **(B)** Representative staining of HLA-C and HLA-E of resting neutrophils (gray) or neutrophils that had been stimulated with CL097/GM-CSF for 20 min (orange). **(C)** Representative staining from one experiment showing expression of HLA-C on resting neutrophils (gray) or neutrophils that had been stimulated with CL097/GM-CSF for 1 h (red). **(E)** Neutrophil staining of indicated HLA class I molecules on transmigrated neutrophils from skin chamber exudates (ePMN) or resting autologous neutrophils (bPMN; paired *t*-test). **(F)** Representative staining from one experiment showing the expression of HLA-C on resting neutrophils (gray) or transmigrated neutrophils (green). Error bars represent SEM.

metalloproteases, and we next addressed to what extent inhibition of specific enzymes could restore HLA expression in activated neutrophils. However, Batimastat, an inhibitor of matrix metalloproteases, did not affect the neutrophil surface expression of HLA class I and neither did α1-antitrypsin nor specific inhibitors of cathepsin G and PR3 (data not shown).

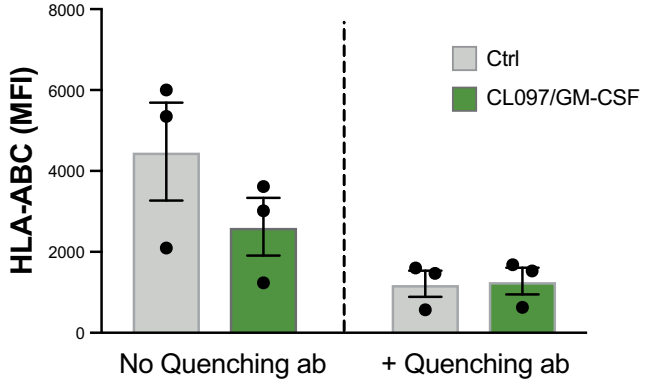

FIGURE 4 | Decrease of surface HLA class I molecules is not explained by internalization. Graph shows resting neutrophils stained with FITC-conjugated HLA-ABC antibody prior to addition of buffer (ctrl) or CL097/GM-CSF for 20 min at 37°C, after which FITC-quenching antibody was added.

Reduced HLA Class I Expression on Activated Neutrophils Translates Into Enhanced Sensitivy to NK Cell Cytotoxicity

As activated neutrophils displayed lower expression of ligands to inhibitory NKRs, and no or modest modulation of their expression of ligands to activating NKRs was detected, we speculated that the outcome of interactions between NK cells and activated vs. resting neutrophils would differ. To investigate the neutrophil sensitivity to NK cell cytotoxicity, the viability of resting or pre-activated neutrophils was determined after co-culture with pre-activated NK cells. As demonstrated in **Figures 5A,B**, both transmigrated skin chamber neutrophils and *in vitro*-stimulated neutrophils were more sensitive to NK cell cytotoxicity compared to resting neutrophils, as reflected by increased binding of Annexin V to extracellular phosphatidyl serine and nuclear staining of cells with compromised plasma membrane integrity with To-Pro-3 (**Figure 5C**). Notably, addition of an HLA antibody, blocking the inhibitory interaction between HLA and KIRs or NKG2A/CD94, resulted in a significantly higher NK cell cytotoxicity against resting neutrophils (**Figure 5A**). By contrast, the cytotoxicity against activated neutrophils, which display low expression of HLA class I molecules, did not further increase upon HLA class I blockade. Blocking of NCR signaling *via* NKp46 resulted in decreased NK cell-mediated killing of *in vitro*-activated neutrophils (**Supplemental Figure 3**), consistent with our previous findings for resting neutrophils (6).

DISCUSSION

After executing their tasks in peripheral tissues, neutrophils, with their cargo of toxins and degrading enzymes, must undergo apoptosis to prevent prolonged inflammation and resulting tissue damage. During the last decade, a growing pile of evidence has identified an important role for NK cells as modulators of the immune response (21, 22, 24) and we have earlier demonstrated that NK cells can induce apoptosis in neutrophils in an NKp46-dependent manner (6). The outcome of NK cell—target cell interactions is dependent not only on cell signaling *via* activating receptors, but also on signaling *via* inhibitory receptors. In this study we investigated the neutrophil surface expression of ligands to inhibitory, as well as activating NKRs, on both *in vitro*-activated neutrophils and *in vivo*-transmigrated neutrophils isolated using a skin chamber model (25). With the exception of certain NKG2D ligands, the expression of ligands to activating NKRs remained largely unchanged upon neutrophil activation. By contrast, we observed a substantial downregulation of HLA class I molecules on activated neutrophils, which included also less abundant HLA structures of importance for NK cell regulation, HLA-C, and HLA-E.

HLA downregulation is a commonly used immunoevasive strategy among virus-infected cells or tumor cells to avoid T cell recognition and elimination. However, the decreased HLA expression will reduce inhibitory signaling in NK cells and thus allow missing-self recognition by NK cells (27–29). The dramatic downregulation of HLA class I molecules observed in this study necessarily involves downregulation of the abundant HLA subtypes, HLA-A and -B which according to previous reports comprise 95% of total HLA class I (26). Interestingly, our analysis demonstrated that the downregulation of HLA class I on activated neutrophils was not confined to the most abundant HLA class I molecules but also included pronounced downregulation of HLA-C and HLA-E. As HLA class I molecules act as ligands to inhibitory NK cell receptors, we hypothesized that the reduced surface expression of NK cell-specific HLA-C and HLA-E would result in higher sensitivity to NK cell cytotoxicity in inflammatory neutrophils. Indeed, the loss of surface HLA class I molecules rendered inflammatory neutrophils more susceptible to NK cell-mediated killing.

Our data suggest that HLA downregulation on inflammatory neutrophils may serve the purpose to increase their susceptibility to NK cell cytotoxicity and thus contribute to resolution of the inflammatory process by NK-induced neutrophil apoptosis. Chronic inflammatory diseases, such as rheumatoid arthritis, are characterized by an ongoing inflammation and presence of high numbers of inflammatory neutrophils (30). A focus of further studies should be to what extent aberrant HLA expression patterns or absence of certain NK cell phenotypes may be related to disease grade and characteristics. In this regard, it has been reported that the immature, less cytotoxic $CD56^{bright}$ NK cells predominate in the inflamed synovium (31). This finding is in line with our data, as the less cytotoxic $CD56^{bright}$ NK cell population may rather fuel the inflammatory process by producing inflammatory cytokines than kill inflammatory neutrophils.

We were not able to pinpoint the exact mechanism for neutrophil HLA downregulation in this study. It is possible that HLA molecules are internalized upon neutrophil activation. To test this hypothesis, we made use of an anti-FITC antibody, which quenches the FITC. As the neutrophils were stained with FITC-conjugated anti-HLA-ABC antibody before priming, internalization would mean that also the FITC-conjugate was internalized. The quenching antibody can only bind to

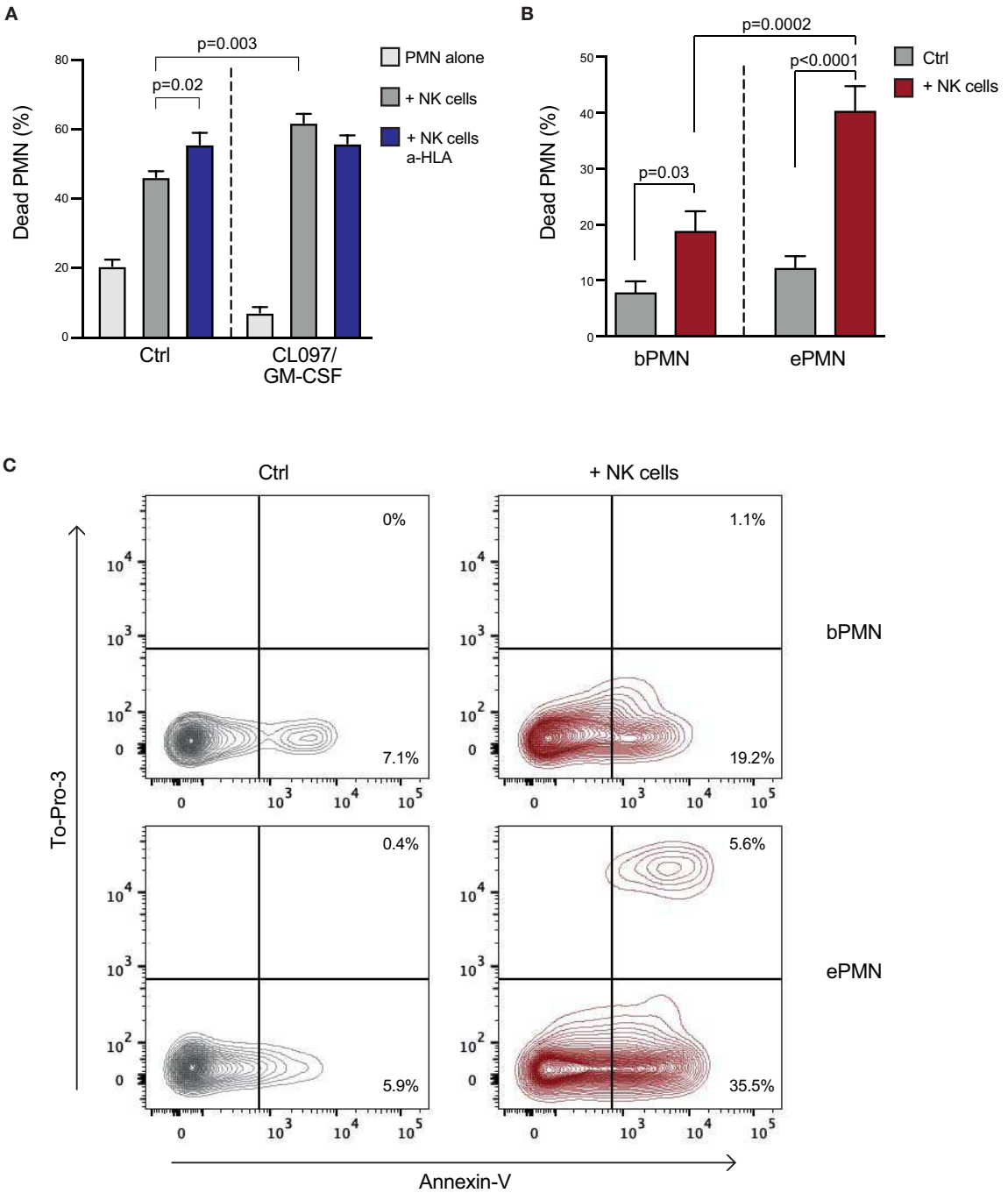

FIGURE 5 | Activated neutrophils are more sensitive to NK cell cytotoxicity compared to resting neutrophils. **(A)** Percentage of dead neutrophils (PMN) after a 3 h co-culture of neutrophils [pre-stimulated *in vitro* with CL097 and GM-CSF, or kept on ice (ctrl)] and bulk-NK cells at an E:T ratio of 10:1, with addition of an HLA antibody as indicated ($n = 7$; one-way ANOVA followed by Dunnett's multiple comparisons test). **(B)** Graph shows percentage of dead neutrophils after a 4 h cytotoxicity assay performed using autologous NK effector cells toward transmigrated (ePMN) or blood neutrophils (bPMN; E:T ratio 10:1; $n = 5$; one-way ANOVA followed by Dunnett's multiple comparisons test). Error bars represent SEM. **(C)** Representative FACS plots showing percentage of dead neutrophils (transmigrated or resting) measured using AnnexinV and To-Pro-3, after a 4 h co-culture with autologous NK cells.

extracellular FITC, and thus, any internal FITC signal would still fluoresce when hit by light in the right wave length. However, the FITC signal was not higher in activated neutrophils than in resting neutrophils, and we could thus not conclude that HLA was internalized. Alternatively, HLA molecules could be shed from the cell surface. Neutrophil granules contain enzymes,

such as the serine proteases, neutrophil elastase, cathepsin G, and proteinase 3, which may be released upon neutrophil transmigration to an inflammatory site (32, 33). Shedding of surface B7-H6 from tumor cells has previously been described as a cause of matrix metalloprotease-mediated shedding (34), and presence of a soluble form of B7-H6 has been reported in supernatants from *in vitro*-stimulated neutrophils (35). This suggests that also neutrophil modulation of HLA molecules may involve proteolytic shedding. Supporting this hypothesis, the metalloprotease inhibitor Batimastat has been reported to inhibit release of soluble HLA class I in activated lymphocytes (19). In this study, however, we were unable to inhibit HLA class I downregulation on neutrophils with Batimastat, although the Batimastat readily inhibited shedding of CD62L from the activated neutrophils. Notably, a fraction of serine proteases from azurophil granules remain associated with the neutrophil cell membrane after granule-mobilization (33), and there are reports suggesting that membrane-bound forms of these enzymes are relatively insensitive to protease inhibitors (36). Thus, the inability of protease inhibitors to prevent HLA class I downregulation does not preclude a role for these proteases in this process.

A third alternative for the observed downregulation of HLA expression could be that neutrophil activation leads to a change in the HLA conformation, disabling antibody recognition of HLA. Several studies have reported open conformers of HLA, with the conformation change leading to a shift in receptor—ligand interactions (37–39). Surface staining of resting and activated neutrophils using an antibody recognizing an open conformation of HLA class I did not reveal any increase in HLA class I open conformers on activated neutrophils (data not shown). Nevertheless, in the study we observed HLA class I downregulation on activated neutrophils using five different antibody clones detecting HLA molecules (clones G46-2.6, W6/32 and A6-136 against HLA-ABC, clone DT9 against HLA-C, and clone 3D12 against HLA-E) strongly suggesting that HLA class I is decreased on the surface of inflammatory neutrophils.

In conclusion, we have demonstrated that both *in vitro*-activated and *in vivo*-transmigrated neutrophils display decreased surface expression of HLA class I molecules. In line with the decreased HLA class I expression, inflammatory neutrophils were more sensitive to NK cell-mediated cytotoxicity, as the loss of inhibitory signaling tips the balance toward NK cell activation and cytotoxicity. Collectively, our study demonstrates that neutrophils dynamically modulate their expression and release of NK cell receptor ligands, and that NK cells may contribute to a controlled resolution of the inflammatory process by killing activated neutrophils.

MATERIALS AND METHODS

Antibodies and Reagents

Following mAbs were used for detection of surface markers: anti-CD11b APC (clone: ICRF44), CD48 BV421 (TÜ145), HLA-ABC FITC (G46-2.6), CD62L APC (DREG-56), CD66 PE (B1.1; all from BD Biosciences, CA, USA), HLA-C (DT9, Merck Millipore), HLA-ABC Alexa Fluor 647 (W6/32) and HLA-E PE-Cy7 (3D12; both from Biolegend), B7-H6 (875002), ULBP1 PerCP (170818), ULBP-3 PE (166510), ULBP-2/5/6 APC (165903; all from R&D Systems, MN, USA), CD56 PE-Cy7 (N901, Beckman Coulter, IN, USA), MICA/B viobright FITC (6D4, Miltenyi Biotech, Bergisch Gladbach, Germany), and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, CA, USA). Anti-B7-H6 (17B1.3) was kindly provided by Prof E Vivier (CMIL, Marseille, France), and anti-PVR (CD155; M5A10) and Nectin-2 (CD112; L14) were received from the lab of Prof. A Moretta (Genova, Italy). Goat serum and α1-antitrypsin were obtained from Sigma-Aldrich (MO, USA), CL097 from InvivoGen (San Diego, CA), GM-CSF from R&D Systems (MN, USA), anti-PR3 from Abcam (UK), and Batimastat and cathepsin G inhibitor from Calbiochem (CA, USA). In experiments where the extracellular FITC signal was quenched, a Fluorescin/Oregon green polyclonal antibody (ThermoFisher, Sweden) was used.

Isolation of Human Leukocytes

Buffy coats or fresh blood obtained from healthy donors were mixed 1:1 with 2% dextran. After sedimentation of erythrocytes, granulocytes and PBMCs were separated by density gradient centrifugation (40, 41). Remaining erythrocytes were lysed in distilled water to obtain a pure granulocyte population. This method provides a granulocyte population of >95% purity. NK cells were isolated from PBMCs using an NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. To obtain activated, polyclonally expanded NK cells (bulk), freshly isolated NK cells were cultured on irradiated feeder cells in the presence of 100 U/mL recombinant human IL-2 (Proleukin; Chiron) and 1.5 ng/ml phytohemagglutinin (PHA) (GIBCO Ltd.).

Isolation of Transmigrated Neutrophils

In order to isolate *in vivo*-transmigrated neutrophils, a skin chamber technique was used, described in detail elsewhere (25, 42). In short, blisters were introduced on the forearm on healthy volunteers using negative pressure. After 2 h, the blister roofs were removed and collection chambers were placed on top of the lesions. Autologous serum was added to the chamber wells. After 24 h, the chambers were removed and *in vivo*-transmigrated neutrophils were harvested from the skin chamber fluid, with a granulocyte purity >90%, and with mainly viable neutrophils (42). Autologous blood neutrophils were isolated from peripheral blood as described above and used as control cells. All neutrophils were diluted in Krebs Ringer Glucose (KRG) supplemented with Ca^{2+}, and stored on melting ice until use. The study was approved by the regional ethical board in Gothenburg (543-07) and voluntary donors gave written informed consent in accordance with the declaration of Helsinki.

Generation of Fusion Proteins for Detection of NKp46 Ligand

HEK293T cells, transfected with a construct encoding for a fusion protein consisting of the extracellular part of NKp46 fused to

an Fc-portion of human IgG, were kindly provided by Prof. O Mandelboim (Jerusalem, Israel). Details of the transfection procedure has been described elsewhere (43, 44). Cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat inactivated fetal calf serum (FCS), 1% PEST, 1% L-glutamine and Puromycin dihydrochloride (5 μg/ml; Sigma-Aldrich). Cell cultures were kept at 37°C in a humidified, 5% CO_2 atmosphere. When reaching 70% confluency, medium was exchanged to low protein medium supplemented with non-essential amino acids, 1% Penicillin-Streptomycin, 1% L-glutamine and 1% Sodium Pyruvate. After 3 days, the cell-free supernatant was collected and fusion proteins were purified with affinity chromatography using a HiTrap Protein G column (GE Healthcare). Proteins were eluted with 0.1 M Glycine/HCl (pH 2,7-3), neutralized in 1 M TRIS/HCl (pH 8), concentrated using an Amicon Ultra 15 centrifugation tube (Millipore) and stored in buffered NaCl in −20°C until use.

Expression of Surface Ligands on Neutrophils

Freshly isolated neutrophils were stimulated with CL097/GM-CSF (1 μg/ml and 100 U/ml, respectively), at 37°C for indicated time. In some experiments, the matrix metalloproteinase (MMP) inhibitor Batimastat (10 μM), α1-antitrypsin (1 μg/ml), cathepsin G inhibitor (1 μM) or anti-PR3 antibody (100 ng/ml) were added to the cells. Supernatants were collected after priming for detection of soluble HLA. Cells were stained with antibodies for 30 min at 4°C. Degranulation/activation was monitored using antibodies directed against the surface markers CD11b, CD62L, and CD66. For detection of unconjugated antibodies (HLA-C, B7-H6, PVR and Nectin-2), cells were saturated in 20% goat serum for 10 min prior to incubation with Alexa Fluor-488 conjugated goat anti-mouse IgG. Samples were collected on a three-laser FACSAria (405, 488, and 633 nm) and data was analyzed using FlowJo software (TreeStar; version 9.7.2 or later).

For detection of NKp46 ligand, neutrophils were treated with the streptoccal enzyme EndoS, kindly provided by Dr. Mattias Collin (Lund University, Sweden), at 37°C for 20 min to digest endogenous IgG bound to the surface of the cells (45). Cells were subsequently incubated with fusion protein (10 μg/ml) for 30 min at 4°C. Cells were saturated in 20% goat serum prior to staining with FITC-conjugated Fcγ-specific goat anti-human IgG (Jackson Immunoresearch Laboratories, PA, USA).

Detection of NK Cell-Induced Neutrophil Apoptosis

Freshly isolated neutrophils were stimulated with CL097/GM-CSF (1 μg/ml and 100 U/ml, respectively), or kept at 4°C without stimuli, for 1 h. After stimulation the neutrophils were co-cultured with polyclonal IL-2 activated NK cells at a 10:1 E:T ratio for 3 h at 37°C in the presence or absence of anti-HLA-I mAb (clone A6136, IgM), or anti-NKp46 (clone KL247, IgM). Transmigrated neutrophils collected from skin chamber exudates or autologous neutrophils isolated from blood were co-cultured with autologous NK cells, stimulated in IL-12 (1 ng/ml) or IL-15 (10 ng/ml) overnight. Apoptosis and lysis of neutrophils were detected with FITC-conjugated AnnexinV (BD Biosciences, CA, USA) and To-Pro-3 (Invitrogen) using flow cytometry (46).

Statistics

For single pairwise comparisons, paired *t*-test was used. For multiple comparisons within a data set, one-way ANOVA followed by Dunnett's multiple comparisons test, was used.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Regional Ethics Board in Gothenburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EB, KC, SP, and FT designed the research. EB, KC, SP, and MP performed the research and analyzed the data. EM, SS, and FT supervised the study. EB, KC, and FT drafted the manuscript. All authors contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02444/full#supplementary-material

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Neutrophils as Suppressors of T Cell Proliferation: Does Age Matter?

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Whereas, neutrophils have long been considered to mainly function as efficient innate immunity killers of micro-organisms at infected sites, they are now recognized to also be involved in modulation of adaptive immune responses. Immature and mature neutrophils were reported to have the capacity to suppress T cell-mediated immune responses as so-called granulocyte-myeloid-derived suppressor cells (g-MDSCs), and thereby affect the clinical outcome of cancer patients and impact the chronicity of microbial infections or rejection reactions in organ transplantation settings. These MDSCs were at first considered to be immature myeloid cells that left the bone marrow due to disease-specific signals. Current studies show that also mature neutrophils can exert suppressive activity. In this study we investigated in a robust T cell suppression assay whether immature CD11b+ myeloid cells were capable of MDSC activity comparable to mature fully differentiated neutrophils. We compared circulating neutrophils with myeloid cell fractions from the bone marrow at different differentiation stages. Our results indicate that functional MDSC activity is only becoming detectable at the final stage of differentiation, depending on the procedure of cell isolation. The MDSC activity obtained during neutrophil maturation correlated with the induction of the well-known highly mobile and toxic effector functions of the circulating neutrophil. Although immature neutrophils have been suggested to be increased in the circulation of cancer patients, we show here that immature neutrophils are not efficient in suppressing T cells. This suggests that the presence of immature neutrophils in the bloodstream of cancer patients represent a mere association or may function as a source of mature neutrophils in the tumor environment but not a direct cause of enhanced MDSC activity in cancer.

Keywords: neutrophils, progenitors, MDSC activity, T cell suppression, cell isolation

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INTRODUCTION

Almost 20 years ago the expansion of immature myeloid cells in bone marrow, spleen and the circulation was described in cancer patients and many animal models (1–6). These cells were found to have a suppressive effect on T cell proliferation and were therefore named myeloid-derived suppressor cells (MDSCs) (7). It is thought that under pathological conditions, such as cancer, a partial block occurs in the differentiation of immature myeloid cells, resulting in the expansion of this population. Their presence and infiltration have been associated with poor prognosis (8–10). There are two subsets of MDSCs described, namely the monocytic-MDSCs (M-MDSC) and

the granulocytic or polymorphonucelar-MDSCs (G- or PMN-MDSCs), which are developmentally immature and found at different stages of myelopoiesis (11–13). The g-MDSC were found to be closely related to neutrophils and are predominantly defined by their capacity to suppress T cell activation and proliferation (3, 5, 14). In fact, many studies have found that also mature neutrophils are able, under certain conditions, to suppress the T cells proliferation. The view of g-MDSCs by some is now more considered as a phenotype or subset of neutrophils (15, 16). Therefore, we wondered if the developmental stage is crucial for neutrophils to exert MDSC activity (i.e., T cell suppression), or in other words, whether "age" of a neutrophil would matter.

In a recent study, we investigated in more depth the mechanism of MDSC activity of mature neutrophils of healthy donors. Here we have shown that MDSC activity is only present upon activation and is dependent on ROS production and release of granule components in a CD11b-dependent manner, facilitated by trogocytosis (Aarts et al. under review). Using the same model, we investigated the capacity of human immature neutrophils derived from the bone marrow to exert MDSC activity. We isolated the different neutrophil progenitors from bone marrow, defined by their expression of the surface markers CD11b and CD16. Isolation of cells based on FACS sorting is common practice, also in the field of MDSC research. Many studies rely on this method to isolate MDSCs. However, we found that FACS sorting caused a major impairment on the functionality of the cells, where also mature neutrophils from blood were not able to perform classical functions upon activation by physiological stimuli such as chemoattractants. Therefore, to circumvent the use of FACS sorting, we isolated the progenitors by density centrifugation, followed by CD16-positive magnetic-activated cell sorting (MACS). We found that only the most mature progenitor neutrophils were able to suppress the T cell proliferation, suggesting that MDSCs are perhaps indeed more a phenotype of neutrophils.

MATERIALS AND METHODS

Study Approval

Bone marrow samples were obtained from individuals considered to be healthy, undergoing surgery for unrelated, non-hematological procedures after obtaining written informed consent in accordance with the Declaration of Helsinki (version Seoul 2008). Heparinized peripheral blood samples were collected from healthy donor volunteers.

Cell Isolation

Bone marrow or peripheral blood was diluted 1:1 with PBS/TSC (tri-sodium citrate) and separated based on density by centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific density of 1.076 g/mL. The interphase fraction derived from the peripheral blood, containing peripheral blood mononuclear cells (PBMC), was harvested for isolation of untouched T cells by magnetic-activated cell sorting with the Pan T cell isolation kit of Miltenyi-Biotec (Bergisch Gladbach, Germany) according to the manufacturer's instructions. The pellet fractions from total bone marrow or whole blood samples contained the neutrophil progenitors or mature neutrophils, respectively. The pellet fractions and total bone marrow samples underwent erythrocyte lysis with hypotonic ammonium chloride solution at 4°C. The cells were kept in Hepes-buffered saline solution (HBSS containing 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose, and 0.5% (w/v) human serum albumin, pH 7.4).

Neutrophil progenitors from total bone marrow were separated by FACS sorting based on FSC/SSC and the expression of CD11b (APC-labeled, clone D12, BD Biosciences) and CD16 (PE-labeled, clone 3G8, BD Biosciences). Neutrophils isolated from whole blood were sorted either based on only FSC/SSC or also in combination with the expression of CD11b and CD16. FACS sorting was performed with either a small (85 μM) or big (100 μM) nozzle under cold or room temperature conditions using BD FACS Aria III (BD biosciences).

Neutrophil progenitors from bone marrow were isolated using discontinuous Percoll fractionation, as described previously (17). In short, after erythrocyte lysis bone marrow aspirate was placed upon a two-layer Percoll gradient of densities 1.065 and 1.080 g/mL and centrifuged at 2,000 rpm, acceleration 3, brake 0, for 20 min. After centrifugation the four cell fractions were collected, washed and resuspended in HEPES+ buffer.

CD16 positive cells were isolated from the pellet fractions after density centrifugation of whole blood or bone marrow by magnetic-activated cell sorting with human CD16 microbeads of Miltenyi-Biotec according to the manufacturer's instructions. The CD16 negative cells were collected from the flow through.

Antibodies and Flow Cytometry

The following directly conjugated antibodies were used for flow cytometry analysis: PB-labeled anti-CD11b (clone ICRF44, BD Biosciences), PECy7-labeled anti-CD16 (clone 3G8, BD Biosciences), FITC-labeled anti-FPR1 (clone 350418, R&D systems), FITC-labeled anti-fMLP, PE-labeled anti-TLR4 (clone 610015, R&D systems), PE-labeled TNFR1 (clone 16803, R&D systems), APC-labeled anti-TNFR2 (clone 22235, R&D systems).

Flow cytometry data were acquired using Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, USA).

T Cell Proliferation Assay

Purified T cells were labeled with CFSE (Molecular probes, Life Technologies, Carlsbad, CA, USA) and cultured in 96-well flat bottom plates (Nunclon Delta Surface, Thermo Scientific, Waltham, MA, USA) for 5 days—or otherwise if indicated—at 37°C in IMDM medium (Gibco, Life Technologies, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal calf serum (Bodinco, Alkmaar, The Netherlands), 10^4 U/mL penicillin, 10 ng/mL streptomycin, 200 mM glutamine, and 0.035% (v/v) β-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA). Proliferation was induced by anti-CD3 (clone 1XE [IgE isotype] hybridoma supernatant, 1:1,000, Sanquin, Amsterdam, The Netherlands) and anti-CD28 (clone 15E8 [IgG1 isotype] at 5 μg/mL, Sanquin) monoclonal antibodies

(moAbs; at 20,000 T cells/well). Mature neutrophils from blood or neutrophil progenitors from bone marrow were added in a 1:3 ratio (60,000 neutrophils/well), in the presence or absence of neutrophil-activating stimuli: fMLF (1 μM, Sigma-Aldrich), TNFα (10 ng/mL, Peprotech EC, London, UK) or LPS (20 ng/mL, *E. coli* 055:B5, Sigma).

After 4–6 days, T cell proliferation, indicated by CFSE dilution, was analyzed by flow cytometry. Cells were harvested from the culture plates and stained with APC-labeled anti-CD4 (clone SK3, BD Biosciences, San Jose, CA, USA) and PerCPCy5.5-labeled anti-CD8 (clone SK1, Biolegend, San Diego, CA, USA) antibodies.

ROS Production

NADPH oxidase activity was measured by assaying the hydrogen peroxide production by mature neutrophils derived from blood or neutrophil progenitors derived from bone marrow in response to various stimuli with the Amplex Red kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Cells (1×10^6/mL). In short, cells were stimulated in Hepes-buffered saline solution with fMLF (1 μM), TNFα (10 ng/mL), LPS (20 ng/mL) + LPS-binding protein (LBP) (50 ng/mL, R&D Systems, Minneapolis, MN, USA) or PMA (100 ng/mL, Sigma) in the presence of Amplex Red (0.5 μM) and horseradish peroxidase (1 U/mL). Fluorescence was measured at 30 s intervals during 4 h with the HTS7000+ plate reader (Tecan, Zurich, Switzerland). Maximal slope of hydrogen peroxide release was assessed over a 2 min interval.

Cytospins and Staining

0.5 or 1×10^5 cells were cytospun (Shandon CytoSpin II Cytocentrifuge) onto 76 × 26 mm glass microscope slides. The slides were air-dried and stained. Cytospins slides were incubated for 5 min in May-Grünwald followed by 15 min in phosphate buffer and subsequent Giemsa solution staining for 30 min. Slides were rinsed in deionized water, air-dried and analyzed with Zeiss Scope.A1 microscope.

Statistics

Statistical analysis was performed with GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, CA, USA). Data were evaluated by one-way ANOVA or unpaired two-tailed student's *t*-test. The results are presented as the mean ± SEM. Data were considered significant when $p < 0.05$.

RESULTS

Sorted Isolated Neutrophil Progenitor Cells From Bone Marrow Do Not Exert MDSC Activity

As previously described (18), mature neutrophils from peripheral blood from healthy donors show MDSC activity (i.e., suppression of T cell proliferation), but only upon activation. To investigate whether immature neutrophils can also exert MDSC activity we tested neutrophil progenitor cells collected from bone marrow samples obtained from patients who underwent cardiac-bypass surgery unrelated to any cancer or underlying hematological or chronic inflammatory disease (other than atherosclerosis). Neutrophil progenitor cells can be divided in four different developmental stages, namely (pro)myelocytes, metamyelocytes, band cells, and segmented neutrophils (19). We isolated the progenitor cells based on the expression of surface markers CD11b and CD16 (**Supplement Figure 1**) via FACS sorting, which is also used as a method to isolate PMN-MDSC from the ring fraction after density gradient centrifugation (20). The four isolated neutrophil progenitor cells were cultured for 5 days in presence of isolated CFSE-labeled T cells from a healthy donor and were left unstimulated or activated with either fMLF, TNFα, or LPS. T cell proliferation was induced by monoclonal anti-CD3 and anti-CD28 antibodies and quantified as relative "precursor frequency": i.e., percentage of naïve cells in the initial population that underwent one or more divisions upon anti-CD3/anti-CD28 antibodies (21), normalized for the condition with only stimulated T cells. See **Supplement Figure 2** for the gating strategy of the flow cytometry analysis. As a control, mature neutrophils were isolated from the same healthy donor as the T cells. None of the isolated neutrophil progenitor cell fractions were able to suppress the T cell proliferation of $CD4^+$ or $CD8^+$ T cells (**Figure 1A**, **Supplement Figure 3**). Only the mature neutrophils isolated from blood were able to suppress the T cell proliferation upon activation. We hypothesized that maybe the lack of MDSC activity could be caused by an absent or lower expression of the receptors (FPR-1, TNF receptor, or TLR4). The expression of the fMLF and TNFα receptor is indeed increasing upon maturation in the bone marrow. However, the presence of the TLR4 is already present at the early progenitor stage onwards and is comparable to the expression of mature neutrophils isolated from blood (**Figure 1B**), indicating that the lack of MDSC activity upon LPS stimulation is not caused by the absence of the receptor as such.

One of the main effector mechanism of human MDSC activity that has been described in the literature is the generation of a large amount of reactive oxygen species (ROS) upon cell activation (Aarts et al. under review) (22). The sorted neutrophil progenitor cells were not able to produce ROS when activated by either fMLF, TNFα or LPS, which then may explain why these immature cells cannot suppress the T cell proliferation in our MDSC activity assay (**Figure 1C**).

FACS Sorting Causes an Impairment in Neutrophil Function

However, and much to our surprise, also the myeloid cells at the most mature progenitor stage in the bone marrow fractions (i.e., the segmented neutrophils of the "reserve pool") were not able to produce ROS. This led us to believe that perhaps the sorting process to isolate the different progenitor cells may cause an impairment in cellular functions. Therefore, we tested multiple sorting conditions using mature neutrophils isolated from blood and investigated their ROS production after sorting. When we sorted mature neutrophils under the same conditions as our bone marrow samples (cold, antibody incubation at 4 degrees, **Supplement Figure 1**), we observed that the sorted neutrophil fraction was not able to produce ROS upon stimulation, apart

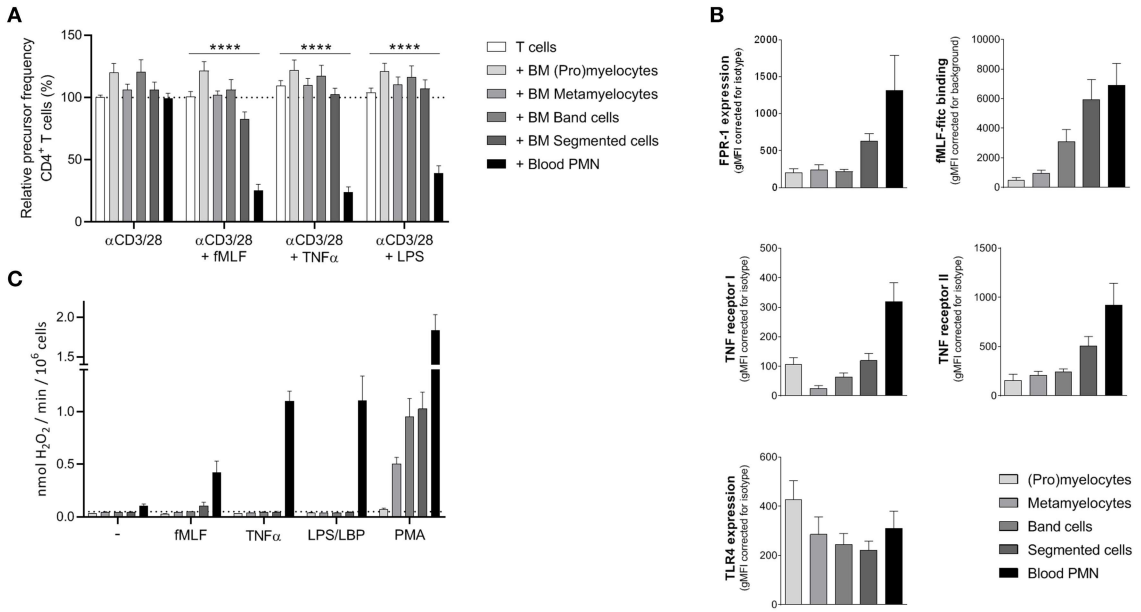

FIGURE 1 | Sorted neutrophil progenitor cells from bone marrow do not exert MDSC activity. Neutrophil progenitors from bone marrow were isolated via FACS sorting based on CD11b and CD16 expression under cold conditions and with a small nozzle. **(A)** Purified CFSE-labeled T cells from healthy donors ($n = 6$) were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of mature neutrophils from control donors (black bars, $n = 6$) or sorted neutrophil progenitors from bone marrow (gray bars, $n = 3$) and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD4^+$ T cells. **(B)** The surface marker expression of FPR-1 and fMLF binding (top panel, $n = 3$–8), TNF receptor I and II (center panel, $n = 4$) and TLR4 (bottom panel, $n = 4$) was measured by flow cytometry analysis of mature neutrophils from blood (black bar) and neutrophil progenitors from bone marrow. **(C)** Mature neutrophils and neutrophil progenitors were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin ($n = 3$). Error bars indicate SEM; $^{****}p < 0.0001$.

from the non-physiological stimulus PMA, a phorbol ester that bypasses surface receptors and directly activates intracellular protein kinase C (**Figure 2A**).

We hypothesized, that the lack of ROS production may be caused by the pressure the cells undergo during sorting, since the temperature nor the labeling did impair the ROS production (**Supplement Figure 4**). To reduce the pressure during sorting we used the biggest nozzle (100 μM) and sorted the neutrophils only based on their size (FSC/SSC) and at room temperature to further exclude any other variable. Once again, the sorted neutrophils showed a lower ROS production and in addition also much less if any MDSC activity in our T cell proliferation assay (**Figures 2B,C**, **Supplement Figure 5**), concluding that FACS sorting is not an ideal method to isolate neutrophils and their progenitors from bone marrow for functional studies, including the detection of MDSC activity.

Neutrophil Progenitors Isolated by Density Centrifugation Show a Less Effective MDSC Activity

Since FACS sorting clearly had an impact on the functionality of neutrophils, we decided to isolate the progenitor cells by Percoll density centrifugation. The pellet fraction includes all the four progenitor populations, where the segmented neutrophils are the most abundant (around 50%, **Supplement Figure 6**). In contrast to the sorted progenitor fractions, the cells from the bone marrow (BM) pellet fraction were able to produce ROS upon stimulation (**Figure 3A**), indicating again that the sorting process indeed impair the cells. Also, in our T cell proliferation assay we observed some inhibition of the T cell proliferation by the BM pellet cells, though this was only observed in the proliferation of $CD4^+$ T cells and not in the $CD8^+$ T cells, where the suppression was not significant (**Figure 3B**, **Supplement Figure 7**). So to conclude, the ROS production and the MDSC activity of the BM pellet cells were not as effective as the mature neutrophils isolated from blood. This could be explained by the fact that the BM pellet fraction is still a heterogeneous cell population of immature (myelocytes and metamyleocytes) and early mature neutrophils (i.e., cells with a nuclear band and segmented shape), where the immature progenitor cells have a lower FPR-1 or TNF receptor expression. Therefore, to fully investigate the MDSC activity of the more immature progenitor cells an extra isolation step is needed for further functional testing.

Only the $CD16^+$ Neutrophil Progenitors Show MDSC Activity

Another technique to isolate the different neutrophil progenitor cells from bone marrow that has been described is discontinuous Percoll fractionation, where the increasing density of the cells with maturity forms the basis of the separation (17). Bone marrow aspirate was placed upon a two-layer Percoll gradient of densities 1.065 and 1.080 g/mL, generating four BM cell

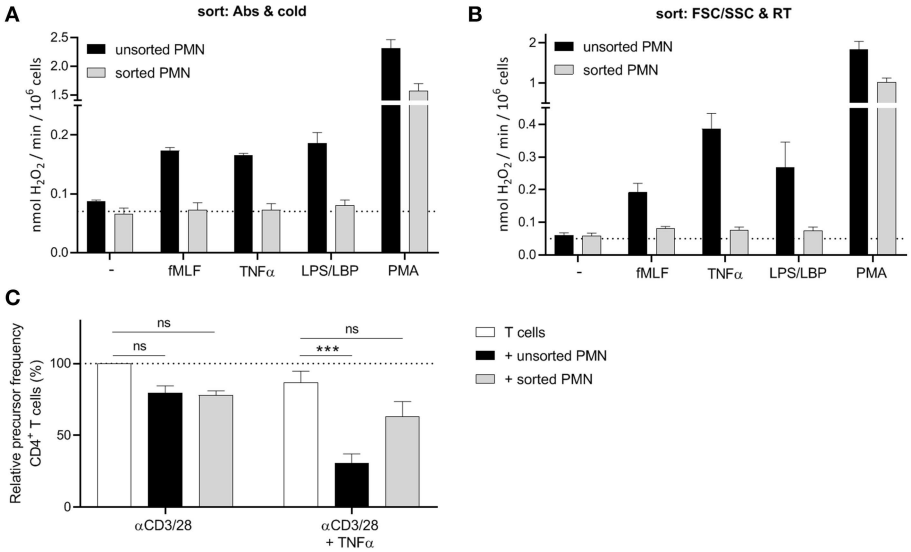

FIGURE 2 | FACS sorting causes an impairment in neutrophil function. **(A,B)** Neutrophils were left unsorted (black bars, $n = 6$) or sorted based on CD11b and CD16 expression under cold conditions and a small nozzle (**A**, gray bars, $n = 3$) or based on size (FSC/SSC) under RT conditions and a big nozzle (**B**, gray bars, $n = 5$). Cells were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin. **(C)** Purified CFSE-labeled T cells from healthy donors were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of unsorted (black bars) or sorted (gray bars) mature neutrophils from control donors and/or indicated stimuli ($n = 3$). Sort was based on size (FSC/SSC) under RT conditions and a big nozzle. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD4^+$ T cells. Error bars indicate SEM; $^{***}p < 0.001$.

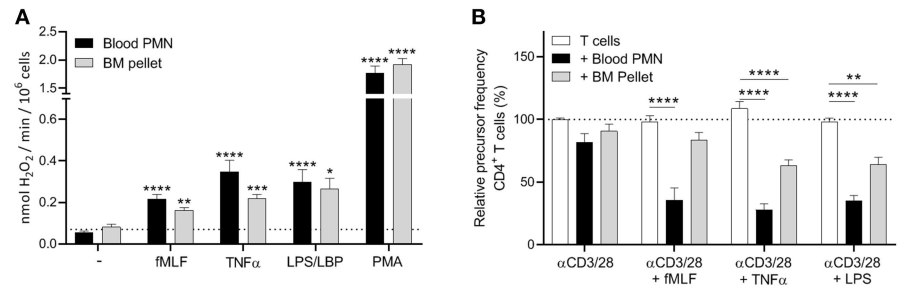

FIGURE 3 | Neutrophil progenitors isolated by density centrifugation show a less effective MDSC activity. **(A)** Mature neutrophils (black bars) and neutrophil progenitors from the bone marrow pellet fraction after density centrifugation (gray bars) were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin ($n = 3$). **(B)** Purified CFSE-labeled T cells from healthy donors were cultured with anti-CD3 and anti-CD28 antibodies (white bars, $n = 6$), and in presence of mature neutrophils from blood (black bars, $n = 6$) or neutrophil progenitors from the bone marrow pellet (gray bars, $n = 3$) and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD4^+$ T cells. Error bars indicate SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

fractions after centrifugation (**Supplement Figure 8A**), where in the first fraction the most immature cells should end up and the most mature cells in the last fraction. However, when we analyzed the four obtained BM cell fractions with the maturation markers CD11b and CD16, we found that each BM cell fraction showed some cell heterogeneity where in each fraction segmented cells were present (**Supplement Figures 8B,C**). Apart from fraction 1, all the BM cell fractions were able to produce ROS upon different stimuli, including fMLF and TNFα which is surprising since the more immature neutrophil progenitor cells do not express the receptors of these ligands as highly as the more mature progenitors (**Figure 1B**). The presence of the ROS production may therefore be due to the presence of the mature segmented cells in the fractions. Because of this cell heterogeneity, we concluded that this method is not suitable for us to investigate the MDSC activity of neutrophil progenitor cells and need a different approach to separate the early immature from the more mature neutrophils in bone marrow.

To divide the progenitors from the BM pellet fraction into immature and "early mature" neutrophils, we isolated the CD16-positive cells from the BM pellet fraction by magnetic-activated cell sorting (MACS) with human CD16 microbeads. The CD16-positive neutrophil fraction from the BM pellet was

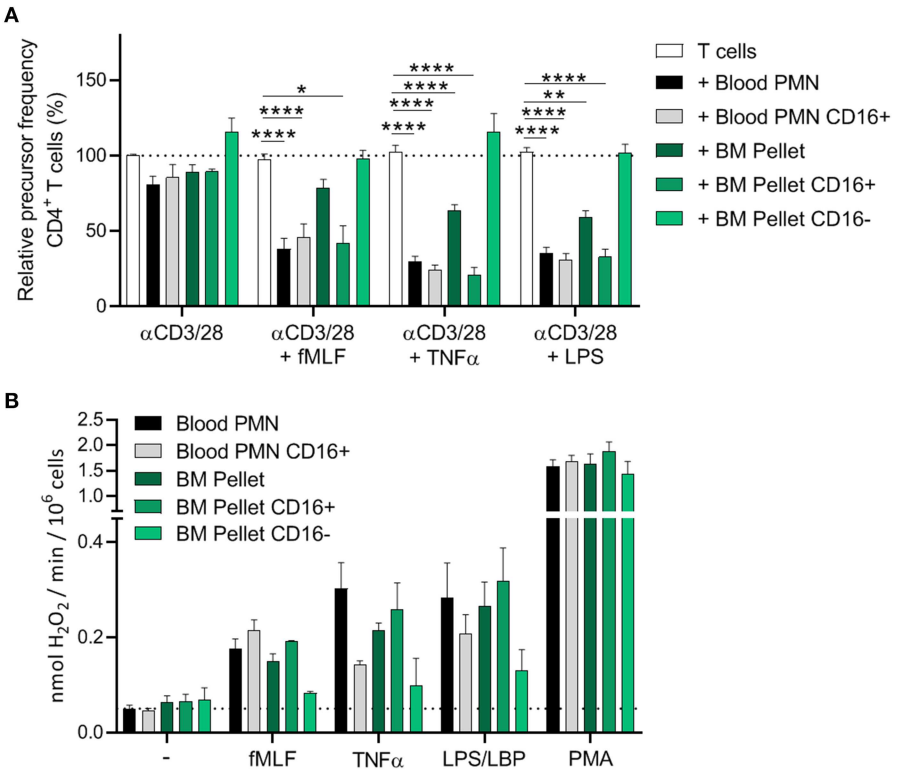

FIGURE 4 | Only the CD16$^+$ neutrophil progenitors show MDSC activity, ROS production and degranulation. **(A,B)** CD16 positive cells were isolated via MACS isolation from mature neutrophils from blood and neutrophil progenitors from BM pellet. **(A)** Purified CFSE-labeled T cells from healthy donors ($n = 4$) were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of mature neutrophils from control donors (black bars, $n = 6$), CD16$^+$ mature neutrophils (gray bars, $n = 6$), total BM pellet fraction (dark green bars, $n = 4$), CD16$^+$ (green bars, $n = 4$) or CD16$^-$ (light green bars, $n = 4$) progenitors from BM pellet and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among CD4$^+$ T cells. **(B)** The indicated cell fractions were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin ($n = 4$–6). Error bars indicate SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

depleted from the immature progenitors, however the CD16-negative fraction still included some "early mature" progenitors though this percentage was significantly lower than in the total BM pellet fraction and did not include the segmented cells as was the problem with the discontinuous Percoll fractionation (**Supplement Figure 9**).

The CD16-positive isolation procedure did not result in a less effective MDSC activity of mature neutrophils from blood (**Figure 4A**), indicating that MACS isolation is a more gentle approach to isolate cells than FACS sorting. Only the CD16-positive progenitor cells from the BM pellet were able to suppress the T cell proliferation (i.e., show MDSC activity). The CD16-negative progenitor cells showed an even higher T cell proliferation than T cells cultured without mature neutrophils or BM progenitor cells but definitely not any indication of enhanced MDSC activity (**Figure 4A**, **Supplement Figure 10**).

The CD16-positive and CD16-negative progenitor cells also differed in ROS production. The CD16-negative progenitors were hardly able to induce ROS production upon (physiological) stimulation, whereas the CD16-positive progenitors showed ROS production more comparable to circulating neutrophils isolated from blood. This could also explain why the total BM pellet shows a decent ROS production upon stimulation, since the BM pellet contains more of the "early mature" (band and segmented cells) neutrophils progenitors (**Figure 4B**).

Though the immature neutrophils seem to have the machinery to produce ROS, as was indicated by the PMA stimulation, the signal transduction pathways within these cells may not be fully developed yet to exert MDSC activity (23). When all these data are taken together, our results suggest that the MDSC activity obtained during neutrophil maturation in the bone marrow correlates with the induction of the well-known highly mobile and toxic effector functions of the fully differentiated neutrophil in the circulation.

DISCUSSION

MDSCs have been described as a heterogeneous subset of immature myeloid cells, defined by their capacity to suppress T cell activation and proliferation. It is thought that progenitor neutrophils exit the bone marrow early, migrate to blood and

have suppressive activity as g-MDSCs with a role during the process of inflammation resolution (24). Tumors represent a chronic state of inflammation, and the presence and infiltration of MDSCs have been associated with poor prognosis (8–10). Although the numbers of immature neutrophils have been reported to be increased in the circulation of cancer patients, we show here that immature neutrophils *per se* are not efficient in suppressing T cells. Obviously, the neutrophil progenitors that we tested derive from patients who are not considered to be suffering from a chronic state of severe inflammation or cancer, which could explain their lack of MDSC activity. However, we were able to induce this activity in the "early mature" neutrophils from bone marrow fractions by activation with physiological neutrophil stimuli. In fact, we have shown in a recent study that MDSC activity by mature neutrophils from healthy donors can only be induced by certain and not all neutrophil activators (Aarts et al. under review), which correlates to the capacity to induce ROS production.

The expansion and accumulation of immature MDSCs in the bloodstream in presence of tumors have been often reported (1, 25). These immature neutrophils that are released in the circulation can perhaps function as a source for mature neutrophils, since it has been shown that immature neutrophils can mature in the circulation (26), or perhaps at the site of inflammation or within the tumor environment itself. It has been shown that adoptively transferred g-MDSCs can enter tumors and differentiate into mature phenotype (27). This could explain on the one hand a role for immature myeloid cells and at the same time explain why we could only observe MDSC activity in the more mature neutrophil and not in any of the immature progenitor fractions from bone marrow. Such a hypothesis is supported by our observation that MDSC activity obtained during neutrophil maturation correlated with the induction of motility and toxic effector functions of the circulating neutrophil required to suppress T cell proliferation (Aarts et al. under review) (22, 23, 28). We may suggest that under certain disease conditions, immature neutrophils can be released from the bone marrow where further differentiation takes place in the circulation or at the site of the inflammation or tumor development to become effective local T cell suppressor cells with MDSC activity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medische Ethische Toetsingcommissie, Amsterdam Universitair Medisch Centra (AUMC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TK and IH are the principle investigators, who conceived, and designed the study. CA, IH, AT, and EM performed the experiments. TB and RB contributed to the design of the study. CA and IH initiated many of the experiments and performed the analysis. CA wrote the manuscript together with TK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02144/full#supplementary-material

Supplement Figure 1 | FACS sorting strategy of bone marrow and neutrophils from blood. Bone marrow and mature neutrophils from blood were sorted based on CD11b and CD16 expression under cold conditions and with a small nozzle. **(A)** Representative flow cytometry images of gating strategy for FACS sorting. **(B)** Representative images of progenitors cells isolated from the bone marrow after FACS sorting stained by May-Giemsa.

Supplement Figure 2 | Read-out of T cell proliferation in absence or presence of fMLF-activated neutrophils. Purified CFSE-labeled T cells from healthy donors were cultured with anti-CD3 and anti-CD28 antibodies in presence or absence of mature neutrophils from control donors. After 5 days of culture, cells were collected for FACS analysis and stained with anti-CD4 and anti-CD8 antibodies. Lymphocytes were gated based on size (first panel) followed by the exclusion of duplet cells (second panel). The CFSE dilution of $CD4^+$ and $CD8^+$ was measured and each cell division was gated for the calculation of the precursor frequency to quantify the proliferation.

Supplement Figure 3 | Sorted neutrophil progenitors from bone marrow do not suppress $CD8^+$ T cell proliferation. Neutrophil progenitors from bone marrow were isolated via FACS sorting based on CD11b and CD16 expression under cold conditions and with a small nozzle. Purified CFSE-labeled T cells from healthy donors ($n = 6$) were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of mature neutrophils from control donors (black bars, $n = 6$) or sorted neutrophil progenitors from bone marrow (gray bars, $n = 3$) and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD8^+$ T cells. Error bars indicate SEM; $^{****}p < 0.0001$.

Supplement Figure 4 | Incubation with FACS antibodies under cold conditions does not impair ROS production. Neutrophils were left unlabeled at RT (white bars) or at 4°C (gray bars) or labeled with anti-CD11b and anti-CD16 antibodies at 4°C (black bars) for 30 min. Cells were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin ($n = 3$).

Supplement Figure 5 | Sorted mature neutrophils do not suppress $CD8^+$T cell proliferation. Purified CFSE-labeled T cells from healthy donors were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of unsorted

(black bars) or sorted (gray bars) mature neutrophils from control donors and/or indicated stimuli ($n = 3$). Sort was based on size (FSC/SSC) under RT conditions and a big nozzle. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD8^+$ T cells. Error bars indicate SEM; $^{**}p < 0.01$.

Supplement Figure 6 | FACS analysis of bone marrow pellet after density centrifugation. The surface marker expression of CD11b and CD16 was measured by flow cytometry analysis of cells in the bone marrow pellet after density centrifugation. Neutrophil progenitors were first gated based on size **(Left)** and then gated based on the expression of CD11b and CD16 **(Right)**. Shown are representative FACS analysis images ($n = 3$).

Supplement Figure 7 | Neutrophils progenitors from BM pellet fraction do not suppress $CD8^+$T cell proliferation. Purified CFSE-labeled T cells from healthy donors were cultured with anti-CD3 and anti-CD28 antibodies (white bars, $n = 6$), and in presence of mature neutrophils from blood (black bars, $n = 6$) or neutrophil progenitors from the bone marrow pellet (gray bars, $n = 3$) and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD8^+$ T cells. Error bars indicate SEM; $^{****}p < 0.0001$.

Supplement Figure 8 | Bone marrow cell fractions obtained by discontinuous Percoll fractionation show cell heterogeneity. **(A)** Schematic drawing of the set-up of the discontinuous Percoll fractionation. Bone marrow was placed upon a two-layer Percoll gradient of densities 1.065 and 1.080 g/mL, generating four fractions after centrifugation. **(B)** Gating strategy of flow cytometry analysis of the four BM cell fractions. Shown are representative FACS analysis images of the granulocyte gating based on size (FSC/SSC). **(C)** The percentage of the different neutrophil progenitors within the cell fractions (indicated by number on the x-axis) were measured by flow cytometry based on CD11b and CD16 expression within the granulocyte gate shown in **(B)**. **(D)** The indicated cell fractions and neutrophils from blood were stimulated with the indicated stimuli and production of H_2O_2 was determined by measuring Amplex Red conversion into fluorescent Resorufin ($n = 2$–4).

Supplement Figure 9 | FACS analysis of mature neutrophils and neutrophil progenitors before and after CD16+ MACS isolation. The surface marker expression of CD11b and CD16 was measured by flow cytometry analysis of both mature neutrophils from blood **(Left)** and neutrophil progenitors from BM pellet **(Right)** before and after CD16 positive MACS isolation. Shown are representative FACS analysis images ($n = 3$).

Supplement Figure 10 | Only the $CD16^+$ neutrophil progenitors can suppress $CD8^+$T cell proliferation. CD16 positive cells were isolated via MACS isolation from mature neutrophils from blood and neutrophil progenitors from BM pellet. Purified CFSE-labeled T cells from healthy donors ($n = 4$) were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in presence of mature neutrophils from control donors (black bars, $n = 6$), $CD16^+$ mature neutrophils (gray bars, $n = 6$), total BM pellet fraction (dark green bars, $n = 4$), $CD16^+$ (green bars, $n = 4$) or $CD16^-$ (light green bars, $n = 4$) progenitors from BM pellet and/or indicated stimuli. Cells were harvested after 5–6 days and analyzed by flow cytometry for CFSE dilution among $CD8^+$ T cells. $^*p < 0.05$, $^{**}p < 0.0001$.

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REVIEW
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Neutrophil Heterogeneity in Cancer: From Biology to Therapies

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Neutrophils have been extensively described in the pathophysiology of autoimmune and infectious diseases. Accumulating evidence also suggests the important role of neutrophils in cancer progression through their interaction with cancer and immune cells in blood and in the tumor microenvironment (TME). Most studies have described neutrophils as key drivers of cancer progression, due to their involvement in various tumor promoting functions including proliferation, aggressiveness, and dissemination, as well as in immune suppression. However, such studies were focusing on late-stages of tumorigenesis, in which chronic inflammation had already developed. The role of tumor-associated neutrophils (TANs) at early stages of tumor development remains poorly described, though recent findings indicate that early-stage TANs may display anti-tumor properties. Beyond their role at tumor site, evidence supported by NLR retrospective studies and functional analyses suggest that blood neutrophils could also actively contribute to tumorigenesis. Hence, it appears that the phenotype and functions of neutrophils vary greatly during tumor progression, highlighting their heterogeneity. The origin of pro- or anti-tumor neutrophils is generally believed to arise following a change in cell state, from resting to activated. Moreover, the fate of neutrophils may also involve distinct differentiation programs yielding various subsets of pro or anti-tumor neutrophils. In this review, we will discuss the current knowledge on neutrophils heterogeneity across different tissues and their impact on tumorigenesis, as well as neutrophil-based therapeutic strategies that have shown promising results in pre-clinical studies, paving the way for the design of neutrophil-based next generation immunotherapy.

Keywords: neutrophil (PMN), cancer, immunotherapy, MDSC (myeloid-derived suppressor cells), G-MDSC (granulocytic MDSC), tumor-associated neutrophils (TANs), subsets

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CIRCULATING NEUTROPHILS IN CANCER

Aside from the molecular signals driving cancer, several studies have demonstrated the contribution of the host-driven inflammatory response to tumor progression and/or to treatment outcome (1–4). Neutrophils are key players in the inflammatory response. They are released into the bloodstream after maturation and differentiation from the bone marrow reservoir (5). The production of neutrophils has been estimated to range from 1 to 2 $\times$ 10^{11} cells per day at steady state in a healthy adult. Neutrophils represent 50–70% of all circulating leucocytes in humans, while they account for 10–25% in mice (6).

Neutrophil Count to Lymphocyte Count Ratio (NLR)

Circulating neutrophil counts are systematically monitored by oncologists during cancer management, owing to chemotherapy-induced neutropenia, which makes patients more vulnerable to life-threatening infections (7, 8). Availability of blood cell counts from retrospective analyses has led to numerous reviews and meta-analyses investigating the prognostic value of the neutrophil count (or preferably neutrophil-to-lymphocyte ratio, also called NLR) in both localized or metastatic contexts (9–12). Hence, over the past decade, literature on NLR has grown steadily. For instance, it is now well-acknowledged that NLR elevation is strongly associated with poor median progression-free survival (mPFS) and median overall survival (mOS) regardless of tumor type, stage of the disease or treatment (9). Interestingly, among metastatic patients, NLR has a prognostic value before chemotherapy and after subsequent lines of treatment in more advanced disease management (11). Optimal NLR cut-off values used to determine increased risk of mortality vary greatly (between 1.9 and 9.21) across studies (12). If we consider NLR as a continuous variable, each of its incremental increases in standard deviation is associated with a 35% increase in the risk of mortality. Moreover, patient follow-up duration influences NLR, as illustrated by the fact that the largest differences observed across patient prognostic groups occur within the first 12 months of follow-up (11). Beside confirming the baseline prognostic value of NLR, its early decrease following only one cycle of chemotherapy appears to be of good prognosis in multiple pathologies: (i) colorectal cancer (13, 14); (ii) mesothelioma; (iii) triple-negative breast cancer (15); (iv) docetaxel-treated patients bearing lung, prostate, head and neck or breast cancers (16); (v) advanced pancreatic adenocarcinoma; and (vi) in peripheral T-cell lymphomas (17).

Conversely, other retrospective studies did not confirm the predictive value of NLR across different randomized chemotherapy arms in colorectal cancer (18), or in advanced biliary tract carcinomas, in which low NLR values during chemotherapy were not associated with significant improvement in survival (only high NLR baseline values that decreased under chemotherapy predicted a significant better mOS) (19). NLR pretreatment values were not predictive of outcome in prostate cancer patients treated with docetaxel (20).

Variation in NLR values during targeted therapy seems to be an interesting biomarker of response in metastatic renal cell carcinoma patients (19). Templeton et al. reported a retrospective analysis of 1,199 patients treated with targeted therapies (bevacizumab, axitinib, sorafenib, sunitinib, temsirolimus) from the metastatic renal cell carcinoma database consortium and highlighted the predictive value of NLR variation between baseline (before targeted therapy commenced) and at 6 weeks ($\pm$2 weeks). They used a validation cohort of 4,350 patients from a prospective clinical trial. Compared to no change, a decrease in NLR exceeding 25% was associated with a significant improvement in the response rate, mPFS and mOS, while an increase was predictive of poor outcome. Of note, the highest response rates were observed in groups with low baseline NLR values that remained low at 6 weeks, though a good response rate was also reported in groups with an NLR superior to 3 which declined below 2.25 after the administration of targeted therapy (21). Similar observations were described in non-small cell lung carcinomas treated with gefitinib or erlotinib (22–24), advanced gastro-intestinal stromal tumors (25), advanced soft-tissue sarcoma treated with pazopanib (26), and hepatocellular carcinomas treated with sorafenib (27, 28).

The emergence of anti-CTLA4 and anti-PD(L)1 immunotherapies provides new hope in cancer management. However, due to an overall response rate below 40% (29, 30) and to treatment costs, stratification of patients to identify the best candidate for immunotherapy has become a challenge. Readily available total blood count has enabled the evaluation of NLR in patients receiving immunotherapy, such as in advanced melanoma in which NLR in pretreated patients was identified as an independent marker of response (31–33), even when NLR was recorded during treatment (34). Similarly, in metastatic non-small cell lung carcinomas under anti-PD-1 therapy (30, 35), higher baseline NLR values were associated with a lower response rate (36). In patients with various advanced solid tumors candidates to phase I trials combining PD-1/PD-L1 inhibitors, a low NLR was correlated with response to treatment and improved OS, but not with increased immune toxicity (37).

Cancer-associated systemic inflammation often characterized by a high NLR, associated with a poor prognosis, was thought to occur only at late stages of tumorigenesis (38, 39). Evidence also suggests that NLR may increase at early-stage (stage I and stage II, separately) before treatment, and retains its poor prognostic significance in various cancer types including colon cancer (40), tongue cancer (41), breast cancer (42), and liver cancer (43). Early systemic modifications may therefore occur at early-stages of tumorigenesis.

Discrepancies across studies may be due to the level of heterogeneity of the populations studied (e.g., primary tumor, stage of disease, patient features: medical history or concomitant medication), limitations of retrospective reports, wide variation in NLR cut-off values, as well as in the dynamic assessment of NLR during treatment, and/or a lack of specificity of NLR (neutrophil count gathering immature neutrophils that might be released in the context of inflammation and expected circulating mature neutrophils). Interestingly, studies assessing the prognostic and/or predictive values of NLR in a wide variety of conditions (disease, stages, histology, treatment…) argue in favor of its utility. This correlation of NLR with clinical outcome suggests that changes in NLR may be linked with broader modifications beyond the tumor microenvironment. Hence, beyond being a relevant clinical biomarker, increase in NLR with disease progression highlights the importance of considering the systemic environment and not simply the tumor for a deeper understanding of biological mechanisms underlying cancer progression. It is possible that the tumor itself secretes factors into the bloodstream, which thereby act on bone marrow to skew hematopoiesis toward granulocytic lineages. Tumor may also release danger/damaged-associated molecules (DAMP) that might be a target for infiltrating neutrophils. Hence, the means by

which an increase in NLR promotes cancer progression remains to be elucidated.

T-Cell Suppressive Circulating Neutrophils

Beyond their increase in the peripheral blood of cancer patients, which was associated with a poor prognosis, subsets of circulating neutrophils were reported to display tumor-promoting functions, inferring a causative role in cancer progression rather than just a consequence of the disease. The most-extensively described tumor-promoting function of circulating neutrophils remains their ability to suppress T-cell proliferation and/or activation *in vitro*. These T-cell suppressive neutrophils are classically termed granulocytic myeloid-derived suppressor cells (G-MDSCs) for both humans and mice. G-MDSCs were documented to expand in tumor-bearing hosts compared to healthy subjects (44, 45). In cancer patients, G-MDSCs are $CD11b^{+}$ $CD14^{-}$ $CD66b^{+}$ $CD15^{hi}$ expressing-cells that are enriched in the low-density neutrophils (LDNs) fraction present within the peripheral blood mononuclear cell (PBMCs) ring, unlike normal density neutrophils (NDNs), which are found in the granulocyte pellet of ficolled blood (46–49). In mice, G-MDSC correspond to $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{hi}$-expressing cells present within the spleen or the tumor (45, 50, 51), and only few studies refer mouse G-MDSCs to LDNs (44). Although LDNs are the best described neutrophil subset(s) in the blood of cancer patients, there is currently no clear LDN-specific biomarker(s). The scavenger receptor Lox1 was recently reported to be expressed by a subset of LDNs (48). Moreover, those LDNs contained both mature and immature neutrophils (44, 49). These findings suggest that LDNs remain a heterogeneous population of circulating neutrophils that need to be further characterized.

Recent studies conducted in human subjects, revealed new subsets of T-cell suppressive circulating neutrophils based on their stage of maturity. Activated mature neutrophils defined as $CD11c^{bright}$ $CD62L^{dim}$ $CD11b^{bright}$ $CD16^{bright}$ cells in healthy volunteers challenged with LPS systemically (52) and $CD66b^{+}$ $CD11b^{bright}$ $CD16^{bright}$ mature LDNs in cancer patients (49) were reported to suppress T-cell proliferation. Both studies show that mature neutrophils inhibit interferon gamma production (IFNγ) by activated T-cells (49, 52). High level of $CD66b^{+}$ $CD11b^{bright}$ $CD16^{bright}$ mature LDNs strongly correlated with adverse outcome in head and neck cancer (49). Interestingly, Evrard et al. demonstrated in a murine model of pancreatic cancer that the concentration of blood $Ly6G^{high}$ $CD101^{-}$ immature neutrophils was significantly greater in mice with a high tumor burden compared to the low tumor burden group, whereas this was not the case for mature neutrophils (53). In the same line, another group showed that the adoptive transfer of unipotent, committed human $CD66b^{+}$ $CD117^{+}$ neutrophil progenitor (hNeP) in immune deficient NSG-M3 mice accelerated osteosarcoma tumor growth compared with the transfer of committed monocyte progenitors (54). Surprisingly in this study, *in vitro* co-culture of either hNeP or mature bone marrow neutrophils with T-cells, activated the latter based on the upregulation of CD69, rather than inhibiting T-cell activation compared to control (54). The precise mechanisms by which immature circulating neutrophils contribute to tumor growth remain unknown.

The suppression of T-cell proliferation by circulating neutrophils has been attributed to the release of different molecules. Reactive oxygen species (ROS) and Arginase 1 are the two most extensively described neutrophil-derived T-cell suppressive factors (46–48, 52, 55). In humans, both factors require a CD18/Mac-1 immunological synapse between neutrophils and T-cells to display suppressive functions (52, 55). Circulating neutrophils appear to suppress T-cell proliferation via reversible cell cycle arrest rather than induction of apoptosis, as the addition of l-arginine or inhibition of arginase in neutrophil/T-cell co-cultures restored T-cell proliferation in G-CSF-treated healthy donors (55) and cancer patients, respectively (49).

Circulating Tumor Cell-Escorting Neutrophils

An emerging tumor-promoting function of circulating neutrophils has recently been unveiled. Neutrophils were shown to entrap circulating tumor cells (CTCs) at metastatic sites to facilitate their extravasation thus contributing to metastasis (56–60). Recent data showed that mouse neutrophils interacted with CTCs to promote their proliferation within the bloodstream and subsequently foster metastasis (61). In breast cancer patient blood, a high level of CTC-neutrophil clusters was associated with a higher risk of developing metastases (61).

Taken together, in addition to the NLR, there is a strong rationale for routinely monitoring CTC-neutrophil clusters with the aim of evaluating their prognostic impact and predictive value in cancer patients.

PHENOTYPIC AND FUNCTIONAL HETEROGENEITY OF TUMOR-ASSOCIATED NEUTROPHILS (TANs)

Neutrophils are able to infiltrate tumor tissue and are termed tumor-associated neutrophils (TANs). In mice, TANs express $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{hi}$, whereas in humans, they are identified as $CD11b^{+}$ $CD14^{-}$ $CD66b^{+}$ $CD15^{hi}$ cells (50).

Identification and Quantification of TANs in Cancer Patients

The clinical relevance of evaluating pro- and anti-tumor functions of TANs is highly pertinent in cancer patients, since TAN infiltration was reported to predict either poor (62–66) or good prognosis (67–69). Conditions that differ between good and poor prognostic TANs will be discussed below. Although the methods of analysis of survival across studies were similar, identification of quantification methods of TANs infiltrating human tumors varied greatly. Hematoxylin & Eosin (H&E) staining remains a good approach to quantify TAN infiltration based on the unique segmented-nucleus morphology of neutrophils. Scanned-tumor slides stained with H&E are accessible from TCGA public database and have already been

used for TAN quantification (70). However, this approach may under-estimate the potential infiltration of immature neutrophils since the banded-nucleus morphology of immature neutrophils is less distinguishable from other immune cells.

TAN infiltration can be also quantified through immunostaining, using antibodies against markers of neutrophils, such as CD66b and CD15. Neutrophils and eosinophils share numerous markers as they are closely related ontologically speaking, and very few studies took this cofounding effect into account. Evidence showed that CD66b is expressed at the same level between eosinophils and neutrophils in peripheral blood of patients with rheumatoid arthritis (71). For CD15 (Sialyl-Lewis X), although expressed on both eosinophils and neutrophils, data showed that it is 10–100 times higher on neutrophils than eosinophils (72). CD15 would therefore be more reliable to distinguish neutrophils from eosinophils, although the search for differentially expressed markers between these two cell types is strongly needed. Some studies also used myeloperoxidase (MPO), neutrophil elastase, CXCR2, or CD33 to identify TANs in human tumors. However, such markers are not specific to TANs and are shared by other immune cells (45, 73–76) or even tumor cells, such as CXCR2.

Bioinformatics approaches inferring the fractions of tumor-infiltrating immune cells from bulk tumor RNAseq data have recently emerged (77). The most classical approach for quantifying neutrophil infiltrate relies on single marker genes specific to neutrophils, such as CSF3R. Different groups have used this approach to classify tumors based on CSF3R expression (78, 79). Other approaches using multiple gene signatures have come to light. Although signatures slightly differ from one another study, algorithms processing these signatures vary greatly. For instance, single sample Gene Set Enrichment Analysis (ssGSEA) was recently used for the stratification of lung cancer patients based on the gene signature of a newly identified pro-tumor neutrophil population. CIBERSORT was used for quantifying neutrophil infiltrate in human tumor based on a signature composed of genes highly expressed in blood neutrophils compared to other blood leukocytes (80, 81).

Pro-tumor TANs

In both murine and human diseases, TANs are mostly described for their ability to promote tumor progression through different mechanisms, such as tumor cell proliferation. Mouse $Gr1^+$ myeloid cells secrete interleukin-1 receptor antagonist (IL-1RA), which were shown to antagonize the anti-tumor effects of cellular senescence in a murine model of $PTEN^{-/-}$ prostate cancer (82). Neutrophil elastase (NE), a protease secreted by neutrophils, accesses the endosomal compartment of tumor cells. There, it degrades the insulin receptor substrate 1 (IRS-1) which increases the interaction between PI3K and PDGFR, thereby promoting their proliferation in a LSL-K-ras model of murine lung adenocarcinoma and in human lung adenocarcinoma cell lines (83). Interestingly, the authors identified in human lung adenocarcinoma an inverse correlation between NE and IRS-1. Neutrophil-derived leukotrienes were also reported to selectively expand the subset of cancer cells that retained high tumorigenic potential (84). Accumulating evidence supports an important role for neutrophil extracellular traps (NETs), composed of DNA, that are associated with proteins such as NE and foster cancer progression. Mechanistically, neutrophils secrete HMGB1 during NETosis, thereby activating the TLR9 signaling pathways in cancer cells to promote their adhesion, proliferation, migration, and invasion (59). A more recent study reported that NET-derived DNA could act as a scaffold for the neutrophil DNA-associated NE and MMP9 proteins during laminin-111 (matrix protein) remodeling which activated downstream integrin $\alpha 3\beta 1$ signaling in disseminated, dormant cancer cells converting them into aggressive lung metastatic cancer cells (85). In addition to their role in tumor cell proliferation, NET-derived molecules such as Cathepsin G (CG) and NE were reported to promote invasion and migration of breast cancer cells (86). Indeed, NETs were observed by intravital imaging *in vivo* in the murine 4T1-derived lung metastasis model. This finding was transferable to humans as they identified the deposition of NETs in triple-negative breast tumors. Recent evidence also implicates neutrophils in the induction of the epithelial to mesenchymal transition (EMT) by sustaining the expression of the EMT transcription factor Snail in cancer cells (87) or via the secretion of the tissue inhibitor of matrix metalloproteinase (TIMP-1) (88), which thereby facilitates metastatic progression. Evidence also suggest that neutrophils could favor angiogenesis and support tumor growth as neutrophil depletion was associated with a decreased number of developed vessels and a lower tumor weight (89). Pro-tumor TANs supporting angiogenesis, were reported to express high level of the proangiogenic factors VEGF and MMP9 in a mouse model of melanoma (89) and in liver tumorigenesis in zebrafish (90). Other groups support the fact that VEGF is highly expressed by pro-tumor TANs and may thus actively promote angiogenesis in mice (91, 92). Evidence in humans showed that fMLF-activated neutrophils induced sprouting of capillary-like structures via VEGF in an *in vitro* angiogenesis assay (93). Another group showed that human neutrophils could promote angiogenesis through NETs (94). In human gastric cancer, tumor cells were showed to make neutrophils produce MMP9, which significantly promoted angiogenic tube formation (95). Further studies in humans will be required to support the idea that proangiogenic neutrophils could promote tumor progression. In contrast, new evidence showed that TANs could foster cancer progression by altering angiogenesis instead, increasing hypoxia which in turn stabilizes the Snail EMT transcription factor, but also contributes to inhibit anti-tumor adaptive immunity (87). Moreover, evidence showed that neutrophils could impair angiogenesis by secreting antiangiogenic factors such as the isoform of VEGF-A, namely, $VEGF\text{-}A_{165b}$ (96, 97).

TANs can also promote tumor progression by interacting with immune cells. TANs were mainly described for their ability to dampen T-cell-mediated anti-tumor immunity. In mice, depending on the tumor model, different TAN-derived soluble factors were reported to suppress proliferation and IFNγ production of intra-tumor $CD8^+$ T-cells, such as ROS (91) or nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) (98). The soluble enzyme arginase 1 derived from tumor-infiltrating myeloid cells was also suggested to mediate T-cell suppression (99). A recent single cell transcriptomic study across

human and mouse lung tumor-infiltrating immune cells showed that arginase 1 was mostly expressed by TANs and tumor-associated macrophages (TAMs) (100, 101). Unlike human blood neutrophils, evidence in mice showed that TANs do not secrete arginase 1 but rather seem to retain it in the cytoplasm to deplete L-arginine intracellularly (99, 102). Other TAN-soluble factors were reported to induce T-cell apoptosis via the secretion of tumor necrosis factor alpha (TNFα) and NO (103). Lately, Fas-Ligand (Fas-L) was shown to be expressed at the cell surface of TANs, leading to T-cell apoptosis in tumors resistant to T-cell based immunotherapies (104).

Anti-tumor TANs

Though largely believed to be pro-tumor, accumulating evidence suggests that TANs also play a role in anti-tumor immunity. Indeed, TANs appear to induce direct tumor cell apoptosis by secreting cytotoxic molecules such as ROS (91, 105). Neutrophil-derived extracellular DNA (NETs) were also reported to induce tumor cell death (106). Another intriguing anti-tumor property of TANs was recently identified. TANs were found to promote the detachment of tumor cells from the basement membrane at an early stage of mouse uterine carcinogenesis, a process known as tumor cell sloughing leading to tumor cell death (78). Accumulating evidence suggests that neutrophils may kill antibody-opsonized cancer cells via antibody-dependent cell cytotoxicity (ADCC), which involves Fc receptors (107–110). Recently, an original mechanism of ADCC, a process termed trogoptosis, was described (111). Indeed, neutrophils establish a synapse with cancer cells, which strongly depends on neutrophil CD11b/CD18 integrins, allowing neutrophils to ingest a fraction of the antibody-opsonized plasma membrane of cancer cells (trogocytosis). A mechanical disruption of the plasma membrane concomitantly occurs, leading to a lytic/necrotic form of cell death. In sharp contrast with the other ADCC-related cytotoxic mechanisms mostly described in NK cells, trogoptosis is independent of granule exocytosis and of the phagocyte NADPH oxidase. Lastly and more importantly, intravital imaging demonstrated that trogoptosis occurs *in vivo* in mice (111).

Another anti-tumor role for TANs in mice and humans was uncovered with their implication in the recruitment and activation of intra-tumor $CD4^+$ and cytotoxic $CD8^+$ T cells (91, 112). A recent study conducted in mice genetically deficient for Tollip, an innate immunity signaling adaptor molecule inhibiting the TLR signaling pathway and potentially other pathways, led to the upregulation of STAT5 and STAT1. This in turn upregulated the co-stimulatory CD80 and downregulated the immune checkpoint PD-L1, specifically in TANs (112). These molecules are both important for T-cell proliferation, IFNγ and granzyme B production. Adoptive transfer of Tollip-deficient neutrophils slowed down colitis-associated cancer progression, thus highlighting a role for Tollip in modulating TAN-mediated cancer immune surveillance (112). In human colorectal cancer, $CD66b^+$ TANs stimulate proliferation and induce secretion of IFNγ from $CD8^+$ T cells *in vitro* (113). Moreover, $CD66b^+$ TANs frequently co-localize with $CD8^+$ T-cells in tumor tissue (113). At an early stage of human lung tumors, TANs were put forward as T-cell antigen presenting cells (APCs), with a high capacity to stimulate T-cell proliferation and IFNγ production (114, 115). A recent study conducted in a murine sarcoma model showed that TANs acting in concert with macrophages, were essential for unconventional αβ T cell type 1 polarization to display anti-tumor potential *in vivo* by secreting IFNγ (79).

TAN Subsets

With such functional heterogeneity of neutrophils, one could hypothesize that different subsets of TANs may be involved. Previous studies on blood and bone marrow neutrophils led to the discovery of new cell-surface markers to distinguish mature from immature neutrophils. Recent evidence showed that both mature and immature neutrophils can infiltrate mouse and human tumors, and were either reported to promote or prevent tumorigenesis (53, 54, 115, 116). Density could also discriminate a subset of blood neutrophils, known as LDNs, isolated by density gradient centrifugation. This method is suitable for blood samples, though its application to tumor samples may not be possible, which remains to be investigated. The search for cell-surface biomarkers of LDNs is thus needed to evaluate the relevance of LDNs in tumors. The previously identified Lox1 marker specific to a subset of LDNs was validated *in situ* in melanoma, colon, head and neck, and non-small cell lung cancer (48). But it remains to be verified if lox1 is not intracellularly expressed by all neutrophils. Characterization of TAN subsets based on cell-surface antigens remains limited to a dozen markers, making the comparison of neutrophil subsets across tissues difficult. Therefore, it remains therefore unclear whether immature blood neutrophils are identical to immature TANs. To further characterize subsets of TANs, transcriptomic profiling of neutrophils is strongly required. A recent single cell transcriptomic study performed in lung cancer patients showed distinct subsets of TANs and blood neutrophils with few overlaps between tissues (101). Whether such a difference can be attributed to differences in processing or isolation of neutrophils between tumor tissues and blood, or whether it is a true biological difference between tissues, remains to be addressed in future investigations. This study demonstrated that different subsets of TANs may co-exist in the same tumor, some being preferentially enriched, while certain TAN subsets seem to be found exclusively either in healthy or tumor tissue (101). The authors also sequenced at the single-cell level TANs from murine lung tumors (101). Unbiased comparison between mouse and human TANs showed conserved subsets allowing scientists to test the functional relevance of distinct subsets in tumor progression and response to therapies in murine models, and eventually apply finding to cancer patients. The work of Zilionis et al. unveiled TAN heterogeneity in the lung tumor context and it remains to be determined whether the same applies other tumor types. Another major ongoing issue is TAN ontogeny, and future investigations are needed to decipher whether TAN subsets correspond to transitional cell states referring to the concept of polarization or whether they are terminally-differentiated distinct cell types, and if TANs present in the tumor microenvironment are derived from circulating G-MDSCs.

A

Prognostic impact of TANs at **early** and **late**-stage of tumorigenesis

Tumor stage	Cancer Type	*In situ* localization of neutrophils	Neutrophil markers	Prognostic impact	OS / DFS / CSS	References
Stage I-II	CRC	Tumor front	CD66b	**Improved**	CSS	Wikberg ML et al. 2017 (117)
	SKCM	Intratumoral	CD66b	Worsened	OS / DFS / CSS	Jensen TO et al. 2012 (119)
	Cervical SCC	Peritumoral	CD66b	Worsened	DFS	Carus A et al. 2013 (120)
Stage II	CRC	Intratumoral	H&E (morphology) and Arginase	**Improved**	OS	Berry RS et al. 2017 (118)
		Intratumoral	CD66b	Worsened	OS	Rao H-L et al. 2012 (63)
Stage III	CRC	Intratumoral	CD66b	Worsened	OS	Rao H-L et al. 2012 (63)
Stage III-IV	HNSCC	Intratumoral	CD66b	Worsened	OS	Trellakis S et al. 2010 (64)

B

Ex-vivo functional analysis of TANs at **early** and **late**-stage of tumorigenesis

Tumor stage	Cancer Type	*Ex-vivo* functionality	Neutrophil markers	References
Stage I-II	NSCLC	TANs **enhance** T-cell proliferation and secretion of IFNγ	CD66b, CD15, CD14, OX40L, 4-1BBL, CD54, CD86, MHC-II	Eruslanov EB et al. 2014 (114) Singhal S et al. 2016 (115)
Stage III-IV	Gastric cancer	TANs **suppress** T-cell proliferation and production of IFNγ	CD66b, CD54, PD-L1	Wang T-T et al. 2017 (121)
Unknown ?	CRC	TANs **enhance** T-cell activation (CD69) and secretion of IFNγ	CD66b, CD54	Governa V et al. 2017 (113)

FIGURE 1 | (A) This figure reviews studies supporting the improved or worsened prognostic impact of TANs *in situ* at early (stage I and stage II; stage II alone) and late-stage (stage III; stage III and IV) of tumorigenesis across different cancer types. Quantification neutrophils based on a particular location *in situ* was precised (intratumoral, tumor front, and peritumoral). Neutrophil markers used for identification of neutrophils *in situ* by immunostaining were either CD66b or Arginase. Others identified neutrophils *in situ* based on their morphology through Hematoxylin and Eosin (H&E) staining. Patient prognostic impact was assessed based on overall survival (OS), disease-free survival (DFS), and cancer specific survival (CSS). Light gray background refers to early-stage tumors whereas dark gray background points out late-stage tumors. Abbreviations were used for the cancer type column: CRC, Colorectal cancer; SKCM, Skin Cutaneous Melanoma; Cervical SCC, Cervical squamous cell carcinoma; HNSCC, Head and Neck squamous cell carcinoma. **(B)** This figure covers studies *ex-vivo* functional analysis supporting the anti-tumor or pro-tumor role of neutrophils in early (stage I and II) or late-stage (stage III and IV) of tumorigenesis in various cancer types. This figure includes markers expressed at protein level by anti-tumor or pro-tumor neutrophils. Light gray background refers to early-stage tumors whereas dark gray background points out late-stage tumors. White background was set for unknown tumor stage. Abbreviations were used for the cancer type column: NSCLC, Non-small-cell lung carcinoma; CRC, Colorectal cancer.

TANs in Early vs. Later-Stage Tumors

Evidence in mice supports this idea that the pro- and anti-tumor role of neutrophils may be strongly linked to tumor stage. Mice with genetic deficiency of G-CSF-R displayed an accelerated tumor initiation at early-stage in a spontaneous murine model of uterine carcinogenesis (78) and 3-methylcholanthrene (3-MCA)-induced sarcomagenesis (79), attesting an anti-tumor role at early-stage of tumorigenesis. In contrast, antibody-mediated neutrophil depletion in established tumors at late-stage, led to decreased tumor growth, supporting a pro-tumor role at late-stage (84, 98).

In humans, evidence for pro- and anti-tumor functions of TANs arose from retrospective studies assessing the prognostic value of tumors highly infiltrated with neutrophils (**Figure 1A**). The prognostic impact of TANs remains controversial as they are associated with either a better or worse outcome. Such discrepancies can be explained by the cancer type, the *in situ* location (peritumoral, intratumoral, or stromal) of neutrophils but also by differences in staining methods such as haematoxylin/eosin (HE), neutrophil elastase (NE), Arginase, MPO, CD66b, or CD15 surface markers. New evidence suggest that the tumor stage may also explain such differences (**Figure 1A**). In colorectal cancer (CRC) patients, the good prognostic impact of TANs is described in early-stage colorectal tumors, especially stage I–II tumors combined (117), or stage II alone (118). Only one study in CRC patients showed that a high intratumoral infiltration of TANs was associated with a worse overall survival (63). The worse prognostic impact of TANs in early-stage tumors was reported in melanoma (119) and cervical squamous cell carcinoma (120). In late-stage tumors, especially in CRC and head and neck squamous cell carcinoma, the prognostic impact of TANs is generally poor (63, 64). To our knowledge, there is no study reporting a good prognostic significance of TANs in late-stage tumors.

Other evidence supporting the link between anti- or pro-tumor function of TANs with the tumor stage came from *ex vivo* functional analysis of TANs (**Figure 1B**). In gastric cancer patients, TANs isolated from fresh tumors co-cultured with purified autologous peripheral blood $CD3^+$ T cells displayed higher immunosuppressive function than neutrophils from healthy adjacent tissue (121). In this study, T-cell suppression is partially explained by PD-L1 expression on TANs since PD-L1 blocking antibodies reverse T-cell proliferation (121). Interestingly, the authors showed that $PD\text{-}L1^+$ TANs were

significantly higher in advanced (stages III–IV) vs. early-stage tumors (stage I–II), suggesting that pro-tumor PD-L1$^+$ neutrophils only emerge at a later-stage of gastric tumorigenesis. Further investigations will be needed to confirm the predominance of pro-tumor TANs in late-stage gastric tumors compared to early-stage tumors. In sharp contrast, in lung cancer patients, TANs, not peripheral blood neutrophils, from early-stage tumors (stages I–II), were reported to enhance the proliferation of autologous T-cells stimulated with anti-CD3/CD28 antibodies (114, 115), suggesting an anti-tumoral function. Nevertheless, the improved prognostic impact of TANs in early-stage lung tumors remains to be assessed. In CRC, co-culture of human TANs from colorectal tumors with autologous CD8 T-cells resulted in an increased expression of the CD69 T cell activation marker and a higher release of IFNγ in culture supernatant (113). However, it remains to be determined if these CD8 T-cell-stimulatory TANs are enriched in early-stage colorectal tumors as compared to late-stage tumors. In line with the CD8 T-stimulatory anti-tumor role of neutrophils in CRC, the majority of studies on favorable outcome of TANs in early-stage tumors are in CRC (**Figure 1A**). Thus, it cannot be excluded that not tumor stage but rather tumor type (and possibly involvement of microbiota), is responsible for this observation, in CRC. Human undifferentiated pleomorphic sarcomas (UPS) recently emerged as another type of cancer in which dense neutrophil infiltrate was found to be associated with better prognosis, regardless of tumor stage (79). The search for cancer type-specific features, especially in CRC and UPS will be the object of future investigations to potentially identify new factors important for the stimulation of anti-tumor neutrophils.

ORIGIN OF PRO- AND ANTI-TUMOR NEUTROPHILS

Having reviewed the heterogeneity of pro- and anti-tumor neutrophils in cancer, it is necessary to discuss their origin.

Neutrophil Activation

The anti- or pro-tumor properties of neutrophils often result from the activation of neutrophils. Neutrophil-derived NETs were previously described to have both pro (85) and anti-tumor properties (106). *In vitro*, NETs can be generated upon acute stimulation of neutrophils with lipopolysaccharide (LPS), Phorbol 12-myristate 13-acetate (PMA), N-Formylmethionyl-leucyl-phenylalanine (fMLF formerly termed fMLP) (83, 106, 122) in both human and mice. Whereas, PMA or fMLF was sufficient to induce NETs (122). Mechanistically, LPS-stimulated platelets via TLR4 which in turn led to the activation of neutrophils thus leading to NETosis. Other platelet activators such as thrombin were found to be equally efficient in producing NETs in presence of platelets (123). The mechanism by which activated-platelets promote NETosis is still not clear and will require further investigations. Cancer cell-derived soluble molecules such as G-CSF were also suggested to induce NETs (86). The engagement of the Fc-alpha receptor (FcαRI/CD89) (124) or Fc-gamma receptor (FcγRIIIB/CD16) (125) was also reported to induce NETs. Neutrophil-derived NET formation is not restricted to *in vitro* stimulation, as it can be reproduced in mouse blood following acute systemic sepsis (58) and in mouse lung tumor after chronic nasal instillation of LPS (84). Neutrophils were also described to display T-cell suppressive functions. Both mouse and human neutrophils acutely stimulated by high doses of IFNγ, up-regulate T-cell suppressing ligands such as PD-L1 which in turn decrease T-cell proliferation (115, 126). The acute induction of endoplasmic reticulum (ER) stress upon thapsigargin (THG) stimulation in human circulating neutrophils was reported to convert these cells into T-cell suppressive G-MDSCs, notably by inhibiting T-cell proliferation (48). Interestingly, in contrast to THG treatment, fMLF and PMA-stimulated neutrophils did not block T-cell proliferation. Instead, PMA-stimulated neutrophils appear to have T-cell suppressive effects by inhibiting the production of T-cell derived IFNγ (127). Activation of the complement receptor 3 (MAC-1/CD11b) signaling pathway also seems important for the induction of T-cell suppressive neutrophils (128).

The engagement of Fc-receptors on neutrophils constitutes another way of activating neutrophils and was reported to lead to killing of antibody-opsonized cancer cells by ADCC *in vitro* (109, 111) and *in vivo* (111). Interestingly, neutrophils were shown to induced ADCC more efficiently with IgA antibodies in comparison to IgG antibodies (107, 108, 110). Using hFcαRI transgenic mice, IgA anti-EGFR antibodies were proven to mediate tumor cell killing *in vivo* (129). Recent evidences support the idea that ADCC could occur *in vivo* as revealed by intravital imaging (111). Taken together, direct acute or chronic stimulation of neutrophils seems to be sufficient to transform naïve neutrophils into pro- or anti-tumor neutrophils. Collectively, neutrophil activation may in turn lead to a variety of pro- and anti-tumor function depending on type, dose of activator, time of stimulation, and tumor model.

Neutrophil Differentiation

By definition, differentiation implies the development of a given progenitor toward several different mature cells with distinct cell fates. Depending on environmental cues, neutrophil progenitors were reported to differentiate into different subsets of neutrophils with either pro- or anti-tumor functions (115, 130, 131).

Mounting evidence indicates that IFNγ and GM-CSF, two cytokines abundantly present in early-stage human lung tumors, are essential for the differentiation of CD11b$^+$ CD15^{hi} CD66b$^+$ CD16$^{int/low}$ CD10$^-$ bone marrow neutrophil progenitors into anti-tumor CD10$^+$ mature MHC-II$^+$ antigen presenting neutrophils, known as "APC-like hybrid neutrophils" (114, 115). Surprisingly, besides expressing APC markers, this TAN subset was shown to express markers of monocytes such as CD14. Both IFNγ and GM-CSF downregulate the expression of the Ikaros transcription factor, known to negatively regulate the development of monocytes/macrophages which may explain the acquisition of CD14 (132, 133). Interestingly, the Ikaros inhibitor, lenalidomide, synergizes with IFNγ and GM-CSF to generate APC-like hybrid neutrophils, but is unable to do so alone, suggesting that other pathways activated by IFNγ and GM-CSF are necessary for this differentiation, though

these latter remain to be unraveled. Moreover, the authors showed that APC-like hybrid neutrophils could also be generated from CD11b^{+} CD15^{hi} CD66b^{+} CD16$^{int/low}$ CD10^{-} low-density immature peripheral blood neutrophils from G-CSF-treated healthy donors (115). Since neutrophil progenitors may also circulate in the bloodstream of cancer patients (54, 55), it is therefore possible that circulating neutrophil progenitors are recruited at the tumor site to differentiate into APC-like hybrid TANs. Neutrophil progenitors used for the generation of APC-like hybrid neutrophils were isolated from bone marrow of cancer patients or from peripheral blood of G-CSF-treated healthy donors (115). It therefore remains unclear whether systemic priming of neutrophil progenitors by tumor-secreted factors, including G-CSF is a prerequisite for their differentiation into APC-like hybrid neutrophils upon exposure to IFNγ and GM-CSF at tumor site.

Recent evidence supports the concept that neutrophils need to be primed systemically to differentiate into pro-tumor TANs. A recent study in mice showed that the differentiation of bone marrow-derived hematopoietic stem and progenitor cells (HSPCs) into pro-tumor SiglecF^{+} TANs (130) required bone marrow osteoblasts. Indeed, *in vivo* cell fate mapping experiments showed that the differentiation of SiglecF^{+} TANs from bone marrow c-Kit^{+} HSPCs was abrogated in osteoblast-deficient mice, suggesting that the bone marrow is essential for the priming of pro-tumor SiglecF^{+} TANs. Extra-medullary tissues such as the spleen were also necessary for the differentiation of HSPCs into T-cell suppressive TANs (131). Splenectomy was indeed reported to blunt T-cell suppressive functions of TANs and synergistically enhanced anti-PD-L1 therapeutic efficacy. Mechanistically, the spleen of tumor-bearing mice mediates the recruitment of circulating HSPCs through CCL2/CCR2 axis, which then differentiate into T-cell suppressive G-MDSC via splenic stromal-derived GM-CSF. This is in line with previous studies showing that T-cell suppressive G-MDSCs expand in the spleen, which is most often characterized by splenomegaly (134). Collectively, although evidence in mice suggests that the systemic environment such as the bone marrow and the spleen are necessary for the differentiation of neutrophil progenitors to pro-tumor TANs, it is still unclear whether this systemic priming is sufficient for differentiation to pro-tumor TANs or whether it requires additional cues at tumor site for example, remains unknown.

In humans, systemic priming appears to be necessary for the generation of T-cell suppressive neutrophils. Indeed, a study recently showed that mature CD10^{+} neutrophils from G-CSF-treated healthy donors display T-cell suppressive properties *ex vivo*, whereas those from untreated healthy donors did not. Interestingly, CD10^{+} neutrophils from untreated healthy donors treated with G-CSF *in vitro* did not display any suppressive activities (55). This suggests that, in humans, G-CSF alone is not sufficient to induce T-cell properties on neutrophils and thus requires other probably systemic cues.

The tumor may also alter the differentiation of neutrophils for its own benefit, by interfering with their maturation toward an anti-tumor phenotype. This concept was initially demonstrated for tumor-infiltrating monocytes. Here, the maturation into anti-tumor macrophages was blocked leading to promotion of tumor growth in a murine model of hepatocellular carcinoma (HCC) (135). Accumulating evidences in humans and mice suggest that immature neutrophils infiltrate tumors, retain their immature phenotype at the tumor site and are correlated with a higher tumor burden (53, 54, 98, 116). Aside from the hypothesis that interfering with neutrophil maturation could alter their differentiation into anti-tumor neutrophils, this data suggest that at least some neutrophil progenitors may also have a naturally occurring T-cell suppressive function *per se*. Indeed, immature bone marrow neutrophils from healthy donors were reported to display spontaneous T-cell suppressive properties, although this remains to be confirmed in further studies (54). The biological characterization of immature TANs and their clinical implication should be the subject of future investigations.

NEUTROPHIL-BASED THERAPEUTIC STRATEGIES

Since neutrophils display various tumor-promoting functions and are predictive of poor patient OS, one potential therapeutic strategy could be the targeting of neutrophils *in vivo*. Pre-clinical studies in the mouse have already reported therapeutic effects of neutrophil depletion using the specific neutrophil-depleting antibody, anti-Ly6G (84, 87, 98, 104). Here, we review the different therapeutic strategies that aimed at targeting neutrophils in cancer (**Figure 2**).

Prevention of Neutrophil Exit From Bone Marrow and Entry to Tumor Tissue

G-CSF/IL-17 Axis Modulation

A number of studies in mice have suggested modulating the level of G-CSF to prevent the expansion of pro-tumor neutrophils. In mice, ablation of G-CSF with anti-G-CSF antibodies was also shown to prevent neutrophil accumulation in bloodstream (61, 98, 136), whereas overexpression of G-CSF induced the expansion of circulating neutrophils and increased metastasis (61, 136, 137). Although G-CSF is being used to stimulate the production of neutrophils in patients who suffer from chemotherapy-induced neutropenia (138), it remains unclear if endogenous G-CSF is responsible for neutrophilia observed in cancer patients. Few studies reported a higher level of serum G-CSF in patients with pancreatic cancer (139), colorectal cancer (140) and non-small cell lung cancer (141), as compared with healthy subjects. None of these studies showed an association between high serum G-CSF concentration and neutrophilia. Only few case report studies reported a link between a high endogenous serum G-CSF level and an increase of white blood cell count consisted primarily of neutrophils (142–144). Taken together, the role of endogenous serum G-CSF as a dominant driver of neutrophilia in cancer patients remains weak. Furthermore, endogenous serum G-CSF levels has not been linked to poorer prognosis to date. Noteworthy, exogenous therapeutic G-CSF does not seem either to worsen survival of cancer patients treated with chemotherapy (145, 146).

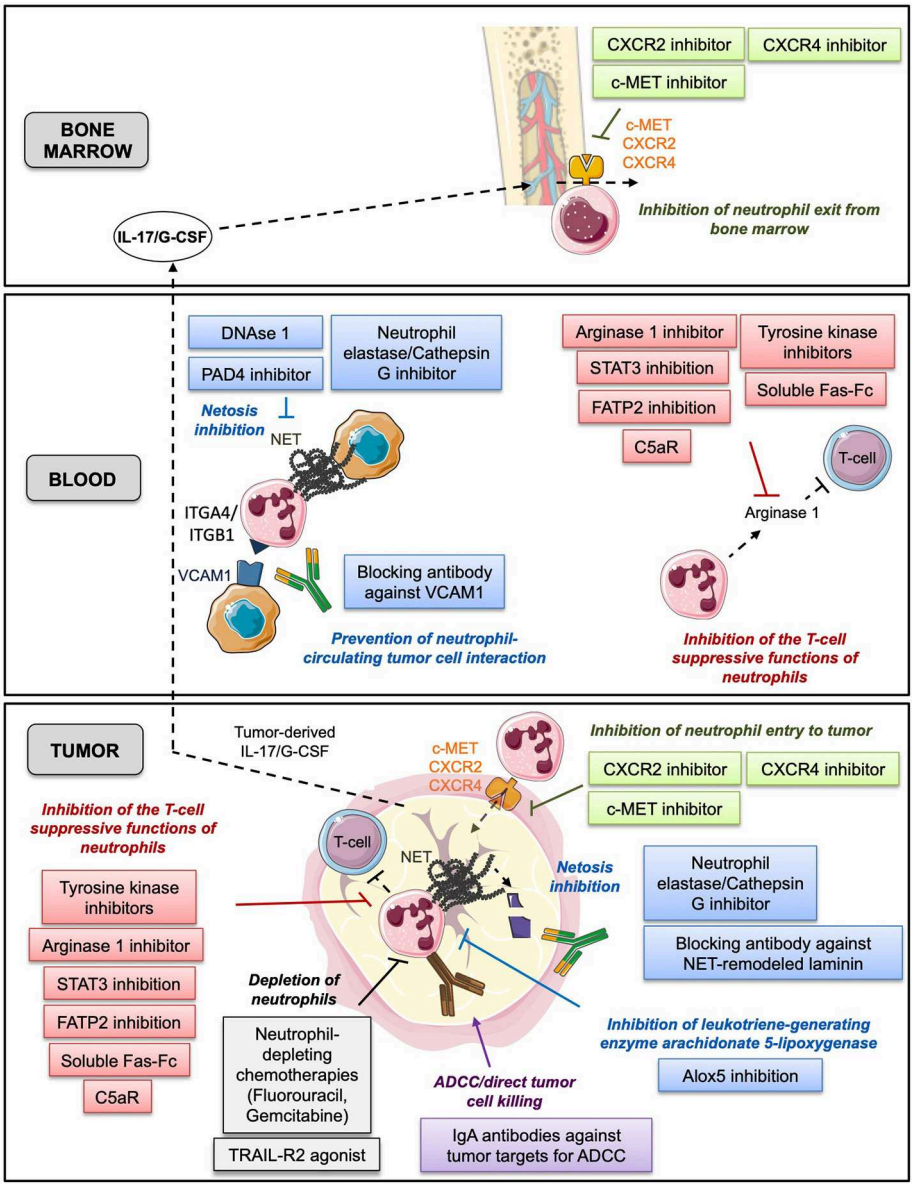

FIGURE 2 | This figure summarizes the different neutrophil-based therapeutic strategies with various mechanisms of action described in bone marrow, blood and tumor. In green: prevention of neutrophil exit from bone marrow and entry to tumor tissue. In gray: depletion of neutrophils. In red: inhibition of the T-cell suppressive functions of neutrophils. In blue: prevention of neutrophil capacity to foster tumor cell proliferation and migration. In purple: promotion of the anti-tumor functions of neutrophils.

Interleukin-17 (IL-17) recently emerged as an upstream regulator of G-CSF and subsequently of neutrophil production *in vivo* (98, 147, 148). In a murine model of lung metastasis, neutralization of IL-17 significantly reduced the level of G-CSF and prevented the systemic expansion of blood neutrophils. Interestingly, studies report an increase of IL-17 in the blood of patients with liver cancer (149), non-small cell lung cancer (148, 150, 151). High level of serum IL-17 was even associated with a worse prognostic for non-small cell lung cancer patients (150, 151). Nevertheless, the link between level of serum IL-17 and neutrophilia remains to be investigated in future studies.

CXCR2/CXCR4 Inhibition

CXCR2 is known to be important for neutrophil migration, controlling their egress from the bone marrow to the bloodstream and their recruitment to sites of inflammation (152–155). Neutrophil acquire CXCR2 to exit bone marrow (153, 154). The genetic or pharmacological inhibition of CXCR2 was reported to decrease primary lung tumor growth (156, 157) and suppress pancreatic cancer metastasis in mice (157). In both studies, specific depletion of $Ly6G^+$ neutrophils recapitulated the therapeutic effect of CXCR2 inhibition. CXCR2 inhibition was followed by an increase in circulating neutrophils due to their

inability to home (156, 157). Although some evidence indicates that CXCR2 inhibition could prevent recruitment of $CD11b^+$ $Gr1^+$ myeloid cells in $PTEN^{-/-}$ prostate tumors (82), it remains unclear whether $Ly6G^+$ neutrophil infiltration at the tumor site is impaired. Since therapeutic effects of CXCR2 inhibition may also be explained by its direct action on tumor cells (158–160), future studies are needed to determine the contribution of neutrophils to CXCR2 inhibition-related therapeutic effects. This would provide a rationale for using CXCR2 as a potent inhibitor of neutrophil recruitment.

CXCR4 was reported to act antagonistically with CXCR2 for bone marrow exit of neutrophils (153, 154, 161). Neutrophils lose CXCR4 to egress from the bone marrow to be released in the circulation (154, 161). However, CXCR4 acquisition seems important for infiltration of neutrophils into the tumor, at least in a preclinical model of colorectal cancer. Hence, blockade of CXCR4 using the FDA-approved agent Plerixafor (AMD3100) inhibited anti-VEGFR2 therapy-induced tumor infiltration of neutrophils and $Ly6C^{low}$ monocytes (162). Increasing number of studies report the therapeutic effects of inhibiting CXCL12/CXCR4 axis by targeting cancer and stromal cells from the tumor microenvironment (163). Very few studies looked at the effects of CXCR4 blockade on tumor-infiltrating immunosuppressive myeloid cells including neutrophils and it remains the object of future investigations. Taken together CXCR4 inhibition appears to be an interesting strategy when used in combination with chemotherapy. This by preventing neutrophils from entering into the tumor while mobilizing bone marrow neutrophils which could compensate chemotherapy-induced neutropenia classically managed by G-CSF injection. Hence, several pre-clinical studies showed the additive therapeutic effects of CXCR4 inhibition with chemotherapies (164–166).

C-Met Inhibition

Capmatinib, an inhibitor of the tyrosine-protein kinase MET (c-MET), was first developed to treat cancer patients with alterations affecting the c-MET pathway in cancer cells due to activating mutations, overexpression, gene amplification, and translocations (167). Interestingly, capmatinib potentiated therapeutic effects of adoptive T-cell transfer and checkpoint immunotherapies in several mouse models of cancer by preventing the reactive mobilization and recruitment of T-cell suppressive neutrophils to tumors and draining lymph nodes (126). Importantly, the therapeutic effect was restricted to c-MET signaling in neutrophils as the genetic ablation of c-MET kinase activity specifically in neutrophils could reproduce the same effect. Moreover, tumor cell lines were insensitive to capmatinib *in vitro* and *in vivo*, excluding any tumor cell-intrinsic c-MET dependency in this study. Another study showed that capmatinib also prevented the recruitment of anti-tumor TANs (168) in different tumor models. Whereas, cancer cell lines knocked down for c-MET had a slower tumor growth rate in mice, the addition of capmatinib countered the therapeutic effect of c-MET knockdown, suggesting that TME-expressing c-MET were involved in the anti-tumor response. TANs appeared to express c-MET which is essential for their recruitment at the tumor site and thereby activation of their anti-tumor function. These studies therefore argue in favor of evaluating the expression of c-MET in both tumor cells and neutrophils in cancer patients who receive capmatinib treatment.

Depletion of Neutrophils

TRAIL-R2 Agonist Antibody

An agonistic antibody of the TNF-Related Apoptosis-Inducing Ligand Receptor 2, known as TRAIL-R2, was proposed to induce cell death of mouse G-MDSC *in vitro* and to potentiate the effect of CTLA-4 immune checkpoint blockade *in vivo*. A recent first-in human clinical trial evaluated the clinical impact of targeting TRAIL-R2. In cancer patients with elevated levels of LDNs before treatment, the TRAIL-R2 agonist antibody selectively depleted LDNs without impacting the number of other peripheral blood myeloid and lymphoid cells, nor showing dose-limiting toxicities (169). However, the selective depletion of LDNs could not be sustained up to 28 days after the start of the treatment. Due to short-term treatment and the small number of patients in the cohort, further studies will be needed to conclude on the selective depletion of LDNs upon TRAIL-R2 agonist antibody treatment.

Chemotherapies

Aside from their potential cytotoxic effects on tumor cells, studies in mice revealed that some chemotherapies may also have side effects on immune cells, including neutrophils. Indeed, fluorouracil (5FU) was reported to have anti-tumor effects *in vivo* in mice and was associated with depletion of splenic and tumor G-MDSCs and monocytic MDSCs (M-MDSCs) (170). In this study, the therapeutic efficacy of 5FU regarding tumor immunity was suggested to most likely be restricted to M-MDSC-depletion since 5FU did not deplete any other immune cell type, nor induce immunogenic tumor cell death. Nevertheless, the same group reported several years later that 5FU and Gemcitabine could also drive M-MDSC-derived IL-1b secretion that induce the release of CD4 T-cell-derived IL-17, which in turn blunts the anticancer efficacy of these chemotherapeutic agents. However, gemcitabine and 5FU were shown to exert higher anti-tumor effects when tumors were established in $Nlrp3^{-/-}$ or $Casp1^{-/-}$ mice or wild-type mice treated with the IL-1 receptor antagonist (IL-1Ra) (171). Therefore, the depleting effect of 5FU on MDSCs, including G-MDSCs, still remains a matter of debate and requires further investigation.

Taken together, studies aiming at evaluating the neutrophil-depleting effects of already-approved therapies remain contrasted. These discrepancies are likely due to differences in doses, timing of administration, location of neutrophil sampling, time of neutrophils detection, as well as mouse and tumor models (172, 173). Future investigations should therefore take into account these parameters to identify in which settings these potential neutrophil-depleting therapies could be the most beneficial. Since neutrophils are constantly monitored during cancer treatment to prevent neutropenia and therefore avoid opportunistic infections, depletion of neutrophils must be restricted to the tumor site (TANs), or only to subsets of neutrophils with tumor-promoting function. To support the rationale that currently-used chemotherapies may offer

additional therapeutic effects by targeting TANs, it will be important to determine whether a higher infiltration of TANs prior to the administration of neutrophil-depleting treatments is associated with a better survival. Interestingly, evidence in stage III colorectal cancer patients all treated with 5FU after surgical removal, showed that a high density of intratumoral TANs before treatment was associated with a longer disease-free survival (68). TAN infiltrate may therefore help identify patients who will likely benefit from 5FU chemotherapy.

Inhibition of the T-Cell Suppressive Functions of Neutrophils

Arginase 1 Inhibitor

L-arginine is an important amino acid that serves as a building block for protein synthesis and as a precursor for multiple intra-cellular metabolites (174). L-arginine is known to be particularly important for T-cell proliferation and survival (175). Moreover, increased intracellular L-arginine in T-cells was shown to favor their differentiation to central memory-like T-cells with enhanced anti-tumor properties (176). L-arginine is mainly catabolized by arginase 1, which is secreted by subsets of myeloid cells, including neutrophils under specific conditions in humans (46, 55). Several reports demonstrated *in vitro* that neutrophil-derived arginase 1 suppresses T-cell proliferation (55), which was rescued by the addition of an arginase 1 inhibitor. The therapeutic effect of the arginase 1 inhibitor on tumor growth was also observed in a pre-clinical mouse model of lung cancer (99, 177). However, the tumor-promoting role of arginase 1 remains controversial as L-arginine also favors tumor cell proliferation and survival. *In vitro*, recombinant human arginase 1 induces cell cycle arrest and apoptosis of human tumor cell lines (178–182). Bioengineered PEGylated arginase 1 for which the half-life was extended through PEGylation, was shown to exert anti-tumor effects in xenograft mouse models (179, 181, 183, 184). Recent evidence suggests that the bioengineered human PEGylated arginase 1 (AEB1102) exerts additive anti-tumor effects when combined to anti-PD-1 or anti-PD-L1 in melanoma, small cell lung cancer (SCLC), and sarcoma patient-derived xenografts (183, 184). Collectively these results demonstrate that disrupting the physiological balance of L-arginine may either inhibit or promote tumor progression depending on T-cell and tumor cell susceptibility to arginine starvation. The synergistic therapeutic efficacy of either recombinant arginase 1 or arginase 1 inhibitor in combination with various chemotherapies or Pembrolizumab (anti-PD-1) is currently being tested in phase 1/2 clinical trials (NCT03371979; NCT02903914; NCT03361228; NCT03314935).

Tyrosine Kinase Inhibitors

In mice, splenic HSPCs were shown to contribute to the T-cell suppressive function of TANs (131) in a murine model of HCC. Low-dose of sorafenib, a c-Kit inhibitor, was associated with increased apoptosis of splenic HSPCs and reduced immunosuppressive function of TANs. Sorafenib synergizes with PD-L1 blockade. The authors showed that the therapeutic effect of sorafenib could be largely attributed to splenic HSPCs depletion as the adoptive transfer of splenic HSPCs following sorafenib treatment abrogated its effects. In contrast, sorafenib promoted expansion of tumor-promoting TANs in the HCC mouse model and therefore limit sorafenib therapeutic efficacy (185). This finding was translatable in HCC patients, since TAN infiltration was higher in patients previously treated with sorafenib prior to liver resection, compared to untreated patients.

Another tyrosine kinase inhibitor, called sunitinib, has also shown promising results in depleting pro-tumor neutrophils. In renal cell carcinoma (RCC) patients, the elevated percentage of the T-cell suppressive $CD15^+$ $CD14^-$ LDNs among PBMCs declined in response to sunitinib treatment. *In vitro*, sunitinib induced the death of $CD15^+$ $CD14^-$ LDNs in a dose-dependent manner (186). Studies in mice showed that sunitinib depleted T-cell suppressive splenic neutrophils, whereas it failed to deplete T-cell suppressive TANs (187, 188). Intra-tumor GM-CSF promotes STAT5 signaling pathway activation, which largely explains the resistance of TANs to sunitinib-induced cell death. In contrast, another group showed that sunitinib depletes tumor-associated $Gr1^+$ MDSC and synergizes with a cancer vaccine to enhance antigen-specific immune responses and tumor eradication (189). However, these authors did not specify which MDSC subset (granulocytic or monocytic myeloid cells) was targeted by sunitinib.

C5aR Blockade

The C5a receptor (C5aR, CD88) becomes fairly well known for its role in immunosuppressive activity of myeloid cells. Lung cancer cells were found to produce C5a which could bind to C5aR expressed by myeloid cells to increase their immunosuppressive functions (190). Concentration of C5a in peripheral blood of lung cancer patients was significantly higher as compared to healthy donors. A recent pre-clinical study showed additional therapeutic effects of C5a pharmacologic inhibition in combination with PD-1 blockade unleashing anti-tumor CD8 T-cell response in a model of lung cancer (191). In this latter study, the C5a inhibitor named NOX-D21 is an l-aptamer that tightly binds to C5a and inhibits the interaction with its receptors. Another group recently reported in a pre-clinical model of squamous cell carcinoma, that the targeting of C5aR1 essentially in macrophages with a peptide antagonist (PMX-53) improved efficacy of paclitaxel chemotherapy and was associated with CD8 T cell response (192). A blocking antibody against C5aR (IPH5401) was recently developed and entered phase 1 clinical trial in combination with PD-L1 blockade for lung cancer patients (NSCLC) and head and neck patients (HCC) (NCT03665129). Recent evidence suggests that not only macrophages but also neutrophils express high level of C5aR (193). Moreover, the authors showed that IPH5401 selectively inhibited C5a-induced activation and migration of human blood neutrophils, suggesting that neutrophils may account as therapeutic target of C5aR blocking antibody.

STAT3 Inhibition

STAT3 transcription factor is well known to be important for the tumor-promoting activities of myeloid cells such as immunosuppression (194–197) or tumor angiogenesis (198). Clinical trials using small molecule inhibitors targeting STAT3 showed limited therapeutic effects and broad side effects (199). Other approach attempting to inhibit STAT3 signaling

pathway by using STAT3 siRNA are currently evaluated in clinical trials (199). The mechanisms by which STAT3 inhibition dampens the immunosuppressive function of neutrophils are not clear. Recent evidences suggest that STAT3 activation leads to the expression of the fatty acid translocase CD36 that in turn increases the uptake of lipids and the oxidative metabolism, and subsequently the immunosuppressive function of myeloid cells (200, 201). Further studies will be needed to evaluate the impact of targeting STAT3 in tumor-promoting neutrophils.

FATP2 Inhibition

Recent evidences suggest that lipid metabolism could contribute to the pathological activation of G-MDSC (201, 202). Human TANs and peripheral blood G-MDSC also upregulate the expression of several lipid transporters such as CD36, Msr1, Ldlr, or Lox1, as compared to neutrophils from healthy donors. Addition of very low-density lipoproteins increased the T-cell suppressive function of MDSC *in vitro* (201). Genetic depletion of the fatty acid translocase CD36 in tumor-bearing mice delayed tumor growth *in vivo* (201). More recent *in vivo* evidence showed that genetic deletion of the SLC27A2 gene (also known as FATP2) encoding the very long-chain acyl-CoA synthetase, specifically in neutrophils using the S100a8-cre mice, abrogated tumor growth in different tumor models (202). FATP2$^{-/-}$ TANs had decreased T-cell suppressive activity as compared to WT TANs (202). The authors further showed that the selective FATP2 inhibitor lipofermata delayed tumor growth in different preclinical mouse models and synergized with either anti-CTLA4 or anti-CSF1R antibody. Human TANs were found to have a higher intracellular lipid content as compared to blood neutrophils from matched cancer patients (202). Future investigations on human samples will be needed to evaluate the clinical relevance of targeting FATP2 in cancer patients.

Soluble Fas-Fc

A pre-clinical mouse study recently reported that neutrophils upregulate Fas-L which in turn triggers apoptosis of tumor-infiltrating lymphocytes, thereby causing resistance to immunotherapies based on checkpoint blockade, cancer vaccines or adoptive T-cell therapy (104). In this study, soluble Fas-Fc neutralized Fas-L *in vivo*, rendering tumors sensitive to T-cell-based immunotherapies. Future studies are required to determine whether high infiltration of Fas-L positive TANs at baseline is associated with unresponsiveness to immunotherapies.

Prevention of Neutrophil Capacity to Foster Tumor Cell Proliferation and Migration

Inhibition of Netosis

A preclinical study showed that NETs promote the development and progression of liver metastases after surgical stress. Patients undergoing curative liver resection for metastatic colorectal cancer, that were characterized by an increased postoperative NET formation, had a >4-fold reduction in disease-free survival (59). Accelerated development and progression of metastatic disease was demonstrated in a murine model that recapitulated NET formation after surgical stress. Local treatment with DNAse 1 or inhibition of the enzyme peptidylarginine deaminase (PAD4), which is essential for NET formation, abolished tumor progression after surgery (59). There are current limitations for the use of the PAD4 inhibitor to prevent NET *in vivo* due to its short-half-life (86). Similar to the PAD4 inhibitor, the short half-life of NE and cathepsin G inhibitors prevents their use *in vivo* for cancer treatment (86). Recently, NET-associated proteases, NE and matrix metalloproteinase 9, were shown to sequentially cleave laminin (85). This proteolytically remodeled laminin induced proliferation of dormant cancer cells by activating integrin α3β1 signaling, and antibodies against NET-remodeled laminin 111 prevented the awakening of dormant cancer cells (85). Engineering of monoclonal antibodies against human NET-remodeled laminin would help determine if such mechanisms occur in humans and whether they are correlated with patient prognosis.

Arachidonate 5-Lipoxygenase (Alox5) Inhibition

Evidences showed that neutrophil-derived leukotriene support breast cancer metastasis by selectively expanding cancer cells that retain high metastatic potential (84). Pharmacologic inhibition of arachidonate 5-lipoxyenase (Alox5) by zileuton (203) could inhibit the pro-metastatic activity of neutrophils. The authors showed that both leukotriene receptor Leukotriene B4 receptor 2 (BLT2) and Cysteinyl leukotriene receptor 2 (CysLT2) were expressed in human breast tumor and matched lymph node metastases. Future investigation will be needed to determine the therapeutic potential of such targets.

Blockade of Neutrophil-Circulating Tumor Cell Interaction

Accumulating evidence indicates that neutrophils interact with circulating tumor cells to promote their proliferation and favor their extravasation to metastatic sites (58, 61, 204). Either NETs or VCAM1 adhesion molecules seem to be important in the interaction between neutrophils and circulating tumor cells. DNAse 1 treatment or VCAM1-deficient tumor cells prevented such interaction and impaired metastasis.

Promotion of the Anti-tumor Functions of Neutrophils

Antigen-Dependent Cellular Cytotoxicity (ADCC)

Mounting evidence strongly supports the concept of targeting Fc-Receptors on neutrophils in anti-tumor immunotherapy. Although they express IgA-Fc-Receptor (CD89) and IgG-Fc-Receptors (CD16; CD32; CD64), evidence showed that neutrophils are more effective at mediating ADCC through IgA antibodies than IgG antibodies *in vitro* (107, 108, 110). Numerous pre-clinical studies conducted in CD89 transgenic mice confirmed its superior ability to induce neutrophil-dependent tumor cell killing for different tumor-associated antigens, such as HER2/neu (on breast carcinoma), EpCAM

(colon carcinoma), EGFR (epithelial carcinoma and renal cell carcinoma), HLA class II (B-cell lymphoma), CD30 (T- and B-cell lymphoma), and CD20 (B-cell lymphoma) (205). Due to its shorter half-life as compared to other serum isotypes, the engineering of IgA antibodies could be a promising therapeutic option to increase effectiveness of currently given monoclonal antibody-based immunotherapies.

CONCLUSION AND PERSPECTIVES

Although immunotherapies held great promise for oncology, only a small percentage of cancer patients showed benefits from these treatments. The search for new therapeutic targets is thus critically needed. Current immunotherapies mostly rely on the adaptive immune system, involving adaptive immune checkpoints such as CTLA-4, PD-1, and PD-L1. Recent pre-clinical studies suggest that innate immunity could offer new therapeutic opportunities. Inhibition of the innate immune checkpoints Tyro3, Axl, and Mertk tyrosine kinase receptors, mostly expressed by TAMs, displayed additional therapeutic effects when combined to the anti-PD-1 monoclonal antibody in a murine model of triple-negative breast cancer (206). Accumulating evidence suggests that neutrophils may be ideal targets for future therapeutic strategies. Several groups reported that the selective depletion of neutrophils could delay tumor growth. Ongoing pre-clinical investigations aim at evaluating the potential synergistic effect of neutrophil depletion with currently approved immunotherapies. In cancer patients, accumulating evidence from retrospective studies supports a rationale for eliminating neutrophils. Indeed, several approved cancer drugs were shown to deplete neutrophils and were associated with a better survival. Nonetheless, we cannot rule-out off-target therapeutic effects of these drugs. Moreover, blood neutrophil count is routinely used as a read-out of cancer treatment-related toxicities, since neutrophils are important in the immune response upon bacterial infection. Further studies are therefore needed to identify pro-tumor neutrophil-associated targets non-overlapping with antibacterial neutrophils, to enhance therapeutic efficacy of immunotherapies while minimizing side effects.

While the elimination of neutrophils seems to provide therapeutic effects in established tumor models, emerging evidence favors an anti-tumor role for neutrophils in early-stage tumors in mice (78, 114, 115). Evidence from retrospective studies and *ex vivo* functional analyses also support the concept of an anti-tumor role for neutrophils in early-stage human tumors. Although challenging, access to early-stage tumors will be original and fundamental to gain new insights into potentially novel yet undiscovered phenotypes and functions of TANs. With such diverse roles, the ideal targeting of neutrophils in oncology would be to promote the enrichment of anti-tumor neutrophils while depleting pro-tumor ones without altering antibacterial neutrophils.

The current dogma in cancer immunotherapy mainly relies on restoring anti-tumor T-cell response. However, a high tumor infiltration by anti-tumor lymphocytes is not sufficient to eradicate all tumor clones during the course of metastasis as for example in colorectal cancer patients, shedding light on tumor-intrinsic and tumor-extrinsic mechanisms of escape that remain to be discovered (207). Resistance to CD8 T cell-induced tumor cell death by ferroptosis can be one possible tumor cell-intrinsic mechanism of escape (194). Neutrophils can kill tumor cells in a non-apoptotic mechanism by a process called trogoptosis, distinct from ferroptosis by its lytic/necrotic form (208) of cell death that may release DAMP and tumor (neo) antigens from the killed cancer cells (111). Neutrophils could therefore improve cytotoxic T cell response to eliminate resistant tumor clones.

Beyond their role at tumor site, neutrophils seem to promote tumor progression by acting systemically as exemplified by the ability of blood neutrophils to escort circulating tumor cells within the bloodstream and facilitate their extravasation to metastatic site (56–61). Moreover, the T-cell suppressive activity of circulating neutrophils is restricted to *in vitro* studies. It remains to be known if such neutrophil-mediated T-cell suppression occurs in blood *in vivo*. Growing evidences also highlight the importance of distant microenvironment away from tumor bed such as the bone marrow and the spleen to educate pro-tumor TANs (130, 131). Future studies should therefore consider the importance of the different microenvironments outside of the tumor bed, regarding the origin and function of neutrophils in cancer, for the benefits of patients.

AUTHOR CONTRIBUTIONS

PL, MS, MP, and JM wrote the original draft of manuscript. PL performed original figures and overall the supervision of writing. LK, CC, NB-V, and M-CM revised and edited the manuscript.

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REVIEW
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Neutrophils—Important Communicators in Systemic Lupus Erythematosus and Antiphospholipid Syndrome

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Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are two autoimmune diseases that can occur together or separately. Insights into the pathogenesis have revealed similarities, such as development of autoantibodies targeting subcellular antigens as well as a shared increased risk of cardiovascular morbidity, potentially due to mutual pathologic mechanisms. In this review, we will address the evidence implicating neutrophils in the pathogenesis of these conditions, highlighting their shared features. The neutrophil is the most abundant leukocyte, recognized for its role in infectious and inflammatory diseases, but dysregulation of neutrophil effector functions, including phagocytosis, oxidative burst and formation of neutrophil extracellular traps (NETs) may also contribute to an autoimmune process. The phenotype of neutrophils in SLE and APS differs from neutrophils of healthy individuals, where neutrophils in SLE and APS are activated and prone to aggregate. A specific subset of low-density neutrophils with different function compared to normal-density neutrophils can also be found within the peripheral blood mononuclear cell (PBMC) fraction after density gradient centrifugation of whole blood. Neutrophil phagocytosis is required for regular clearance of cell remnants and nuclear material. Reactive oxygen species (ROS) released by neutrophils during oxidative burst are important for immune suppression and impairment of ROS production is seen in SLE. NETs mediate pathology in both SLE and APS via several mechanisms, including exposure of autoantigens, priming of T-cells and activation of autoreactive B-cells. NETs are also involved in cardiovascular events by forming a pro-thrombotic scaffolding surface. Lastly, neutrophils communicate with other cells by producing cytokines, such as Interferon (IFN) -α, and via direct cell-cell contact. Physiological neutrophil effector functions are necessary to prevent autoimmunity, but in SLE and APS these are altered.

Keywords: neutrophils, reactive oxygen species, neutrophil extracellular traps, systemic lupus erythematosus, antiphospholipid syndrome

KEY POINTS

- The neutrophil effector functions; phagocytosis, oxidative burst and formation of NETs, are all important for a successive host defense against pathogens.
- In SLE, neutrophils are more activated, have a lower phagocytic capacity, a decreased production of NOX2 ROS, an increase in mitochondrial ROS, and are more prone to spontaneously release NETs.
- In APS, neutrophils have an activated phenotype with increased aggregation, mitochondrial dysfunction with increased mitochondrial ROS production, and display enhanced spontaneous NET release.
- Dysregulated neutrophil functions are shared pathogenetic mechanisms in both SLE and APS, contributing to loss of tolerance.

INTRODUCTION

The neutrophil is the most abundant leukocyte and important in most aspects of the immune system. Long thought of as a non-specific cell at the front line of defense against infections, often causing tissue damage on its way, the neutrophil is becoming increasingly recognized as a more sophisticated cell of vital importance for immune homeostasis. The innate immune system is the first line of host defense against invading microorganisms. Neutrophils are phagocytes circulating in the blood awaiting to be recruited to sites of infection with a primary role in the clearance of extracellular pathogens (1). The homing to inflamed tissues is dependent on interaction with endothelium and several adhesion molecules, such as selectins and integrins, for successful extravasation and migration. Neutrophils have a wide range of receptors that recognize microbial and fungal structures as well as complement receptors that facilitate the phagocytosis of pathogens. After the engulfment, the phagosome fuses with neutrophil granules containing antimicrobial peptides and proteins. The increased oxygen consumption, called the oxidative burst, results in formation of toxic superoxide anions and reactive oxygen species (ROS) enabling activation of the antimicrobial peptides and proteins. The neutrophils may also defend against pathogens by releasing their granules and chromatin, so they form extracellular fibers (2). These neutrophil extracellular traps (NETs) degrade virulence factors and kill bacteria. An overall decrease in neutrophils are deleterious and neutropenic patients are at high risk for mortality from severe and recurrent infections (1).

The neutrophil effector functions; phagocytosis, oxidative burst and formation of NETs, are all renowned for their importance in host defense, but abnormalities in these functions are also associated with development of autoimmune disease. The specific mechanisms connecting each neutrophil effector function with autoimmune reactions in the context of systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) will be discussed in detail in this review. The influence of neutrophils on the pathogenesis of SLE is more explored, less is known about the potential effect on the closely related pathogenesis of APS. In this review we will highlight unifying factors and by utilizing knowledge from studies in SLE, we anticipate a better understanding also of neutrophil impact on APS pathogenesis. See **Figure 1** for a graphical overview of neutrophil contribution to the pathogenesis of both conditions.

SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

SLE is a heterogenous inflammatory autoimmune disease with increased mortality and low quality of life, involving most organ systems, including joints, skin, kidneys and heart (3). Despite best available treatment, flares and disease activity are seen in most patients over time and the chronic inflammation contributes to the development of irreversible organ damage. Cardiovascular disease (CVD) represents the most common cause of morbidity and mortality (4). SLE mostly affects women in childbearing age and the prevalence in e.g., Sweden is estimated to 65 in 100,000 (5). APS is an autoimmune disease characterized by thrombosis and pregnancy related complications such as miscarriage and preeclampsia, in the presence of antiphospholipid antibodies (aPL). An APS diagnosis is considered if at least one of the clinical criteria (i.e., thrombosis or pregnancy morbidity) and one of the

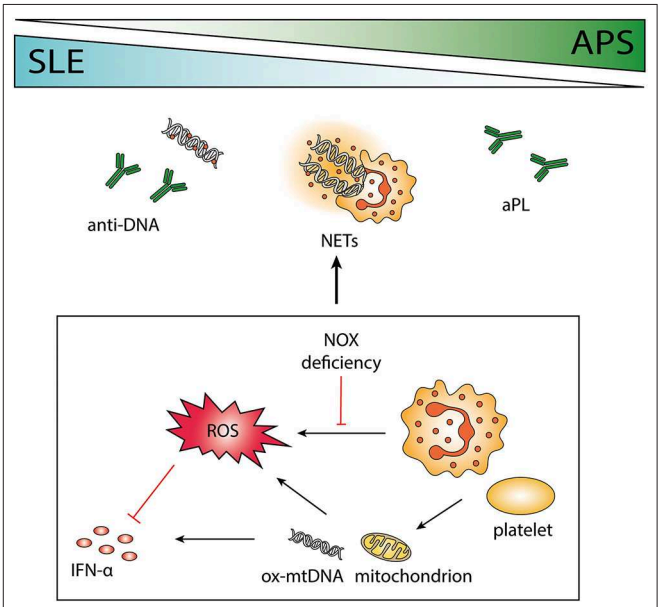

FIGURE 1 | The role of neutrophils in systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). SLE and APS are two autoimmune diseases that can occur together or separately in a patient with overlapping pathogenesis. Antibodies to phospholipids and DNA are characteristic of the two disorders, and the release of neutrophil extracellular traps (NETs) may contribute to the antigenic burden. Neutrophils are important immune regulators via the release of reactive oxygen species (ROS), e.g., by impeding interferon (IFN) -α. Polymorphisms causing deficient ROS production by NOX is associated with autoimmunity, and absence of functional NOX may force cells to use mitochondrial ROS instead. Both neutrophils and platelets can release mitochondria and mitochondrial DNA (mtDNA), highly potent in inducing IFN-α production. Neutrophil-platelet interactions increase ROS production and facilitate NET release.

laboratory criteria (i.e., persistently positive lupus anticoagulant test and/or presence of anti-cardiolipin or anti-β2-glycoprotein-I antibodies at moderate to high titer) are met (6). In addition to the typical manifestations of thrombosis, there are other "non-criteria" manifestations (7). Stagnation of blood flow can lead to livedo reticularis, a red or bluish net-like discoloration of the skin. Heart valve abnormalities, such as thickening of the heart valve occur in up to a third of all APS patients. The clinical spectrum also includes thrombocytopenia, cognitive disorder, seizures, and renal vasculopathy, resembling lupus and similar to lupus APS may have the features of a multiorgan systemic disease. The prevalence is 40–50 in 100,000 and in about half of the cases APS occurs as a primary condition, whereas in the remaining half, it occurs secondary to another autoimmune disease, most notably SLE (8, 9). APS shares both pathogenetic and clinical features with SLE. One or more of the major aPL or lupus anticoagulans (LA) are found in ∼20–30% of SLE patients (10, 11) and aPL are more frequent in first-degree relatives of SLE or primary APS patients, suggesting a genetic susceptibility (12). Familial clustering of primary APS and SLE associated APS has been observed and identified predisposing genetic factors include HLA variants (primarily DR4 and DR7) (13, 14). There are conflicting data regarding non-HLA gene association with APS, including IRF5 and STAT4 (15–17). Thus, the two diseases share genetic predisposition and clinical manifestations, suggestive of shared pathogenetic mechanisms.

Inflammation plays a central role in the development of CVD and patients with chronic inflammatory diseases in general are predisposed. SLE patients have a 2–10-fold increased risk of developing CVD, such as stroke and myocardial infarction, compared to the general population (18), and premenopausal women have the greatest fold difference with a 50-fold increased risk (19). Traditional risk factors such as smoking, dyslipidemia and hypertension are associated with atherosclerosis, but cannot fully explain the increased risk seen in SLE patients. Inflammatory processes driving the SLE pathogenesis also contributes to the development of CVD. An activated type I interferon (IFN) system has effects on the endothelium contributing to endothelial dysfunction, an early step in the development of atherosclerosis (20). The underlying mechanisms of how presence of aPL can result in clinical manifestations are still not fully understood, but alterations of the endothelia, formation of aPL immune complexes, activation of platelets and the complement system have all been suggested to contribute to the pro-coagulative state causing clinical manifestations (7). SLE patients, also without APS, have a 2–5-fold increased risk of venous thrombosis (21), and an even higher risk is seen in patients positive for aPL (11). The interplay between SLE and APS is complex and immune dysregulation appears to play a key role in driving both of the pathogeneses and contributing to CVD and venous thrombosis.

Both SLE and APS are characterized by antibodies directed against self-antigens, normally found within cells, but exposed during cell death. In SLE, apoptotic cells that are not sufficiently eliminated may undergo secondary necrosis, thereby releasing autoantigens (e.g., nucleotide containing structures) and endogenous danger signals that promote inflammation (22). Furthermore, autoantibodies and nucleic acid containing antigens form immune complexes (ICs) which can induce production of IFN-α by plasmacytoid dendritic cells (pDCs) via Toll-like receptor (TLR) -7 or -9 (23). Neutrophils may also produce IFN-α in response to chromatin under certain circumstances (24). Binding of IFN-α to its receptor initiates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway with activation of IFN regulated genes (25). The majority of patients with SLE express increased levels of IFN-inducible genes, i.e., "a type I IFN signature," and/or have raised circulating levels of IFN-α (26, 27). The role of type I IFN in SLE is well established, however, there is evidence of an active type I IFN system also in APS. An IFN signature is found in primary APS and associates with endothelial progenitor dysfunction (28). Similar to SLE, development of transient features of APS may evolve during IFN-α treatment. The development of low titers of anti-cardiolipin antibodies during interferon alpha therapy in chronic hepatitis C has been reported (29), and APS may evolve during PEG IFN-α therapy (30). The possible importance of IFN-α in APS pathogenesis is illustrated by the fact that aPLs may also contribute to IFN-α production, by inducing translocation of TLR7 to the endosomes in pDCs, thereby priming them to internalize RNA (31). Of relevance for both SLE and APS, IFN-α is linked to increased activation of antigen presenting cells, augmented antibody production, and increased apoptosis (26). Deficient physiologic clearance of circulating ICs can lead to IC-deposition in tissues and cause inflammation. In this way, a vicious circle of increased apoptosis, impaired clearance, autoantigen exposure, autoantibody production, chronic inflammation, and tissue damage may proceed.

NEUTROPHILS AND THEIR EFFECTOR FUNCTIONS IN SLE AND APS

Neutrophil Phenotypes

Despite neutrophils being short lived and terminally differentiated cells, heterogeneity among neutrophil phenotypes, both regarding function and expression of surface markers, exist. In the circulation, there are naturally occurring differences between neutrophils depending on their age. Aged neutrophils will express less of adhesion molecule L-selectin and more of activation marker CD11b (32). In tissues, neutrophils have certain ability to polarize depending on the tissue specific milieu and in similarity with monocytes and macrophages neutrophils might polarize into a pro- [tumor necrosis factor (TNF) α-driven] or anti-inflammatory [transforming growth factor (TGF) β-driven] phenotype (32).

In SLE, neutrophils display an activated phenotype with increased aggregation and platelet-neutrophil complex formation compared to neutrophils of healthy controls, and SLE neutrophils are more prone to undergo apoptosis (33–36). Increased neutrophil activation is also seen in APS, and neutrophils from APS patients have an increased expression of cell adhesion genes and proteins resulting in increased neutrophil adhesiveness (37, 38). In models of fetal injury, aPLs

generates complement protein C5a via the classical pathway, and C5a is a potent chemotactic factor and activator of neutrophils. C5a-mediated neutrophil infiltration is observed at sites of fetal resorption and depletion of neutrophils protects mice from aPL-mediated fetal injury (39).

A specific subset of granulocytes with low density and different properties compared to normal density granulocytes have been identified in several chronic inflammatory conditions, first discovered in SLE in 1986 (40). These neutrophil-like cells are found within the peripheral blood mononuclear cell (PBMC) fraction after density gradient centrifugation of whole blood, separated from normal neutrophils present in a higher density fraction. Two types of low-density neutrophils have been described in SLE; low-density granulocytes (LDGs) associated with a proinflammatory phenotype and neutrophil-like myeloid derived suppressor cells (PMN-MDSC) which have an anti-inflammatory phenotype (41, 42). These cell types share several features including density, morphology and CD-marker expression but differ markedly in their role in inflammation (**Table 1**). There are currently no consensus regarding whether these cells belong to the same or different cell types.

LDGs are characterized by proinflammatory features such as production of cytokines and spontaneous release of NETs containing oxidized mitochondrial DNA (43, 44, 49, 55). Compared to normal neutrophils, LDGs have impaired oxidative burst and phagocytosis, but an enhanced ability for NET release and cytokine production (43, 48). Proinflammatory cytokines produced by LDGs include type I IFN, IFN γ, IL-6, IL-8 and TNFα, all of importance in SLE pathogenesis (43). NETs released from LDGs induce endothelial damage by activation of endothelial matrix metalloproteinase-2 via matrix metalloproteinase-9 present in NETs (31). Moreover, LDG NETs contain enzymes such as myeloperoxidase and nitric oxide synthase which oxidize high density lipoprotein, making it proatherogenic (56, 57). In SLE, LDGs are associated with vascular damage (43, 58) and with disease activity in juvenile lupus (59). In APS, LDGs are enriched especially in patients with high titers of anti-β2-glycoprotein-I (60), antibodies capable of inducing NETosis (61, 62).

An increased NET release by LDGs may contribute to the high cardiovascular morbidity in both SLE and APS, and the importance of NETs will be discussed further in this review.

First described in cancer, MDSCs are defined as myeloid progenitor cells with suppressive effects on T-cells (51) and can be divided into two groups, monocyte-like (M-MDSC) and neutrophil-like (PMN-MDSC), both subtypes being immunosuppressive. PMN-MDSC exert their immunosuppressive effects mainly via the production of ROS (52, 63). In murine models of SLE, PMN-MDSCs have been demonstrated to induce expansion of regulatory B- and T-cells, decrease T-cell activation, suppress B-cell differentiation and autoantibody production, as well as ameliorate SLE symptoms (50, 53, 64, 65). Despite several studies on PMN-MDSCs in murine autoimmunity, they have not been characterized in human disease. Two studies investigating MDSCs in SLE patients demonstrate that levels of cells with PMN-MDSC phenotype correlate with increased disease activity (66), and interferon signature (67), but without suppressing T-cell proliferation or activation, thus being LDGs rather than MDSCs. To our knowledge no work regarding MDSCs in APS is published. Clearly, MDSCs in the context of APS and SLE needs further attention to scrutinize their role in humans.

Neutrophil Phagocytosis and Clearance

Clearance deficiency of dying cells is involved in the etiology of autoimmunity and there is an observed increase of apoptotic neutrophils in combination with an impaired phagocytosis by macrophages in SLE (36, 68). In the absence of a proper clearance, apoptotic cells may turn into secondary necrotic cells (SNECs), releasing autoantigens and danger signals (22). The first neutrophil abnormality described in SLE was the discovery of the so called LE-cell (lupus erythematosus cell) first reported in 1948 in the bone marrow of SLE patients (69). The LE-cell is a blood granulocyte in which the nucleus after excessive phagocytosis of opsonized apoptotic cell remnants, closely resembling SNEC, become outstretched and pushed toward the edges of the cell (70, 71). A combination of antibodies to several different histone proteins promotes this phenomenon, increasing the uptake of nuclear material (72, 73).

Nuclear remnants in the circulation of healthy individuals are not phagocytosed, but rapidly degraded by DNases and C1q via the reticuloendothelial system. In SLE, impaired DNase activity or deficiency of complement proteins is common. Nuclear material, opsonized by antinuclear antibodies (ANA) and complement, is instead dependent on removal by phagocytosis by e.g., neutrophils (74), and autoantibodies recognizing SNECs promote neutrophilic phagocytosis (75).

Apoptotic cells which are not cleared become decorated with β2GPI and cardiolipin is reportedly translocated from the inner mitochondrial membrane to the cell surface during apoptosis, exposing targets for APS-related antibodies (76–78). Opsonization of apoptotic bodies may shift the clearance toward proinflammatory pathways. Thus, the reduced removal of apoptotic cell remnants may be an explanation to why SLE and APS are syndromes often occurring simultaneously and an indication of shared pathogenic mechanisms.

Neutrophil Reactive Oxygen Species

There are two sources of ROS in neutrophils (**Figure 2**). The most important source of ROS in neutrophils is generated from the NADPH oxidase 2 (NOX2) enzyme complex, which is selectively expressed in phagocytic cells and produces large amounts of ROS during oxidative burst. In addition, as in other cell types, neutrophil ROS can be formed by oxidative phosphorylation during mitochondrial ATP production (79). The role of ROS in autoimmune diseases is highly complex, where a normal production of ROS is necessary both for host defense during infection and redox regulation of the immune system. ROS may be toxic for host cells and have therefore traditionally been considered harmful, but the last decade of research have clearly demonstrated that NOX2-derived ROS are important second messengers and immune regulators, suppressing excessive inflammation (79).

TABLE 1 | Phenotypes and functions of neutrophils, LDG and PMN-MDSC.

	LDG	PMN-MDSC	Neutrophil	References
Density	Low (PBMC-fraction)	Low (PBMC-fraction)	Normal (PMN-fraction)	(43)
CD-markers	$CD15^+CD14^-$ $CD10^+CD14^-$ $CD10^+CD15^+CD14^-$	$CD11b^+CD14^-CD15^+$ $CD11b^+CD14^-CD66^+$ $CD11b^+Gr\text{-}1^+$ $CD15^+LOX1^+$	$CD11b^+CD14^{low}$ $CD15^+CD16^+$ $CD62L^+$	(43–47)
Morphology	Neutrophil-like Less segmented nucleus	Neutrophil-like Less segmented nucleus	Neutrophil Segmented nucleus	(27, 44)
ROS	+	+ + +	++	(43, 48)
NETs	+ + +	+	++	(49, 50)
Phagocytosis	+	?	++	(43)
Immune suppression	-	++	+	(44, 51, 52)
Cytokine production	IFN-α, TNFα, IL-8, IL-6	IL-10		(43, 53)
Gene expression	Granule enzymes Cytokines	Granule enzymes Cell cycle-related proteins		(27, 49, 54)

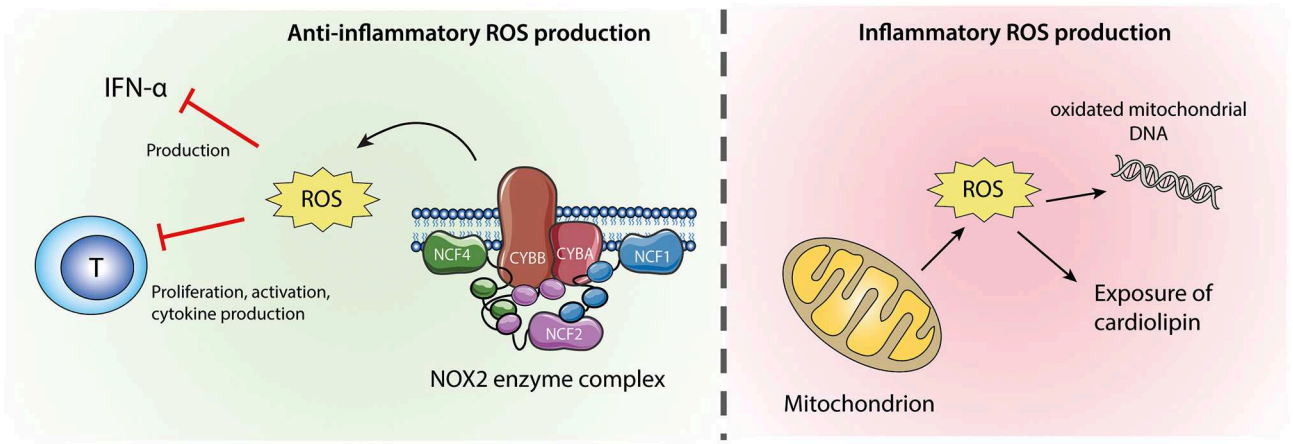

FIGURE 2 | Neutrophil ROS from NOX2 and mitochondria. ROS produced by the NOX2 enzyme complex **(left)** are anti-inflammatory via their inhibitory effects on IFN-α production and T-cell activation. Mitochondrial ROS **(right)** might cause oxidation of mitochondrial DNA which if released from the cell is highly inflammatory. Excessive mitochondrial ROS and apoptosis can also result in translocation of cardiolipin from the inner to the outer mitochondrial membrane and the plasma membrane.

NOX2-Derived ROS

The process of oxidative burst is highly controlled and only occurs in primed or stimulated cells, as the multicomponent NOX2 complex in resting cells is inactive, separated between the plasma membrane and the cytosol. The membrane proteins NOX2 and cytochrome b-245 alpha polypeptide (CYBA) form the catalytic flavocytochrome b_{558}, using NADPH to produce superoxide (80). However, the production of reactive oxygen is only possible if the membrane proteins are associated with the cytosolic components neutrophil cytosolic factor 1 (NCF1), NCF2 and a small GTPase (i.e., Rac 1 or 2). Upon neutrophil activation, NCF1 is heavily phosphorylated by protein kinase C and the subsequent conformational change enables binding to NCF2 and NCF4. The cytosolic complex is transported to the plasma membrane where it docks to flavocytochrome b_{558} resulting in activation of the NOX2 complex (80, 81).

Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by mutations in the NOX2 subunits (82), characterized by recurrent severe infections and lupus-like features due to impaired ROS production via NOX2. Genetic variations in several NOX2 complex components have been associated with multiple autoimmune diseases (83–87), highlighting the importance of a functional NOX2 complex for maintenance of a healthy immune system. Several independent studies have found a connection between impaired ROS production and SLE (83–85, 88), and in animal models of SLE deficiency of NOX2 function results in excacerbated disease (89, 90). The NCF1-339 single nucleotide polymorphism (SNP), where the minor allele T reduces the burst capacity of neutrophils, is one of the strongest identified genetic associations with SLE with an OR of 3.7 and an allele frequency of 17% (83). The NCF1-339 T allele has been shown to increase the expression of type I IFN regulated genes and associate with a younger age at

SLE diagnosis. Genetic variants in NCF2 and NCF4 generating decreased ROS production have also been associated with SLE and/or other rheumatic diseases (85, 87).

In NOX2 expressing cells such as neutrophils, ROS produced by the NOX2 complex are important for regulation of autophagy (91), clearance associated LC3 (microtubule-associated protein 1 light chain 3α)-mediated phagocytosis (92), pH-regulation in endosomes and phagosomes for quiescent handling of necrotic material (73, 93), as well as degranulation (94) and release of NETs (95, 96). Perhaps even more important are the use of ROS as a messenger molecule. NOX2 derived ROS have suppressive effects on CD4+ and CD8+ T-cell proliferation and pro-inflammatory cytokine production, which is abrogated in the presence of a ROS scavenger (63, 97). When released within the immunological synapse, ROS can downregulate T-cell reactivity by inhibiting T-cell receptor activation (98). Additionally, although not investigated in neutrophils, macrophages can induce the expansion of regulatory T-cells in a NOX2 dependent manner (99). This may be relevant since regulatory T-cells are important for maintaining tolerance, and the number of cells and the function is reduced in SLE (100), while studies in APS show contradictory results (101, 102). Of particular importance in SLE is the inhibitory effect ROS has on the production of IFN (103, 104). Interestingly, neutrophils from APS patients demonstrate a proinflammatory signature with overexpression of IFN signaling genes (37, 105) and both mice and CGD patients, which lack NOX2 activity, display an IFN signature and upregulation of STAT1 (106). Deficiency of ROS may oppose tolerance and increase IFN-α, potent in driving the SLE pathogenesis and possibly also APS.

Little is known about how NOX2-derived ROS are related to APS. Oxidative stress unfolds the ring conformation of β2GPI and increases the immunogenicity by exposing domain I, an epitope for pathogenic autoantibodies (107). The activation of NOX2 by aPL in monocytes and dendritic cells upregulates TLR-7 and−8 with subsequent proinflammatory cytokine production (108). Moreover, aPL activation of NOX2 also results in induction of tissue factor. Some studies suggest that high, rather than low, NOX2-derived ROS are contributing to APS (109). More research is needed to clarify the role of ROS in APS.

Mitochondrial ROS

In absence of a functional NOX2 complex, neutrophils may become more reliant on mitochondrial ROS. Proinflammatory LDGs have weak oxidative burst but enhanced mitochondrial ROS production and release mitochondria-derived NETs (55). The shifted balance from NOX2 to mitochondrial ROS is probably of importance for the proinflammatory phenotype of LDG. Enhanced production of mitochondrial ROS cause oxidation of the unprotected mitochondrial DNA. In SLE neutrophils, oxidized mitochondrial DNA is not properly disposed of and the neutrophils instead extrude oxidized mitochondrial DNA with potent IFN-stimulatory effect on pDC (110). Additionally, inhibition of mitochondrial ROS in a lupus mouse model reduces disease severity and type I IFN response (55).

In APS, mitochondrial ROS may be of pathogenetic importance. Healthy monocytes exposed to aPL leads to mitochondrial dysfunction and inhibition of mitochondrial ROS reduces the expression of prothrombotic and proinflammatory markers (111). Cardiolipin is exclusively expressed by mitochondria and becomes exposed on the outer membrane upon mitochondrial dysfunction (112). Hence, decreased NOX2 activity, increased mitochondrial ROS production and mitochondrial dysfunction may expose cardiolipin, hypothetically leading to development of APS. Mitochondria decorated in NETs or as a consequence of impaired mitophagy (55, 110) are important for autoimmune reactions toward this antigen, and anti-mitochondrial antibodies are detected in both SLE and APS (113, 114). Thus, an altered mitochondrial function may drive the disease toward APS.

Neutrophil Extracellular Traps

NETs were first described in host response via the ability to capture and kill bacteria (2), but has later been found to be implicated in a myriad of different conditions ranging from coagulation to cancer to autoimmunity (115, 116). We now know that the formation of NETs is equally important as the other more studied neutrophil effector functions in the immune system (**Figure 3**).

NETs are released from neutrophils in response to a wide variety of stimuli such as microbes and pathogen associated molecular patterns (PAMPs), but also sterile stimuli including cytokines, antibodies, immune complexes and chemicals such as protein kinase C activator phorbol-myristate-acetate (PMA) and calcium ionophores (2, 95, 96, 117–119). Several intracellular NET-inducing signaling pathways have been described (120), most of which include either generation of superoxide via the NOX2 complex or citrullination of proteins by PAD4 (95, 121), and in some instances by mitochondria derived ROS (55, 119). The process of NET release is often accompanied by neutrophil cell death (NETosis), but "vital NETosis" where the neutrophil remain viable after NET release is also described (122, 123). Depending on stimulus and intracellular pathway involved, the content of released NETs varies. DNA in NETs might origin either from the nucleus, the mitochondria or both (2, 55, 124, 125), and proteins may vary both in quantity and presence of post-translational-modifications such as citrullination (126, 127). NETs seem to have different immunogenic effects depending on their composition. Integration of multiple signals, such as environmental triggers, metabolic state and signals from the tethered phagocytic cargo, determine the decision of neutrophils to phagocytose or generate NETs (128). Defects in phagocytosis may lead to intravascular generation of NETs, promoting vascular inflammation seen in diseases characterized by defective clearance.

NETs expose epitopes normally shielded by the plasma membrane, which if not sufficiently cleared contributes to the autoantigenic burden, as demonstrated in several diseases including SLE, RA and small-vessel vasculitis (129–132). Enhanced NET release and impaired clearance of NET components are seen in both SLE and APS (62, 131, 133, 134). In SLE there is more and more evidence of involvement of NETs, as

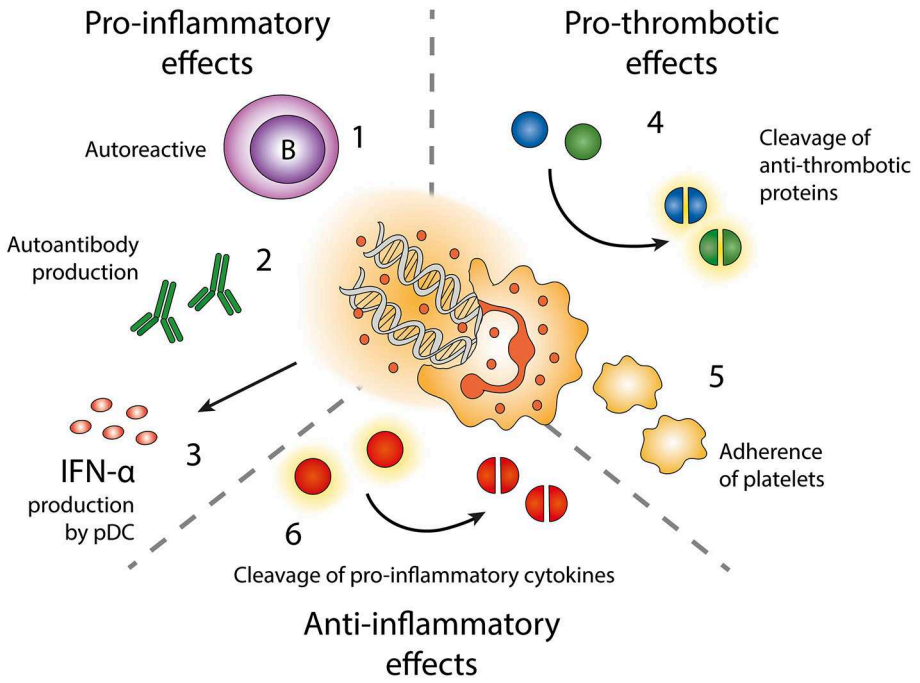

FIGURE 3 | Effects of neutrophil extracellular traps (NETs). Exposure of autoantigens leads to activation of autoreactive B-cells (1) and autoantibody production (2). NETs and NET-containing IC activate pDC to produce IFN-α (3). NETs contain enzymes cleaving anti-thrombotic proteins (4) resulting in activation of the coagulation cascade. NETs also form a scaffold onto which platelets can adhere and form thrombi (5). NET-related enzymes can also cleave and inactivate pro-inflammatory cytokines (6).

neutrophils of patients with SLE are more prone to spontaneously release NETs (33, 49), NET-remnants can be found in the circulation (135) and depositions in skin and kidney glomeruli (49). The NETs have several downstream effects on other cell types, such as priming of T-cells by reducing their activation threshold and activation of autoreactive B-cells (136, 137). NET-derived immune complexes trigger polyclonal B cell activation via TLR9, but also expand self-reactive memory B cells (136). Moreover, immune complexes formed by autoantibodies and NET-components can activate pDCs, resulting in production of IFN-α (55, 117, 125). Thus, NETs do not only contribute to SLE by antigen exposure but also by its impact on other immune cells.

Similarly, patients with primary APS also display enhanced spontaneous NET release (62). Plasma of patients with APS (both primary and secondary) as well as aPL alone can induce NETs from healthy neutrophils (61, 138), and NET release correlates with circulating levels of aPL (62). Deposition of NETs can be found in intervillous tissue of pre-eclampsia pregnancies (139), possibly contributing to the pregnancy morbidities present in APS. In a study by Meng et al. mice treated with IgG isolated from APS patients demonstrated exaggerated NET formation and thrombosis, where NETs were found within the thrombi (140).

In the vasculature, NETs can form a scaffolding structure onto which platelets aggregate and form thrombi (141, 142). Activated platelets release the damage-associated molecular pattern high-mobility group box protein 1 (HMGB1) to neutrophils, committing them to NET generation (142). Neutrophils stimulated with antiphospholipid antibodies release NETs and promote thrombosis (140). NETs are also found in both arterial and venous thrombosis without underlying autoimmune disease [reviewed in Laridan et al. (143)]. NETs can bind factor XII and cooperate with platelets to activate the intrinsic pathway (144). Neutrophil proteases elastase and cathepsin G present in NETs cleave the anti-coagulant proteins antithrombin III, heparin cofactor II and tissue factor pathway inhibitor (TFPI) (145–147), resulting in a pro-coagulant microenvironment, and NET-associated oxidative enzymes oxidize high density lipoprotein making it proatherogenic (57). Moreover, SLE serum induces neutrophil autophagy and NETosis by upregulating expression of hypoxia-response and stress response protein REDD1 (148). Through this pathway NETs are decorated with tissue factor and IL-17A, making them highly prothrombotic.

However, there are controversies regarding NETs and their role in autoimmune diseases (120). Independent studies have demonstrated that SLE mouse models with knocked-out or inhibited NOX2 or PAD4, thus with deficient NET formation, display either a more severe or unchanged disease phenotype compared to mice with normal NET releasing ability (89, 90, 149, 150). In the case of NOX2 pathway blockade, this effect is probably dependent on the many effects of NOX2-derived ROS as regulators of inflammation, as discussed previously in this review. Potential protective effects of NETs include aggregation and

degradation of inflammatory cytokines via NET-related serine proteases (151).

Neutrophil Interactions With Other Cells of the Immune System

Neutrophils cross-talk with most cells of the immune system, reviewed in Scapini and Cassatella (152). As previously discussed in this article, neutrophils produce large amounts of ROS during oxidative burst, which has several immunoregulatory effects on other cells of the immune system.

Neutrophils produce several different cytokines, both pro- and anti-inflammatory, as well as immunomodulatory and chemotactic cytokines (153). The cytokine profile of patients with both SLE and APS differ from that of healthy individuals, with increased levels of many pro-inflammatory mediators (60, 154), which might be a consequence of the many neutrophil abnormalities seen in SLE as discussed in this review. IFN-α, a key cytokine involved in many aspects of both SLE and APS can be produced in neutrophils (24). The amount of IFN produced by a neutrophil is only a fraction of what can be produced by a pDC but considering the vast amount of neutrophils present in the circulation the neutrophil is probably a more important source of IFN than previously considered. IFN produced by neutrophils in the bone marrow in SLE has been demonstrated to disturb B-cell development (155). Neutrophils also produce B-cell activating factor (BAFF/BLyS) which promotes activation of autoreactive B-cells and antibody production in SLE (156, 157). T-cells represent a key checkpoint for autoreactive B-cells and BAFF affects classic T-B cell interactions. Activated CD4+ cells migrate to B-cell follicles and transforming into follicular T-cells, which promotes formation of germinal centers and sustains the activation of B-cells and eventually long-lived plasma cells (158). Neutrophils also possesses the capacity of antigen presentation (159, 160). Antigen presentation by professional antigen presenting cells activates T-cells producing e.g., IFN-γ. It has been shown that these cytokines stimulate upregulation of MHC II and costimulatory molecules on neutrophils, enabling them to present antigens to T-cells, and an upregulation of MHC II on neutrophils is found in rheumatoid arthritis and systemic small vessel vasculitis (161, 162). Thus, neutrophils may also play a role in the pathogenesis of autoimmune diseases by presenting autoantigens.

Neutrophils also interact with other immune cells via direct cell-cell contact. Especially platelets communicate with neutrophils in this manner (163). Increased platelet-neutrophil interaction has been detected in SLE and activated platelets binding to neutrophils facilitates NET release (164, 165), suggesting that neutrophils and platelets act together in this process in SLE (166). Neutrophils are also dependent on platelets during migration, as clusters of P-selectin glycoprotein ligand 1 (PSGL-1) needs to bind P-selectin on activated platelets in order to express receptors driving neutrophil migration (167). Blockade of PSGL-1 mediated neutrophil-platelet interactions results in decreased neutrophil migration and protection against thrombo-inflammatory injury. PSGL-1 is upregulated in APS patient neutrophils and PSGL-1 deficient mice are protected from antiphospholipid antibody mediated thrombosis (37). Activated platelets release their granules with proinflammatory chemokines and cytokines capable of promoting chemotaxis of neutrophils (168). Neutrophil-platelet interactions are important in thromboinflammation and probably involved in the cardiovascular manifestations in SLE and APS by mechanisms mentioned earlier; generation of NETs and release of neutrophil constituents such as elastase, cathepsin G, and tissue factor. However, the communication with platelets may also contribute to resolution of inflammation. Platelets potentiate intra- and extracellular generation of ROS and myeloperoxidase from stimulated neutrophils and enhance Fcγ receptor-mediated phagocytosis (169).

CONCLUDING REMARKS

Neutrophils have a limited amount of effector functions, but an imbalance in any of them will have large effects on the whole immune system. SLE and APS are two autoimmune diseases that can occur together or separately in a patient with overlapping pathogenesis and may be considered as a continuum with a sliding scale of clinical manifestations. Patients suffering from SLE or APS have an increased cardiovascular morbidity compared to the healthy population, which to a significant extent is related to abnormal neutrophil function. Autoimmunity is generally thought of as a phenomenon mainly dependent on activation of an autoreactive adaptive immune system, but in SLE and APS a functional neutrophil is important for preventing loss of tolerance via immune regulation and clearance.

AUTHOR CONTRIBUTIONS

LW and AB contributed to the original idea and manuscript writing. SA and PL participated in the planning and writing of sections of the manuscript.

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CASE REPORT
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The Pathogenicity of BPI-ANCA in a Patient With Systemic Vasculitis

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Objective: ANCA associated vasculitis (AAV) is characterized by systemic necrotizing vasculitis with the presence of ANCA. Although BPI-ANCA is one of the atypical ANCAs and is occasionally seen in patients with vasculitis, the pathogenicity of BPI-ANCA remains unclear. This study was performed to examine the pathogenic role of BPI-ANCA against neutrophils.

Methods: A 76-year-old Japanese man showed BPI-ANCA positive systemic vasculitis with a medical history of *Pseudomonas aeruginosa* infection. BPI-ANCA IgGs were eluted from the patient serum using an immunoadsorbent column. *In vitro* experiment, healthy donor neutrophils were treated with BPI-AAV IgGs, MPO-AAV IgGs, healthy control IgGs under TNFα stimulation. After 3 h incubation, neutrophil extracellular trap (NET) was assessed by immunofluorescent imaging. To determine the pathogenicity of BPI-ANCA, TNFα-primed neutrophils were incubated with monoclonal BPI-ANCA in the presence or absence of recombinant BPI.

Results: BPI-AAV IgGs-treated neutrophils showed NET formation with histone citrullination. Interestingly, the monoclonal BPI-ANCA did not induce NET, but the immune complexes (ICs) of recombinant BPI and BPI-ANCA induced TNFα-dependent NET formation with hypercitrullination. Furthermore, TNFα increased the expression of BPIs in neutrophils and the BPIs were translocated to cell surface.

Conclusion: BPI-ANCA could affect neutrophils leading to NET formation and may play a role in the development of systemic vasculitis as pathogenic autoantibody.

Keywords: ANCA-associated vasculitis, neutrophil extracellular traps (NETs), Bactericidal/permeability-increasing protein (BPI), BPI-ANCA, immunity

KEY MESSAGE

1) BPI-ANCA activates neutrophil via forming immune complexes with BPI, leading to NET formation.
2) BPI-ANCA-induced NET may play role in the pathogenesis of vasculitis.
3) Gram-negative infections might be involved in the development of BPI-ANCA.

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INTRODUCTION

Bactericidal/permeability-increasing protein (BPI) is an antibacterial neutrophil cytoplasmic protein that plays an important role in the immune system. BPI has anti-endotoxin properties, which allow it to work against Gram-negative bacteria (GNB) infection. In addition, BPI delivers components of GNB to dendritic cells for processing. Anti-neutrophil cytoplasmic autoantibodies (ANCAs) against neutrophil granule BPI (BPI-ANCA) have been reported in various kinds of diseases, such as cystic fibrosis and inflammatory bowel diseases (IBD), in which opportunistic infections with GNB are common. During persistent GNB infections, complexes of BPI and GNB are processed and presented by dendritic cells, potentially leading to the breakdown of immunological tolerance against BPI (1). Although BPI-ANCAs are known to be involved in the pathogenesis of systemic vasculitis (2), it remains unclear whether they play a pathogenic role in the development of vasculitis. Here, we report that BPI-ANCAs in a patient with systemic vasculitis affect neutrophils to undergo neutrophil extracellular trap (NET) with hypercitrullination, thus contributing to the development of systemic vasculitis.

CASE PRESENTATION

A 76-year-old Japanese man who had experienced recurrent chronic bronchiolitis with *Pseudomonas aeruginosa* infection for over 10 years presented with a 6-month history of haematuria/proteinuria and purpura. A skin biopsy revealed cutaneous leukocytoclastic vasculitis, and a renal biopsy showed pauci-immune crescentic glomerulonephritis. Serum examination by immunofluorescence showed the patient was negative for P-ANCA, but positive for C-ANCA. Routine enzyme-linked immunosorbent assay (ELISA) revealed that he was negative for both MPO-ANCA and PR3-ANCA. The titer of serum immune complexes (C1q binding assay) was 50.0 μg/mL (normal range; < 3.0 μg/mL). Further ELISA assay (ANCA panel kit, Euro Diagnostica) revealed that the antigen for C-ANCA was BPI (titer; 6.5 O.D. ratio, **Table 1**) and other atypical ANCAs including azurocidin, cathepsin G, elastase, lactoferrin, and lysozyme were negative. Based on these findings, the patient was diagnosed with BPI-ANCA-associated systemic vasculitis (AAV). He was treated with prednisolone with antibiotics for GNB infections and his clinical findings were recovered (**Figure 1**).

TABLE 1 | Laboratory data.

Complete Blood Count	
WBC	12,000 /μL
RBC	4.33 x 10^6 /μL
Hemoglobin	11.2 g/dL
Platelet	29.4 × 10^4 /μL
Biochemistry	
BUN	17 mg/dL
Cr	0.7 mg/dL
Cysc	1.43 mg/L
Urinalysis	
Haematuria	≥100 /HPF
WBC	5–9 /HPF
Proteinuria	0.76 UP/UCr ratio
Immunoserological test	
CRP	5.14 mg/dL
IgA	477 mg/dL
IgG	1,838 mg/dL
IgM	61 mg/dL
C3	42 mg/dL
C4	38 mg/dL
CH5O	11.0 U/mL
Immune complexes	50 μg/mL
(normal range; < 3.0 μg/mL)	
ANA	×160
RF	41.1 IU/mL
Anti DNA Ab	(–)
PR3 ANCA	<20.0 RU/mL
MPO-ANCA	<20.0 RU/mL
Anti GBM Ab	(–)
P-ANCA (IIF)	(–)
C-ANCA (IIF)	(+)
BPI ANCA	(+)/ 6.5 O.D.Value

WBC, white blood cell; RBC, red blood cell; UP/UCr, urine protein to urine creatinine ratio; ANA, antinuclear antibody; RF, rheumatoid factor.

The Pathogenicity of BPI-ANCA in *in vitro* Experiments

To evaluate the significance of BPI-ANCAs in vasculitis, healthy donor neutrophils were treated *in vitro* with BPI-ANCA immunoglobulin (Ig)Gs derived from the patient. BPI-AAV IgGs-treated neutrophils underwent Sytox Green-positive NET formation with histone citrullination under TNFα stimulation in a manner similar to that of MPO-AAV IgGs-treated neutrophils (**Figures 2A,B**). Since the polyclonal ANCA-IgGs were extracted from patient sera using Protein G column, to elucidate the pathogenicity of BPI-ANCA, a TNFα-primed neutrophils were treated with monoclonal BPI-ANCA and control antibody in the presence of recombinant BPI. Although the monoclonal BPI-ANCA did not induce NET formation, the immune complexes (ICs) of recombinant BPI and BPI-ANCA induced TNFα-dependent NET formation with hypercitrullination (**Figure 2C**). To elucidate the phenomenon that TNFα accelerated the ICs-induced NET formation, the expression of BPI in neutrophils with or without TNFα stimulation was evaluated by immunostaining. TNFα up-regulated BPI expression in neutrophils and the overexpressed BPIs were translocated to cell surface (**Figure 2D**).

DISCUSSION

We demonstrated that BPI-ANCAs in a patient with systemic vasculitis activated neutrophils to undergo NET formation *in vitro* studies, suggesting that BPI-ANCAs contribute to the pathogenicity during the development of vasculitis.

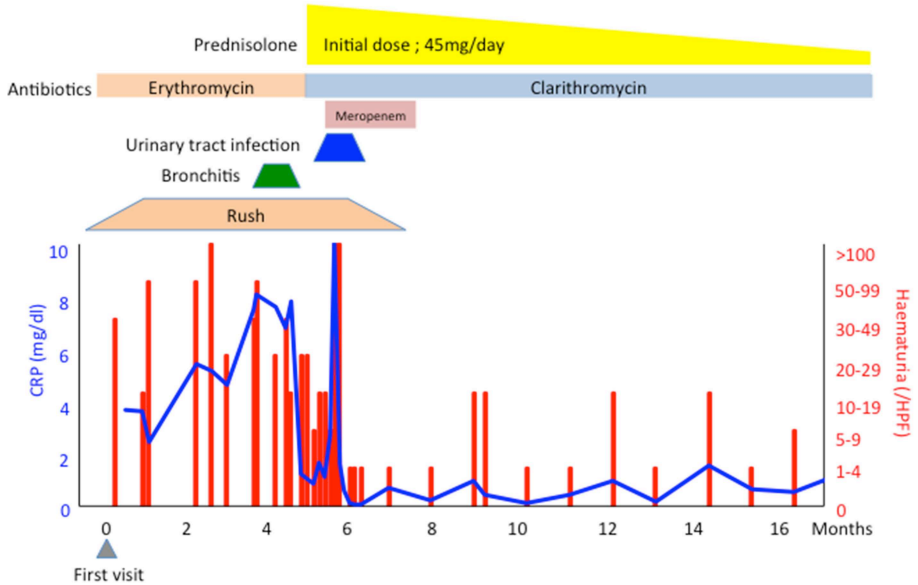

FIGURE 1 | Clinical course of a patient.

BPI-ANCAs have been frequently detected in various kinds of disease, including cystic fibrosis and inflammatory bowel diseases, and these auto-antibodies were found to be involved in organ damage and excessive inflammation (1). Meanwhile, Zhao et al. reported that BPI-ANCAs were detected in 40% of patients with double-negative ANCA (negative for MPO- and PR3-ANCA) vasculitis (2). Although MPO- and PR3-ANCAs are known as pathogenic antibodies that can activate neutrophils (3, 4), the role of BPI-ANCAs remains unclear.

Here, we found that BPI-ANCA IgGs activated neutrophils under TNFα stimulation, leading to NET formation with hypercitrullination. Furthermore, monoclonal BPI-ANCA induced NETs with hyper-citrullination via forming ICs of BPI and BPI-ANCAs in accordance with TNFα stimulation. The C1q binding ICs were detected by ELISA method in this patient (**Table 1**), implying that the patient-eluded BPI-ANCAs may form ICs with BPI in the absence of additional exogenous BPIs and IgGs themselves could have NET inducibility. In addition, BPIs on neutrophils were translocated to cell surface and overexpressed by TNFα stimulation. These phenomena indicate that BPI-ANCAs may be produced during chronic inflammatory diseases, such as cystic fibrosis and IBD, and consequently (1) BPI-ANCAs bind to circulating excessive BPIs that are exposed by infections, inducing the formation of ICs, (2) the circulating ICs may bind to neutrophils with overexpressed BPI, (3) TNFα facilitated ICs mediated signaling such as spleen tyrosine kinase (5), leading to NET formation, and (4) these contribute to the development of systemic vasculitis.

In addition, Traaij et al. (5) reported that ANCA-IgG-depleted serum induced NET formation and that the NET inducibility was associated with the vasculitis activity. Therefore, we conducted NET experiments using both whole serum and IgG-depleted serum. In contrast with healthy samples, the serum and IgG-depleted serum from patients with either MPO-AAV or BPI-AAV induced NET formation (**Figure 2E**), suggesting that some humoral factors as well as BPI-ANCAs might influence NET formation and the pathogenesis of disease.

Although most patients with cystic fibrosis have BPI-ANCAs, it remains controversial why only a few patients of BPI-ANCAs positive develop systemic vasculitis. There are several possible explanations, including the amount of exposed BPI during infection, the presence of refractory infection, and the characteristics of BPI-ANCAs (e.g., affinity to antigen and IgG subclasses). While, BPI-ANCAs IgG derived from patients in this study.

CONCLUDING REMARKS

This study showed that BPI-ANCAs could influence NET formation and may play a role in the development of systemic vasculitis as pathogenic autoantibodies.

MATERIALS AND METHODS

Patients and Healthy Controls

Blood samples were obtained from the patient with BPI-AAV, MPO-AAV and healthy control. This study was approved by the Institutional Review Board of Hokkaido University Hospital. We obtained written informed consent for the publication from the patient.

Nets Induction Assay

Neutrophils were extracted from a peripheral blood sample of a healthy volunteer using Polymorph Prep. After 30 min of 5 ng/mL tumor necrosis factor (TNF)α pretreatment at 37°C, the neutrophils were treated with 3% serum, or 3% IgG depleted

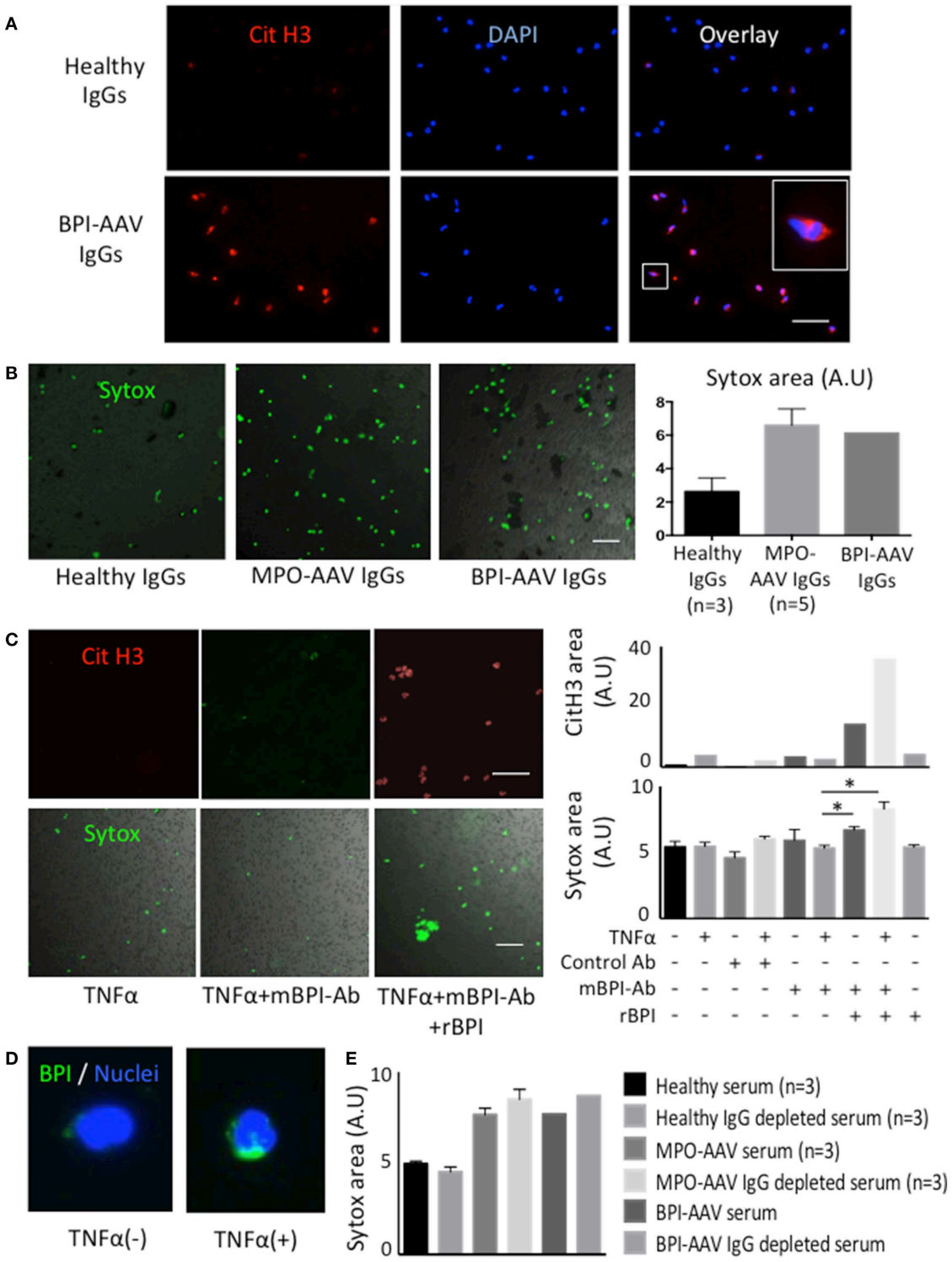

FIGURE 2 | BPI-ANCAs induce NETs with hyper citrullinated histones. **(A)** Representative NETs staining of healthy or BPI-AAV patient IgGs-stimulated neutrophils using CitH3 (red) and DAPI (BLUE). **(B)** Representative Sytox-positive neutrophils live image. From the left, healthy IgGs ($n = 3$), MPO-AAV patient IgGs ($n = 5$), BPI-AAV patient IgGs. Right figure shows Sytox-positive areas quantified by Image J. **(C)** Representative CitH3-positive NETs and Sytox-positive neutrophils treated with TNFα, TNFα+mBPI-Ab, TNFα+mBPI-Ab+rBPI. Right figures show CitH3-positive area (upper) and Sytox-positive area (lower) under each situation. Data represent the mean±SEM of three independent experiments and were analyzed using unpaired *t*-test (PRISM software, GraphPad). *$p < 0.05$. **(D)** BPI expression

(Continued)

FIGURE 2 | with or without TNFα stimulation. Green; BPI, BLUE; DAPI. **(E)** Sytox positivity of neutrophils treated with 3% serum, or 3% IgG depleted serum of patients with BPI-AAV, MPO-AAV ($n = 3$) and healthy control ($n = 3$). Scale bar in all figures, 50 mm. CitH3, citrullinated histone3; DAPI, 49,6-diamidin-2-phenylindol; BPI, bactericidal/permeability increasing protein; ANCA, anti neutrophil cytoplasmic antibody; AAV, ANCA associated vasculitis; IgG, immunoglobulin G; TNFα, tumor necrosis factor alpha; mBPI-Ab, monoclonal BPI antibody; rBPI, recombinant BPI.

serum, or 400 μg/mL IgGs eluted from serum of patients with BPI- AAV, MPO-AAV, healthy control. In experiment using monoclonal antibody, mouse BPI monoclonal antibody (10 μg/mL) and recombinant BPI (Santa Cruz) were used. The neutrophils were seeded on chamber slides or 96-well plate and incubated for 3 h at 37°C *in vitro*.

IgGs were eluted from serum using an immunoadsorbent column (Protein G HP SpinTrap; GE Healthcare, Tokyo, Japan) according to the manufacturer protocol. Contamination of endotoxin in samples was ruled out using the Limulus test kit (Wako Pure Chemical, Osaka, Japan). NETs were evaluated by immunofluorescent staining and by live imaging.

Immunofluorescent Staining for NETs and Neutrophil BPI

After incubation, neutrophils were fixed by 4% paraformaldehyde (PFA). After blocking (2%BSA for 30 min at room temperature), citrullinated histone 3 (CitH3) antibodies (rabbit IgG, 1:200, dilution, for 24 h at 4°C, Abcam) and BPI antibody (mouse IgG, 1:100, for 24 h at 4°C, Santa Cruz) were used. DNA was stained using DAPI.

Live Imaging *in vitro*

To evaluate neutrophil death including NETs, sytox green reagents (final concentration = 15 nM, Thermo Fisher Scientific) were applied to neutrophils and Sytox-positive neutrophils and NETs were visualized and quantified by Fluorescence microscopy (Keyence). The CitH3-positive NETs and Sytox-positive NETs/dead neutrophils were quantified using Image J software.

ETHICS STATEMENT

All procedures performed in the study were in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patient presented here.

AUTHOR CONTRIBUTIONS

ST and DN wrote and summarized the manuscript. KW-K and DN conducted *in vitro* neutrophil experiments. ST, KW-K, YK, SN, and TA managed and followed up the patients' care. All authors reviewed the manuscript and approved the final version.

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Nox2 Regulates Platelet Activation and NET Formation in the Lung

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The mortality rate of patients with critical illness has decreased significantly over the past two decades, but the rate of decline has slowed recently, with organ dysfunction as a major driver of morbidity and mortality. Among patients with the systemic inflammatory response syndrome (SIRS), acute lung injury is a common component with serious morbidity. Previous studies in our laboratory using a murine model of SIRS demonstrated a key role for NADPH oxidase 2 (Nox2)-derived reactive oxygen species in the resolution of inflammation. Nox2-deficient ($gp91^{phox-/y}$) mice develop profound lung injury secondary to SIRS and fail to resolve inflammation. Alveolar macrophages from $gp91^{phox-/y}$ mice express greater levels of chemotactic and pro-inflammatory factors at baseline providing evidence that Nox2 in alveolar macrophages is critical for homeostasis. Based on the lung pathology with increased thrombosis in $gp91^{phox-/y}$ mice, and the known role of platelets in the inflammatory process, we hypothesized that Nox2 represses platelet activation. In the mouse model, we found that platelet-derived chemokine (C-X-C motif) ligand 4 (CXCL4) and CXCL7 were increased in the bronchoalveolar fluid of $gp91^{phox-/y}$ mice at baseline and 24 h post intraperitoneal zymosan-induced SIRS consistent with platelet activation. Activated platelets interact with leukocytes via P-selectin glycoprotein ligand 1 (PSGL-1). Within 2 h of SIRS induction, alveolar neutrophil PSGL-1 expression was higher in $gp91^{phox-/y}$ mice. Platelet-neutrophil interactions were decreased in the peripheral blood of $gp91^{phox-/y}$ mice consistent with movement of activated platelets to the lung of mice lacking Nox2. Based on the severe lung pathology and the role of platelets in the formation of neutrophil extracellular traps (NETs), we evaluated NET production. In contrast to previous studies demonstrating Nox2-dependent NET formation, staining of lung sections from mice 24 h post zymosan injection revealed a large number of citrullinated histone 3 (H3CIT) and myeloperoxidase positive cells consistent with NET formation in $gp91^{phox-/y}$ mice that was virtually absent in WT mice. In addition, H3CIT protein expression and PAD4 activity were higher in the lung of $gp91^{phox-/y}$ mice post SIRS induction. These results suggest that Nox2 plays a critical role in maintaining homeostasis by regulating platelet activation and NET formation in the lung.

Keywords: neutrophil, NET formation, platelet, Nox2, lung injury

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INTRODUCTION

Despite numerous technological advances in intensive care in recent years, mortality from sepsis and the systemic inflammatory response syndrome (SIRS) remains static. The World Health Organization made sepsis a global health priority in 2017 based on the burden of disease with an incidence of 750,000 cases/year in the USA. Although the mortality rate has decreased substantially, more than 25–30% of patients with sepsis will die from complications of multi-organ injury (1). Among many organ systems at risk during SIRS, acute lung injury and the resultant acute respiratory distress syndrome is a common cause of severe morbidity. Mechanical ventilation and treatment of the inciting stimulus are the most common therapies, but there are no current effective therapies targeting underlying mechanisms (2, 3). Acute respiratory distress syndrome is characterized by acute and diffuse inflammation in the lung with rapid neutrophil infiltration and pulmonary edema. The contribution of neutrophils to the lung injury process is well-recognized with exuberant cell activation and the release of both reactive oxygen species (ROS) and proteolytic granule contents (4).

Early studies in our laboratory suggested a novel anti-inflammatory role for the neutrophil NADPH oxidase 2 (Nox2) in the pathogenesis of acute respiratory distress syndrome as demonstrated by the severe lung pathology that developed in mice lacking Nox2 after a systemic inflammatory stimulus (5). Wild type (WT) and Nox2 deficient ($gp91^{phox-/y}$) mice develop SIRS in response to intraperitoneal injection of zymosan, but only $gp91^{phox-/y}$ mice develop acute and severe lung pathology with inflammation, disseminated thrombosis, and hemorrhage. Margination of neutrophils is seen in the lungs of both WT and $gp91^{phox-/y}$ mice, but only the mice lacking Nox2 have infiltration of neutrophils in the alveolar space (6). A decrease in circulating platelet concentration is seen within 2 h in $gp91^{phox-/y}$ mice with concomitant generation of microthrombi in the lung and hemorrhagic bronchoalveolar (BAL) fluid (7). Considered in combination, these studies suggested that Nox2 derived ROS serve to repress the inflammatory response in the lung.

The notion that Nox2 deficiency could lead to a pro-inflammatory state is certainly not novel. Patients with chronic granulomatous disease lacking Nox2 function display a number of hyperinflammatory phenotypes; *in vitro* analysis of PMN demonstrates upregulation of CD11b and phosphorylation of p38 MAPK at baseline (8). *In vivo,* patients with chronic granulomatous disease experience frequent infections and chronic inflammation suggesting a role for Nox2 in limiting and resolving inflammation. Chronic granulomatous disease is characterized by the presence of granulomas, masses of immune cells at the sites of inflammation and infection. Granulomas have been described in numerous tissues and organs including the lung, bladder, eyes, and gastrointestinal tract (9). In broad terms, neutrophil recruitment to tissues and organs, whether in response to sterile inflammation as in a granuloma or in response to infection, is governed by a coordinated interplay of chemoattractants and adhesion molecules. Ideally, neutrophils are rapidly recruited to a site of infection and then abruptly called off with resolution of the inciting stimulus.

Our laboratory demonstrated upregulation of alveolar macrophage chemokine production in Nox2-deficient mice (7), but these tissue macrophages are not the only source of chemokine production. CXC chemokine ligand 4 (CXCL4) and CXCL7, chemokines released by activated platelets, are strong neutrophil chemoattractants (10). P-selectin glycoprotein 1 (PSGL-1) is expressed on leukocytes and plays an important role in cell adhesion and migration through the endothelium. PSGL-1 binds to the cell adhesion molecule, P-selectin, expressed on activated endothelial cells and activated platelets (11). CXCL4, CXCL7, and P-selectin (12, 13) are stored in the alpha granules of platelets and released or upregulated to the platelet surface upon activation (11, 14). In addition, to functioning as a chemokine, CXCL4 has been demonstrated to induce the formation of neutrophil extracellular traps or NETs (15, 16).

NETs were first described 14 years ago as a novel mechanism of cell death that served an antimicrobial function (17). A much broader role for NETs is now recognized including involvement in the pathophysiology of chronic inflammatory processes ranging from rheumatoid arthritis to gout, suggesting NET formation may contribute to tissue damage (18, 19). NET formation has been observed *in vivo* and in response to numerous stimuli *in vitro*, and the activation pathways leading to NET formation vary by stimulus (18, 20). Although it was initially perceived that NADPH oxidase generated ROS were required for NET formation, it is now recognized that ROS-dependence is stimulus dependent (21). Histone H3 citrullination (H3CIT) by active peptidylarginine deiminase 4 (PAD4) is required for NET formation to some stimuli but not all (18). Among the various stimuli, activated platelets can elicit NET formation in the absence of ROS (18). In addition, there is recent evidence that NETs contribute to the pathogenesis of lung injury and that NET formation in the lung is mediated by platelet-neutrophil interactions (22).

In this study, we extend our previous findings of a protective role for Nox2 in the development of lung injury and provide a better understanding of the underlying mechanisms whereby Nox2 limits and resolves inflammation in the lung. We found that Nox2 limits platelet activation and the upregulation of adhesion molecules. In our model, NET formation occurred only in the lungs of Nox2-deficient mice, suggesting that Nox2 specifically represses NET formation under homeostatic conditions. In the absence of Nox2, multiple cell types lose this homeostatic repression; platelet and macrophages release neutrophil chemoattractants and there is rapid infiltration of activated neutrophils in the setting of activated platelets. These concurrent events allow the formation of NETs that lead to extensive tissue damage. A better understanding of the role of Nox2-derived ROS in limiting and resolving inflammation is

Abbreviations: BAL, Bronchoalveolar Lavage; H3CIT, Citrullinated Histone H3; CXCL, CXC Chemokine Ligand; Nox2, NADPH Oxidase 2; NET, Neutrophil Extracellular Trap; PAD, PeptidylArginine Deiminase; PSGL-1, P-selectin Glycoprotein 1; ROS, Reactive Oxygen Species; SIRS, Systemic Inflammatory Response Syndrome.

critical to developing targeted therapies for the treatment of lung injury secondary to systemic inflammation.

MATERIALS AND METHODS

Materials

Zymosan A from Sigma-Aldrich (St. Louis, MO, USA) was prepared as previously described (7). Ly6G (clone 1A8), CD41 (clone MWReg30), CD45 (clone 30-F11), PSGL-1 (clone 4RA10), and ICAM-1 (clone HA58) antibodies were purchased from BD Biosciences (San Jose, CA, USA). Collagenase D, DNase I, and tetramethylbenzidine were purchased from Sigma-Aldrich. CXCL4 (clone 140910), CXCL7 (clone 159703), and myeloperoxidase (clone 392105) antibodies, streptavidin HRP, and recombinant mouse P-selectin (CD62P Fc chimera recombinant protein) were purchased from R&D Systems (Minneapolis, MN, USA). PAD4 (clone EPR20706) and H3CIT (ab5103) antibodies were purchased from Abcam (Cambridge, MA, USA). Ly6G-biotin antibody, MicroBeads, and columns were purchased from Miltenyi Biotec (San Diego, CA, USA). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other reagents and supplies were purchased from Fisher (Waltham, MA, USA).

Animals

All studies were approved and conducted under the oversight of the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. C57BL/6J (WT) and Nox2-deficient (B6.129S-*Cybbtm1Din*/J) mice ($gp91^{phox-/y}$) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and kept in a barrier facility with free access to standard rodent chow and water. *CYBB* encodes $gp91^{phox}$ and is present on the X chromosome. CGD resulting from a mutation in CYBB is inherited in an X-linked recessive pattern; therefore, only male mice were used. Mice were age matched for all experiments.

Zymosan-Induced Generalized Inflammation

Sterile systemic inflammation was induced using the zymosan-induced generalized inflammation model, in which mice received an intraperitoneal injection of zymosan (0.7 mg/g) as previously described (5). Mice were sacrificed at 2 and 24 h post injection.

Evaluation of Lung Injury

BAL was performed as previously described (7). Following BAL, the pulmonary vasculature was perfused through the right ventricle with 5 ml of phosphate buffered saline (PBS). Lungs were removed, cut into small pieces with scissors, and subjected to digestion in digestion buffer (1 mg/ml collagenase D and 0.1 mg/ml DNase I in PBS) for 30 min at 37°C with continuous shaking. Lungs were mechanically digested using a Miltenyi gentleMACS™ Dissociator from Miltenyi Biotec (San Diego, CA, USA) according to the manufacturer's directions. Homogenized lungs were passed through a 70 μm nylon filter, and the lung homogenate was centrifuged at 500 g for 5 min at 4°C. The resultant supernatant was collected and stored at −80°C. Miltenyi MicroBeads were used to isolate neutrophils from the digested lung according to manufacturer's directions.

ELISA for CXCL4 and CXCL7

96-well NUNC Maxisorp microplates were coated with antibodies against CXCL4 or CXCL7 in carbonate buffer (100 mM $NaHCO_3$, 34 mM Na_2CO_3, pH 9.6) overnight at room temperature. Wells were blocked with PBS containing 1% BSA and 5% sucrose for 1 h. Standards and samples were diluted in assay diluent (PBS with 0.1% BSA), loaded into duplicate wells, and incubated for 2 h at room temperature. Biotinylated antibody against the target was added and allowed to incubate for 2 h followed by streptavidin-HRP for 20 min. Tetramethylbenzidine was added to the wells for 10–30 min for color development and 0.5 M H_2SO_4 was added to stop the reaction. All incubations were performed at room temperature and wells were rinsed three times with wash buffer (PBS with 0.05% Tween 20) between each step. Absorbance at 450 nm was measured on a Clariostar Omega from BMG Labtech (Cary, NC, USA).

Flow Cytometry

Neutrophil-platelet aggregates—Antibodies were added to whole blood and incubated on ice for 1 h. FACS lysis buffer from BD Biosciences (San Jose, CA, USA) was added and cells were mixed on ice for 10 min. Cells were then washed with 3 ml of cold PBS. Secondary antibodies were added where appropriate and incubated for another 30 min on ice. Cells were washed and resuspended in cold PBS and taken immediately to the flow facility for analysis. Cell surface markers—Isolated cells were fixed with 4% paraformaldehyde for 30 min on ice and blocked with PBS containing 4% normal goat serum and 2% non-fat dry milk on ice for 20 min. Cells were incubated with primary antibodies on ice for 1 h followed by washing and a 30 min incubation on ice with secondary antibody as needed. All samples were washed and resuspended in PBS prior to flow analysis. All data acquisition was performed on a BD FACSCalibur from BD Biosciences (San Jose, CA, USA) in the Flow Cytometry Facility at the University of Texas Southwestern Medical Center with ≥10,000 events collected per analysis. Data were analyzed using FlowJo Software, version 10.0.08 from Treestar (Ashland, OR, USA). Ly6G was used to identify neutrophils where appropriate.

Microfluidic Flow Chamber Assays

Microfluidic flow chamber assays were performed to measure differences in the formation of neutrophil-platelet aggregates. For these experiments, the microfluidic flow chambers consisted of polydimethylsiloxane stamps that were permanently sealed to a glass coverslip by pretreatment with an air plasma (23). The microfluidic channels had a cross-sectional dimension of 300 μm wide by 50 μm tall and were fabricated by standard photolithography procedures described previously (23, 24). To measure the frequency of neutrophil-platelet aggregates, the microfluidic chambers were initially filled with Hanks' balanced salt solution and then coated with recombinant mouse P-selectin

by perfusing a 0.2 μg/ml P-selectin solution through the channel at 1,600 s^{-1} for 15 min (25). The microfluidic channels were then blocked by filling the channels with a solution of 0.5% human serum albumin in Hanks' balanced salt solution and incubating for 30 min at room temperature before placing the microfluidic device on a Zeiss AxioVert A1 microscope equipped with a 63× Plan-Apochromat (NA 1.4) objective and a Hamamatsu ORCA-Flash 4.0 scientific CMOS camera. Whole mouse blood anti-coagulated with 10 units/mL of heparin was then perfused through the channels at a shear rate of 100 s^{-1} and the rolling of leukocytes on the P-selectin coated surface was visualized by differential interference contrast microscopy and recorded at 30 frames per second. For each video recording (∼5 s) we quantified the number of rolling leukocytes with and without adherent platelets. For those leukocytes with platelets adhered, we counted the total number of platelets.

Confocal Microscopy

Mouse lungs were harvested 24 h post SIRS induction following BAL and circulatory perfusion as described above. The lung samples were rinsed with PBS immediately and fixed in 10% neutral buffered formalin overnight at 4°C. The fixed lungs were embedded in paraffin. Paraffin blocks were sectioned in 5-μm slices and deparaffinized followed by antigen retrieval with antigen unmasking solution from Vector Laboratories (Burlingame, CA, USA). The sections were blocked with 3% bovine serum albumin and incubated with primary antibodies at 4°C overnight. Sections were washed and incubated in appropriate secondary antibody for 2 h at room temperature followed by mounting. Images were acquired using a Zeiss LSM880 confocal microscope with Plan-Apochromat objectives at room temperature with Zeiss Immersol 518 F halogen free/fluorescence free imaging medium and processed using ZEN software from Carl Zeiss Microscopy (Thornwood, NY, USA). A 10×/0.8 air objective was used to acquire images for quantification of H3CIT staining. 10 fields at 10× magnification per section were evaluated and H3CIT positive cells were counted and expressed as total number per field. A 40×/1.4 oil objective was used for the representative H3CIT images (**Figures 6B–E**), and a 63×/1.4 oil DIC objective was used for the representative PAD4 images (**Figures 8A,B**).

Immunoblotting

Lung tissue was homogenized in protein lysis buffer (20 mM imidazole, 2 mM EGTA, 100 mM NaCl, 1% Triton X-100, 1 mM PMSF, 2% leupeptin/pepstatin A) using a Polytron Immersion Disperser from Kinematica (Bohemia, NY, USA) on medium to high speed for 20 s on ice. Homogenized samples were spun at 15,000 g for 10 min at 4°C to remove particulates. The protein lysate was collected, mixed with sample buffer and heated at 70°C for 10 min to denature proteins. TGX gels from Biorad (Hercules, CA, USA) were used to resolve proteins by SDS-PAGE followed by transfer to a nitrocellulose membrane. Membranes were blocked with 3% bovine serum albumin in tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature followed by overnight incubation in primary antibody at 4°C. Secondary antibodies were conjugated to horse radish peroxidase and detected by chemiluminescence using a ChemiDoc XRS Imager from Biorad. Bands were analyzed using Image Lab software from Biorad.

Peptidylarginine Deiminase 4 Activity

Lung digest supernatant was harvested as described above. The PAD4 Inhibitor Screening Assay Kit (AMC) from Cayman Chemical (Ann Arbor, MI, USA) was used to assess PAD4 activity according to the manufacturer's instructions. Briefly, a fluorescent substrate consisting of an arginine, a carboxybenzyl group, and a fluorophore was incubated with the sample. In the absence of active PAD4, the substrate is unaltered and the fluorophore is released upon addition of the developer. In the presence of active PAD4, the substrate is citrullinated preventing the release of the fluorophore upon addition of the developer. Fluorescence was measured in a Clariostar Omega from BMG Labtech (Cary, NC, USA) with excitation set to 360 ± 15 nm and emission set to 450 ± 20 nm. Fluorescent signal is inversely proportional to PAD4 activity. Sample buffer alone was set to 0% activity and recombinant PAD4 enzyme was set to 100% activity. Sample activity was compared to the activity of the recombinant enzyme. Cl-amidine, an irreversible and cell-permeable pan PAD inhibitor, was used at a final concentration of 100 μM per the manufacturer's instructions. Cl-amidine potency is unstable and loses activity very quickly over time (21). For this reason, we used the inhibitor to validate specificity of the assay and not in a function blocking capacity.

Statistics

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 7 for Windows from GraphPad Software (La Jolla, CA, USA). Results were considered statistically significant with a p-value ≤0.05. Comparisons between groups were performed using one-way ANOVA with multiple comparison. In some cases, direct comparisons between genotypes were made using unpaired Student's t-tests. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

RESULTS

Nox2 Regulates Platelet Activation

In view of the findings of hemorrhagic BAL fluid and extensive thrombosis in the lungs of $gp91^{phox-/y}$ mice, we sought evidence of platelet activation in our model. We measured two platelet-derived chemokines, CXCL4 and CXCL7. We found an increase in both chemokines in the BAL fluid of $gp91^{phox-/y}$ mice at baseline and after induction of SIRS by intraperitoneal zymosan injection (**Figure 1**). Although these neutrophil chemoattractants are increased at baseline, there were minimal/no neutrophils in the BAL fluid of $gp91^{phox-/y}$ mice under unstimulated conditions (7). These data suggest that the presence of CXCL4 and CXCL7 alone is not sufficient to stimulate tissue damage or neutrophil infiltration in the lung. There is a significant decrease in CXCL4 and CXCL7 in the BAL fluid of WT mice 24 h after SIRS induction. CXCL7 but not CXCL4 is

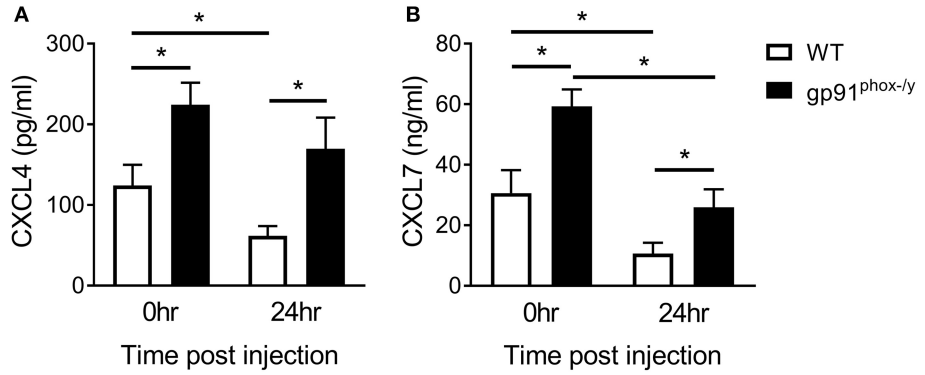

FIGURE 1 | Nox2 regulates platelet activation. Platelet-derived chemokines **(A)** CXCL4 and **(B)** CXCL7 are elevated in the BAL fluid of gp91$^{phox-/y}$ mice at baseline and 24 h after induction of SIRS by peritoneal injection of zymosan, $n \geq 8$ for CXCL4, $n \geq 5$ for CXCL7, $^{*}p < 0.05$.

reduced in the BAL fluid of gp91$^{phox-/y}$ mice 24 h after SIRS induction (**Figure 1**).

Decreased Interaction Between Neutrophils and Platelets in the Peripheral Blood of Mice Lacking Nox2

Based on the suggestion that platelets are activated under resting conditions in the Nox2-deficient state, we assessed interactions between neutrophils and platelets in the peripheral blood post-SIRS using two complementary approaches: flow cytometry and a microfluidic adhesion assay. We observed greater frequency of neutrophil-platelet aggregates in the peripheral blood of WT mice at baseline and at 24 h after the induction of SIRS consistent with sequestration of activated platelets in the lung of gp91$^{phox-/y}$ mice. In addition, neutrophil-platelet aggregates were further decreased in the peripheral blood of gp91$^{phox-/y}$ mice post-SIRS while remaining steady in WT mice (**Figure 2**). Previous results from our lab demonstrate a reduction in the platelet count of gp91$^{phox-/y}$ mice following SIRS (7) consistent with sequestration in the lung and decreased interaction with neutrophils in the peripheral blood of mice lacking Nox2.

Neutrophil Infiltration in the Alveolar Space of Mice Lacking Nox2

Based on previous studies in our lab (5) demonstrating significant immune cell infiltration in the lung of gp91$^{phox-/y}$ mice, we sought to determine if this infiltration was primarily in the lung interstitium or the alveolar space. In the previous study, lung sections were prepared from lungs without prior BAL. For this study, lungs were harvested after BAL and circulatory perfusion to remove cells from the alveolar space and the vasculature. We counted the total number of cells isolated from the lung digest 24 h post-SIRS and found no difference in total cell number or total neutrophil number between the two genotypes, although there was a trend toward increased neutrophils in the gp91$^{phox-/y}$ mice (**Figures 3A,C**). In contrast, the total cell number and the total neutrophil number were significantly greater in the BAL fluid of gp91$^{phox-/y}$ mice 24 h following SIRS induction (**Figures 3B,D**) demonstrating neutrophilic infiltration in the alveolar space.

Neutrophil ICAM-1 Expression Increases as a Result of the Systemic Inflammatory Response

We next sought to better understand the mechanisms driving migration of neutrophils to the alveolar space. Leukocyte-endothelial interactions mediated by leukocyte integrins and endothelial ICAM-1 have been well-described (26). Neutrophils are marginated in the lung of both WT and gp91$^{phox-/y}$ mice within 2 h of SIRS induction, and the neutrophils from gp91$^{phox-/y}$ mice express higher levels of CD11b (7). Although neutrophils are marginated in the lung of both WT and gp91$^{phox-/y}$ mice, there is a rapid and significant infiltration of neutrophils into the alveolar space of gp91$^{phox-/y}$ mice only, within 2 h post zymosan injection, and these cells express higher levels of CD11b (7). In contrast to endothelial ICAM-1, relatively little is known about ICAM-1 expression in neutrophils. ICAM-1 upregulation has been demonstrated in response to lipopolysaccharide both *in vitro* and *in vivo* in murine neutrophils (27) and *in vitro* in human neutrophils (28). In the current study, we measured a significant increase in ICAM-1 expression on neutrophils in the peripheral blood of both WT and gp91$^{phox-/y}$ mice 2 h following SIRS induction (**Figure 4A**). After 24 h of systemic inflammation, neutrophils isolated from the lung of gp91$^{phox-/y}$ mice expressed more ICAM-1 than at baseline (**Figure 4B**). ICAM-1 expression on neutrophils from the blood or the lung was not different between the two genotypes at the time points tested suggesting that ICAM-1 expression does not account for the difference in neutrophil migration.

Neutrophils Expressing High Levels of PSGL-1 Migrate to the Alveolar Space in the Absence of Nox2

Based on the difference in neutrophil-platelet interactions in the peripheral blood, we next sought to measure the neutrophil

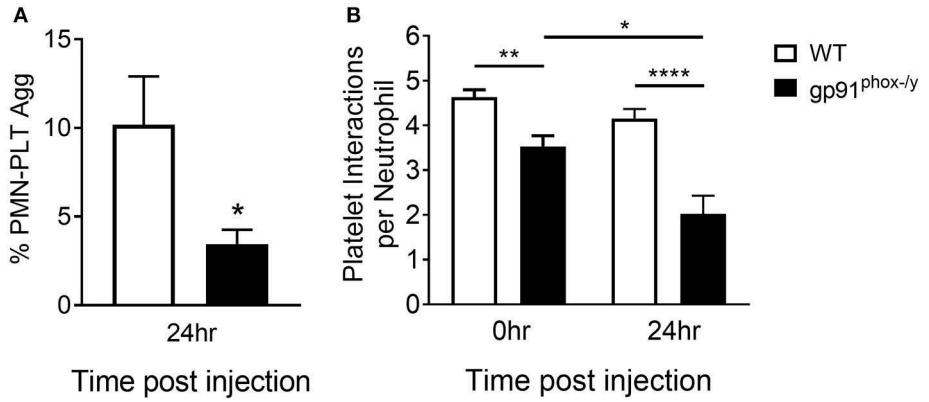

FIGURE 2 | Decreased interaction between neutrophils and platelets in the peripheral blood of mice lacking Nox2. **(A)** A greater percentage of circulating neutrophils in WT mice display platelet markers as demonstrated by dual positivity for CD41 and CD45 by flow cytometry 24 h following induction of SIRS by peritoneal injection of zymosan, $n = 8$, $^{*}p < 0.05$. **(B)** Using a microfluidic adhesion assay, there are more platelets per neutrophil in the peripheral circulation of WT mice both at baseline and after induction of SIRS, and the interaction is unchanged in WT mice. In contrast, neutrophils from gp91$^{phox-/y}$ mice demonstrate fewer neutrophil-platelet interactions at baseline and the number of aggregates decreases further after SIRS induction, $n \geq 47$ cells per condition, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

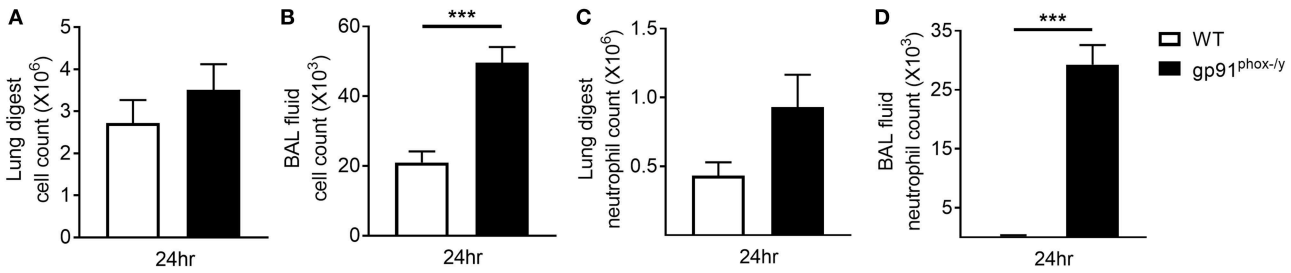

FIGURE 3 | Neutrophilic infiltration in the alveolar space of gp91$^{phox-/y}$ mice following SIRS induction. Total cell count from the lung digest **(A)** and BAL fluid **(B)** of WT ($n = 8$) and gp91$^{phox-/y}$ ($n = 12$) mice 24 h after SIRS induction. Total neutrophil count from the lung digest **(C)** and BAL fluid **(D)** of WT ($n \geq 3$) and gp91$^{phox-/y}$ ($n \geq 5$) mice 24 h after SIRS induction, $^{***}p < 0.001$.

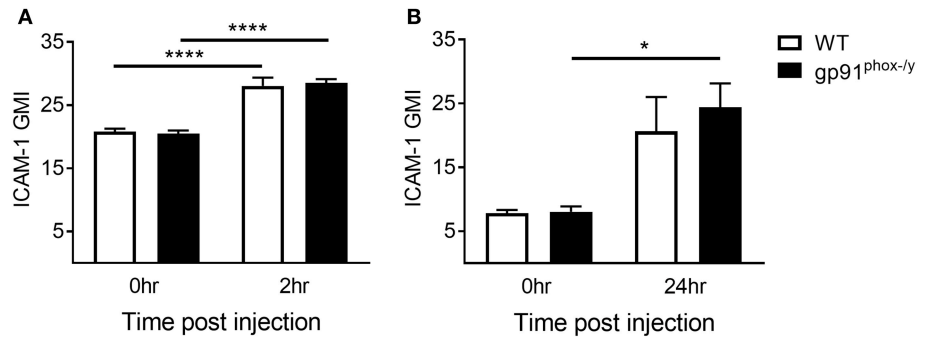

FIGURE 4 | Increase in neutrophil ICAM-1 expression following systemic inflammation. **(A)** Peripheral blood neutrophil ICAM-1 expression at baseline and 2 h following SIRS induction, $n \geq 4$. **(B)** ICAM-1 expression on neutrophils isolated from the lung at baseline and 24 h following SIRS induction, $n \geq 3$, $^{*}p < 0.05$, $^{****}p < 0.0001$.

surface expression of PSGL-1, a receptor of P-selectin on the platelet surface, analyzing circulating PMN, PMN that marginated in the lung, and those that transmigrated to the alveolar space. Although there is no difference in PSGL-1 expression on neutrophils in the peripheral blood of WT and gp91$^{phox-/y}$ mice under resting conditions, there is a rapid decrease in PSGL-1 expression on circulating neutrophils in the gp91$^{phox-/y}$ mice by 2 h post SIRS, with a concomitant increase in PSGL-1 expression on neutrophils in the BAL fluid, suggesting that the "higher" expressing neutrophils have

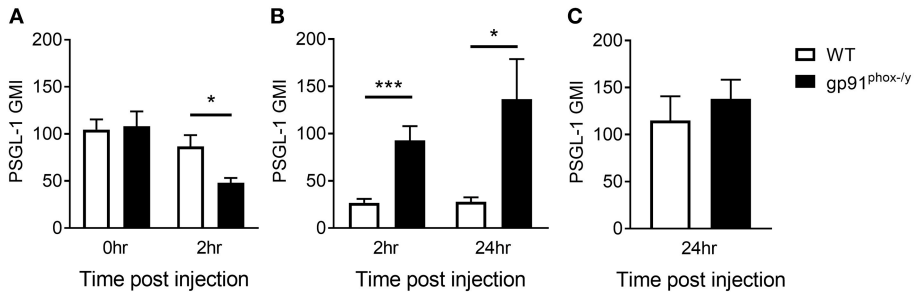

FIGURE 5 | Nox2-deficient neutrophils expressing high levels of PSGL-1 migrate to the alveolar space. **(A)** Peripheral blood neutrophil PSGL-1 expression at baseline and 2 h post zymosan injection, $n \geq 3$. **(B)** Neutrophils from the BAL fluid of gp91$^{phox-/y}$ mice express more PSGL-1 following SIRS induction than neutrophils from WT mice, $n \geq 7$. **(C)** PSGL-1 expression on neutrophils isolated from the lung digest 24 h after SIRS induction, $n \geq 4$, $^{*}p < 0.05$, $^{***}p < 0.001$.

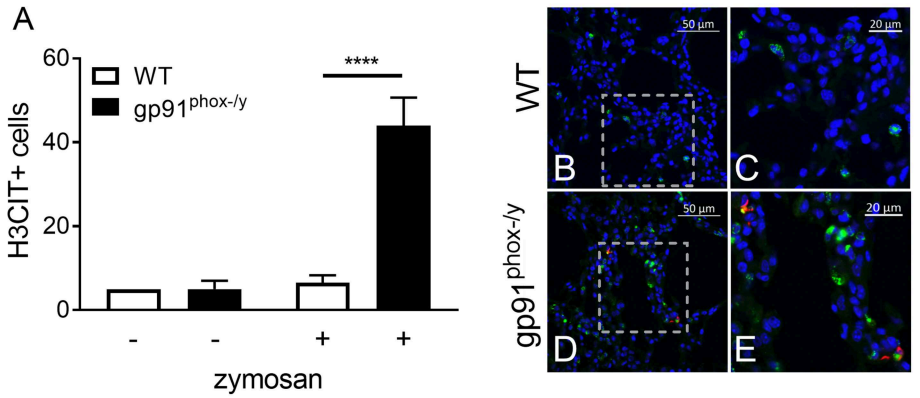

FIGURE 6 | Nox2 represses NET formation in the lung following systemic inflammation. **(A)** Enhanced NET formation is evident as measured by the number of H3CIT positive neutrophils in the lungs of the gp91$^{phox(-/y)}$ mice 24 h after SIRS induction by intraperitoneal injection of zymosan as compared with WT mice or saline injection, $N = 2$ saline injected, and 5–7 zymosan injected, $^{****}p < 0.0001$. **(B–E)** Representative lung sections demonstrating dual staining for myeloperoxidase (green) and H3CIT (red) in WT **(B–C)** and gp91$^{phox(-/y)}$ **(D–E)** mice 24 h after SIRS induction. DAPI was used to identify the nucleus.

migrated into the alveolar space (**Figures 5A,B**). At 24 h post SIRS induction, neutrophils in the BAL fluid of gp91$^{phox-/y}$ mice express more PSGL-1 (**Figure 5B**). There is no difference in PSGL-1 expression on neutrophils isolated from the lung at 24 h (**Figure 5C**). PSGL-1 on neutrophils binds to P-selectin on activated endothelial cells and platelets resulting in adhesion and migration of cells into inflamed tissues. Considered in combination, these data suggest an environment in the alveolar space of gp91$^{phox-/y}$ mice with highly activated platelets and neutrophils creating optimal conditions for NET formation and tissue damage.

Nox2 Regulates NET Formation in the Lung

Given the severe lung pathology, neutrophil-platelet crosstalk, and the presence of CXCL4 and CXCL7, chemokines implicated in NET formation, in the alveolar space of gp91$^{phox-/y}$ mice, we investigated lung sections for evidence of NETs. NET formation is characterized by decondensation of chromatin followed by protrusion of nuclear contents outside the cell. Primary granule contents including elastase and myeloperoxidase are commonly found associated with the DNA web (18). NET formation is a regulated inflammatory process resulting in tissue damage and blood vessel occlusion in extreme cases (18, 20, 29, 30). We observed a striking increase in the number of myeloperoxidase and H3CIT dual positive cells in the lung of gp91$^{phox-/y}$ mice 24 h after SIRS induction (**Figure 6**). H3CIT positive cells were seen in both the interstitium and blood vessels. In addition, H3CIT was increased in whole lung lysate of gp91$^{phox-/y}$ mice 24 h after SIRS induction as compared with saline injected mice and WT mice that were injected with zymosan. Although there is a trend toward increased H3CIT in the lung lysate of WT mice 24 h after SIRS induction, it is not significantly different than H3CIT in the lung lysate of saline injected mice (**Figure 7**). The increase in NET formation and H3CIT in the lung of gp91$^{phox-/y}$ mice is not due to an overall increase in neutrophils. Although there is a trend toward increased neutrophils in the lung of gp91$^{phox-/y}$ mice 24 h following SIRS induction, it does not reach the level of significance (**Figure 3C**). Based on the increase in H3CIT in the lung of gp91$^{phox-/y}$ mice, as well as numerous studies demonstrating a requirement for PAD4 in the

citrullination of H3 (31), we studied the activity of PAD4 in our model system.

Nox2 Regulates PAD4 Activity in the Lung

PAD4 activity has been mechanistically implicated in NET formation in response to multiple stimuli including ROS-dependent and ROS-independent mechanisms (20, 30–32). In our mouse model of systemic inflammation, PAD4 was observed in a nuclear distribution in neutrophils present in the lung of gp91$^{phox-/y}$ mice 24 h following SIRS induction, as expected (**Figures 8A,B**). Consistent with greater citrullination of H3 in the lung of gp91$^{phox-/y}$ mice following induction of SIRS, PAD4 activity was greater in the lung of mice lacking Nox2 following SIRS induction, and PAD4 activity in the lung of mice lacking Nox2 was significantly higher at 24 h than at baseline (**Figure 8C**). There was no difference between the genotypes in PAD4 activity at baseline.

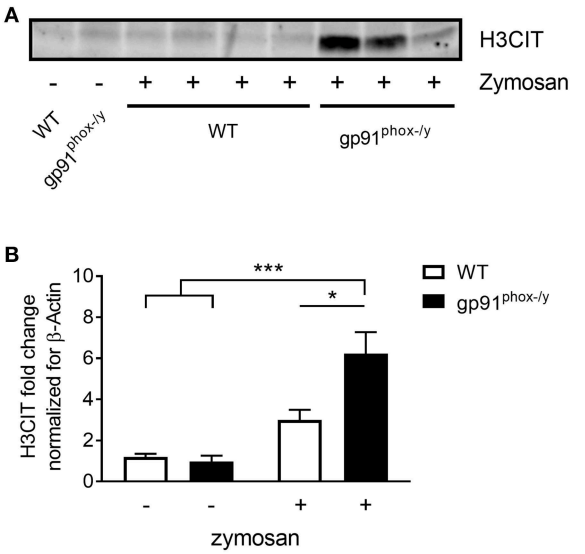

FIGURE 7 | Increased citrullinated H3 in the lung of gp91$^{phox-/y}$ mice following SIRS induction. **(A)** Representative immunoblot showing H3CIT protein from the lung of WT and gp91$^{phox-/y}$ mice 24 h after saline injection or SIRS induction by intraperitoneal zymosan. **(B)** Quantitation of H3CIT in the lung showing an increase in expression in the lung of gp91$^{phox-/y}$ mice, $n = 5$ saline injected and 7 zymosan injected, $^{*}p < 0.05$, $^{***}p < 0.001$.

DISCUSSION

Advances in modern intensive care technology have decreased mortality during the acute inflammatory phase of numerous illnesses. Although most patients now survive the initial insult, a significant subset develop life-threatening inflammatory multi-organ failure, and there are no directed therapies to treat these organ-specific dysfunctions. A better understanding of the underlying mechanisms that govern organ-specific inflammation will help define targeted therapies for this vulnerable population of patients. In previously published work, we demonstrated a critical protective role for the NADPH oxidase 2 in the development of systemic inflammation and multi-organ injury in the setting of sterile SIRS (5, 6). In the current study, we sought to unravel the specific mechanisms by which Nox2 might specifically protect the lung, and to characterize the events that result in neutrophilic infiltration in the alveolar space, resulting in severe thrombosis, hemorrhage, and mortality.

The lung has been recognized as a unique organ in terms of the signaling mechanisms governing neutrophil migration and activation (33–35). We recently demonstrated that Nox2 in alveolar macrophages plays a homeostatic role in repressing basal chemokine secretion into the alveolar space (7). Here, we significantly expand current understanding of the protective role of Nox2 in lung homeostasis with three novel findings. First, we demonstrate that Nox2-derived ROS participate in the repression of platelet activation in the lung under resting and stimulated conditions. This role likely alters neutrophil-platelet interactions and has downstream impact on neutrophil-mediated tissue damage. Second, neutrophilic inflammatory injury to the lung is characterized by widespread alveolar NET formation in the absence of Nox2. This display of NET formation in the Nox2-deficient state is critically important to the way we think about oxidants. Finally, we demonstrate enhanced PAD4 activity in the lung of Nox2 deficient mice under conditions that promote NET generation.

The initial report of NETs, and what was initially termed NETosis, described an extrusion of nuclear contents decorated with primary granule proteases (17). The early studies of NETs characterized NETosis as a unique mechanism of cell death with a role in bacterial killing and a requirement for Nox2-derived ROS (36). Numerous laboratories extended these findings for Nox2-dependent NETs, although in many cases the phorbyl ester, PMA, was utilized as the primary stimulus for NET generation. There is a growing list of stimuli that induce NET formation, and a broad range of intracellular signaling pathways involved in these processes. Moreover, it is now recognized that NETs have a role extending beyond host defense, with NET mediated tissue damage playing a role in chronic inflammatory diseases and a growing understanding of involvement in autoimmunity (18, 37). Relevant to our work, it is clear that not all NET formation is Nox2-dependent, and there may, in fact, be completely unique signaling pathways governing these distinctive forms of NET generation (20, 30, 32, 38). To our knowledge, our report provides the first description of Nox2-mediated repression of NET formation under inflammatory conditions.

Whereas, much of the published literature surrounding NET formation utilizes reductionist *in vitro* analyses, we sought to understand the mechanisms of lung injury in an animal model. In our *in vivo* model of systemic inflammation, there are likely diverse and interdependent cell signaling pathways involved. The importance of integrin-mediated signaling via CD11b has been demonstrated in sterile inflammation (39). Our studies have demonstrated upregulation of CD11b on the neutrophil surface in the absence of Nox2 function *in vitro* and *in vivo* (7, 8), specifically in neutrophils reaching the alveolar space in mice. In the current study, we extend our observations, by focusing

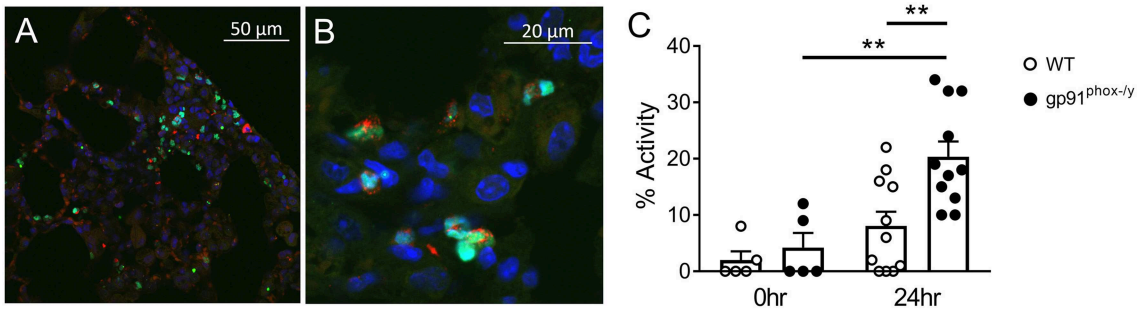

FIGURE 8 | Nox2 represses PAD4 activity in the lung following systemic inflammation. **(A,B)** Lung sections from a gp91$^{phox-/y}$ mouse 24 h following injection of intraperitoneal zymosan demonstrate nuclear localization of PAD4 (green), co-localizing with DAPI (blue) and granular localization of myeloperoxidase (red) staining. **(C)** PAD4 activity in the supernatant recovered from whole lung digest of mice at baseline ($n = 5$) and 24 h ($n = 11$) following injection of zymosan, $^{**}p < 0.05$.

on neutrophil-platelet interactions. The finding of enhanced levels of platelet chemokines in the alveolar space of Nox2-deficient mice under unstimulated conditions suggests that Nox2 has an active role in repressing platelet activation. Importantly, platelet-derived CXCL4 has been demonstrated to be critically involved in the pathogenesis of acute lung injury from both viral and bacterial etiologies (40–42). CXCL4 is involved in platelet aggregation and plays a role in coagulation. CXCL7 exists in monomer, dimer, and tetramer form. The monomer form is a potent neutrophil agonist, and the dimer form has been implicated in neutrophil-platelet crosstalk (43). The expression of these platelet chemokines, and also alveolar macrophage derived chemokines (7) is not sufficient, in our model system, to elicit neutrophil migration under resting conditions. However, once systemic inflammation is initiated, additional changes in the neutrophil activation state become apparent allowing the progression of lung injury. PSGL-1 is an adhesion molecule expressed on neutrophils that binds to P-selectin expressed on activated endothelial cells and platelets. This binding of PSGL-1 to P-selectin facilitates neutrophil adhesion and has been implicated in NET formation (14–16, 30, 44). In our model, while there are no differences in PSGL-1 expression on neutrophils in healthy mice, there is significant upregulation on neutrophils that have migrated to the alveolar space of gp91$^{phox-/y}$ mice. We postulate that the presence of macrophage-derived cytokines (7), activated platelets, NET-inducing chemokines, and the upregulation of adhesion molecules combine to create an environment favorable to NET formation.

The notion that platelets are directly involved in NET formation is not novel (14). In murine models of both ventilator-induced lung injury and transfusion-related lung injury activated platelets and chemokine release are implicated in the development of NETs (15, 16, 29, 39). Human *in vitro* studies also support direct involvement of platelets in the activation of NET formation (45). Microparticles from activated platelets express high-mobility group box 1 protein, a damage-associated molecular pattern, and promote vasculopathy and NET formation in patients with systemic sclerosis and in a murine model (46). Moreover, platelet-mediated NET formation has already been demonstrated to be Nox2-independent (47). The specific signaling pathways allowing platelet activation to promote NET formation have not been clearly elucidated and likely involve the interplay of numerous interrelated pathways. Our *in vivo* model of SIRS advances our understanding of NET formation in the setting of systemic inflammation and provides evidence for Nox2-mediated repression of NET formation.

Although there are vast arrays of signaling intermediates implicated upstream of NET formation induced by different stimuli, a growing literature supports a critical role for PAD4 as a key mediator of chromatin decondensation. PAD4 is one of five known PADs and the only one with a nuclear localization sequence, and is expressed primarily in granulocytes, whereas the other PADs have broader expression. PADs catalyze a post-translational modification resulting in the de-imination or citrullination of arginine (48). Histone citrullination appears to be the essential step directly upstream of the extrusion of nuclear contents, but the signals leading to PAD4 activation are not fully understood. Using murine knockout models, neutrophils lacking PAD4 cannot generate NETs *in vitro* (49) or *in vivo* (50), and display increased susceptibility to certain bacterial infections (48). As further demonstration of the physiologic relevance of these observations, a PAD4 inhibitor, Cl-amidine, provided protection in a lethal model of polymicrobial sepsis in mice (51). *In vitro,* a highly selective PAD4 inhibitor, GSK 484, inhibits citrullination in human and murine neutrophils and blocks NET formation in response to ionomycin and bacterial stimuli (31). These recent studies have attracted greater attention based on the potential for these inhibitors to be used therapeutically in inflammatory disease. There are several lines of evidence suggesting that dysregulation of PAD4 activity plays a role in the pathogenesis of rheumatoid arthritis in both murine models of arthritis and human neutrophil studies. Moreover, several single nucleotide polymorphisms in PAD4 have been associated with susceptibility to rheumatoid arthritis (52). Our data suggest that off target inhibition of Nox2-derived ROS by broad spectrum anti-oxidant therapies might increase PAD4 activity thus enhancing inflammation.

In summary, the current study significantly advances our understanding of the critical role of Nox2-derived ROS in the maintenance of immune homeostasis in the lung and in the

resolution of inflammation following a systemic inflammatory insult. The lung is a vital organ where immune cells come in very close contact with one another and the external environment. There is the potential for the introduction of inflammatory stimuli on a routine basis. In this study, we demonstrate a role for Nox2 in the repression of basal platelet chemokine secretion, in the suppression of neutrophil adhesion molecule upregulation, and in the regulation of a key neutrophil enzyme known to participate in the formation of NETs. The absence of Nox2 alone is insufficient to induce tissue injury. However, the absence of Nox2 creates an environment of low level inflammation that predisposes vulnerable organs to severe injury in the setting of systemic inflammation. In order to improve outcomes for patients who experience significant inflammatory organ injury, it is critical to consider the multifaceted role of ROS in the inflammatory response.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

All studies were approved and conducted under the oversight of the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

AUTHOR CONTRIBUTIONS

JH, MC, RP, and NA planned and conducted experiments. MC performed all microscopy and image analysis. JH, RP, NA, DS, and JM analyzed data and contributed to the study design. JH, MC, and NA prepared figures for the manuscript. JH and JM wrote the manuscript.

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Regulatory Interactions Between Neutrophils, Tumor Cells and T Cells

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Apart from their activity in combating infections, neutrophils play an important role in regulating the tumor microenvironment. Neutrophils can directly kill (antibody-coated) cancer cells, and support other immune anti-tumoral strategies. On the other hand, neutrophils can also exert pro-tumorigenic activities *via* the production of factors which promote cancer growth, angiogenesis and metastasis formation. The balance of anti- and pro-cancer activity is influenced by the particularly delicate interplay that exists between neutrophils and T lymphocytes. In murine models, it has been reported that γδ T cells are a major source of IL-17 that drives the recruitment and pro-tumorigenic differentiation of neutrophils. This, however, contrasts with the well-studied anti-tumor activity of γδ T cells in experimental models and the anti-tumor activity of human γδ T cells. In this article, we first review the reciprocal interactions between neutrophils, tumor cells and T lymphocytes with a special focus on their interplay with γδ T cells, followed by the presentation of our own recent results. We have previously shown that zoledronic acid (ZOL)-activated neutrophils inhibit γδ T-cell proliferation due to the production of reactive oxygen species, arginase-1 and serine proteases. We now demonstrate that killing of ductal pancreatic adenocarcinoma (PDAC) cells by freshly isolated resting human γδ T cells was reduced in the presence of neutrophils and even more pronounced so after activation of neutrophils with ZOL. In contrast, direct T-cell receptor-dependent activation by γδ T cell-specific pyrophosphate antigens or by bispecific antibodies enhanced the cytotoxic activity and cytokine/granzyme B production of resting human γδ T cells, thereby overriding the suppression by ZOL-activated neutrophils. Additionally, the coculture of purified neutrophils with autologous short-term expanded γδ T cells enhanced rather than inhibited γδ T-cell cytotoxicity against PDAC cells. Purified neutrophils alone also exerted a small but reproducible lysis of PDAC cells which was further enhanced in the presence of γδ T cells. The latter set-up was associated with improved granzyme B and IFN-γ release which was further increased in the presence of ZOL. Our present results demonstrate that the presence of neutrophils can enhance the killing capacity of activated γδ T cells. We discuss these results in the broader context of regulatory interactions between neutrophils and T lymphocytes.

Keywords: bispecific antibody, cytotoxicity, gamma/delta T cells, neutrophils, pancreatic ductal adenocarcinoma, T lymphocytes, tumor microenvironment, zoledronic acid

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INTRODUCTION

Polymorphonuclear neutrophils are bone marrow-derived white blood cells which account for 50 to 70% of leukocytes circulating in the peripheral blood. They are highly mobile and are generally considered to be short-lived but can increase their longevity upon activation during infection (1). Despite their characteristic morphology, neutrophils display an enormous functional plasticity which correlates to some extent with the expression of cell surface markers and the production of cytokines, chemokines, antimicrobial peptides (AMP) and bioactive molecules like serine proteases, arginase-1 and reactive oxygen species (ROS) (2). Neutrophils constitute perhaps the most important cellular component of innate immunity, playing an indispensable role in the immune defense against microbes like bacteria and fungi (3). They attack microbes through phagocytosis followed by degranulation or through the release of noxious substances, including granule-derived compounds like antimicrobial peptides, reactive oxygen species (ROS), and nitric oxide species (NOS), in addition to the extrusion of extracellular fibrillary networks termed neutrophil extracellular traps (NETs) (4). NETs are composed of nuclear material like DNA and histones and are decorated by proteins from neutrophil granula. Trapping of microorganisms and subsequent exposure to granule-derived proteins leads to their disposal, a process termed NETosis (4). Beyond their immediate role in innate immunity, it has become increasingly clear that neutrophils can also directly interact with other cells of the innate (e.g., Natural Killer [NK] cells and dendritic cells [DCs]) and adaptive (B cells, T cells) arms of the immune system. As such, neutrophils have been shown to produce, in a context dependent manner, a plethora of cytokines and chemokines (2, 5). Similar to other immune cells, the local micromilieu shapes the functional differentiation pathways of neutrophils. Thus, they can acquire a pro-inflammatory (type 1) phenotype associated with the production of cytokines such as IL-1, IL-6, TNF-α, or IL-17, or an anti-inflammatory (type 2) phenotype associated with production of cytokines like IL-1Rα (receptor agonist) and TGF-β (2, 4). It should be emphasized, however, that there is some controversy with respect to the reported expression/production of some factors (like IL-17) by human neutrophils. Important technical issues have to be taken into account (2, 6).

Neutrophil Interactions With Tumor Cells

Neutrophils play important roles in cancer biology which may include both pro-tumorigenic and anti-tumorigenic activities, depending on the tumor type, the cellular microenvironment, and the constellation of immune modulating factors present. These multifaceted aspects have been summarized in several excellent recent review articles (7–10). Interestingly, increased numbers of neutrophils are frequently present in the peripheral blood of patients with various cancer types, correlating with less favorable prognosis (11). The phenotypic diversity and plasticity of circulating neutrophil subpopulations in cancer patients is reflected by physical properties such as density. Sagiv and coworkers identified three populations of neutrophils in the blood of cancer patients, consisting of low density large immature granulocyte-like myeloid-derived suppressor cells (G-MDSC) and mature neutrophils which exert pro-tumorigenic activity, and high density small mature neutrophils with anti-tumor activity (12). Conventional Ficoll-Hypaque density gradient centrifugation separates these neutrophil subsets as low density pro-tumorigenic neutrophils remain on top of the gradient together with monocytes and lymphocytes (i.e., the mononuclear fraction) while high density neutrophils with anti-tumor properties sediment to the bottom together with red blood cells (13). How can neutrophils mediate anti-tumor activity? It was noted already in the early 1980's that neutrophils can kill various tumor cell lines upon extended *in vitro* co-culture with tumor cells (14). More recently, it was observed that neutrophils from certain healthy donors were capable of killing several established human tumor cell lines but not primary epithelial cells; whereas neutrophils from lung cancer patients were much less active (15). Further analysis revealed that the activation of signaling pathways including PI3 kinase and p38 kinase increased the sensitivity of the selected tumor cells to neutrophil killing. In this study, cytotoxicity was determined by the Real-Time Cell Analyzer (RTCA) system which measures the decrease of impedance over time when adherent target cells detach from the bottom of culture wells as a consequence of lysis. Attempts to identify the mechanism of neutrophil killing of tumor cells in these studies pointed to a role of hydrogen peroxide (H_2O_2) since catalase significantly reduced the extent of tumor cell lysis (15). Recently, it was discovered that H_2O_2 secreted by neutrophils induces a lethal influx of Ca^{2+} in tumor cells which is mediated by the transient receptor potential cation channel, subfamily M, member 2 (TRPM2), a ubiquitously expressed H_2O_2-dependent Ca^{2+}-permeable channel that is frequently upregulated in cancer (16). Interestingly, the expression of TRPM2 (and thus the sensitivity to neutrophil killing) is up-regulated during the epithelial-to-mesenchymal transition (EMT), rendering mesenchymal cells more susceptible to neutrophil cytotoxicity, while cells expressing lower levels of TRPM2, as observed during mesenchymal-to-epithelial transition (MET), are protected from neutrophil killing (17). In addition to the H_2O_2-dependent "spontaneous" cytotoxicity, neutrophils are potent mediators of Fc receptor-dependent antibody-dependent cellular cytotoxicity (ADCC) against antibody-opsonized tumor cells [discussed in (7)]. The antibody isotype plays an important role in triggering efficient ADCC. It appears that IgA antibodies targeting the FcαRI (CD89) expressed on neutrophils are most effective in this respect (9, 18). The mechanism of how neutrophils actually execute ADCC has been recently identified as trogoptosis; a process which involves intimate CD11b/CD18-dependent conjugate formation facilitating neutrophil antibody-opsonization leading to necrotic tumor cell death (19).

As briefly discussed, subsets of neutrophils can exert anti-tumor activity. However, a large body of evidence indicates that neutrophils actually promote tumorigenesis and metastasis formation through a plethora of mechanisms (6). This is supported by studies showing that the presence of tumor-associated neutrophils (TANs) correlates with a poor prognosis in different cancers (9, 10, 20–22), although this is not a generally

valid observation (7, 23). An important aspect to consider when dissecting pro- vs. anti-tumorigenic neutrophilic functions is that results obtained from well-defined murine model systems may not always reflect the same role of neutrophils in corresponding human cancer diseases (24). Like macrophages, neutrophils can be categorized into type 1 and type 2 subsets. Type 1 neutrophils (N1) are pro-inflammatory and produce, amongst other factors, IL-12 and CCL3; whereas, N2 neutrophils are immunosuppressive and produce IL-10, CCL2 and high amounts of arginase (2). In the context of the tumor microenvironment, neutrophils are recruited and polarized into tumor-promoting N2 cells by tumor-derived factors, of which TGF-β has a major role (25). N2-polarized TANs possess a broad arsenal of effector mechanisms to support cancer growth, tumor metastasis and angiogenesis. These include (but are not limited to): the production of elastase, arginase-1, prostaglandin E_2; the formation of NETs; and the secretion of pro-angiogenetic factors, like matrix metalloprotease 9 (MMP9) and VEGF (6, 9). There is also a role of neutrophils in promoting metastasis formation and tumor progression outside the primary tumor. A recent study reported that ovarian tumor-derived factors stimulated the neutrophil influx into the omentum and the local protrusion of NETs which were found to bind to ovarian cancer cells and thereby to promote metastasis to the omentum. NET formation is known to depend on peptidyl arginine deiminase 4 (PAD4) (26, 27). In their experimental model, Lee et al found that reduced NET formation, as observed in PAD4-deficient mice or following pharmacological inhibition of PAD4, was associated with reduced omental metastasis (28). Szczerba and coworkers recently described another pathway in which circulating neutrophils might contribute to metastasis formation (29). It was found that neutrophils in the peripheral blood can associate with rare circulating tumor cells. In comparison to the transcriptome of unassociated tumor cells, the tumor cells associated with neutrophils had up-regulated the expression of genes involved in cell cycle progression which lead to more efficient metastasis formation (29). Together, these new data broaden our understanding on the role of neutrophils in tumor metastasis formation and may develop new avenues for therapeutic intervention.

Neutrophil Interactions With T Cells

T cells require two signals for activation, i.e., antigen recognition *via* the T-cell receptor (TCR) and a co-stimulatory signal typically provided by interaction between CD28 on T cells with corresponding B7 family members (CD80, CD86) on antigen-presenting cells (APC) (30). However, there are other co-stimulatory pathways such as ICOS/ICOS-ligand or CD40/CD40-ligand (CD154) which similarly play important co-stimulatory roles. The most potent APC are dendritic cells (DCs) which express high levels of MHC class II molecules (in addition to MHC class I), CD80 and CD86, and offer ample contact areas to T cells through their conspicuous dendrites. DCs process and present endogenous (e.g., viral) antigens to CD8 T cells *via* the MHC class I presentation pathway, but they also take up exogenous (e.g., bacterial) antigens and present those to CD4 T cells *via* the MHC class II presenting machinery. Notably, DCs can also "cross-present" exogenous antigens and bring them into the MHC class I pathway for presentation to CD8 T cells (31). While activated human T cells upregulate MHC class II molecules, and thereby can bind peptides and bacterial superantigens for presentation to other T cells, antigen processing and specifically cross-presentation is the domain of professional APCs, particularly DCs. T cell activation needs to be tightly controlled. To this end, T cells upregulate inhibitory receptors like CTLA-4 and PD-1, which upon interaction with their ligands CD80/86 and PD-1 ligand (PD-L1), respectively, deliver negative signals resulting in T cell growth arrest and exhaustion (32). Given that tumors as well as immunosuppressive cells in their microenvironment, like MDSC, frequently upregulate PD-L1 as a strategy to dampen efficient T-cell responses, the introduction of antibodies interfering with such pathways ("checkpoint inhibitors") has been a major breakthrough in the treatment of certain cancers (33, 34). The use of checkpoint inhibitors may also interfere with the cancer-associated fibroblast (CAF)-induced PDL-1 expression on neutrophils, which functions to impair T cell-responses against hepatocellular carcinomas (35). In addition to the negative impact of PD-1 and CTLA-4 signaling, T cell activation is controlled by regulatory circuits involving FoxP3-positive regulatory T cells (Treg) and anti-inflammatory M2 macrophages, which are recruited by pro-tumorgenic neutrophils, and contribute to further suppressing cytotoxic T cell function (10). Multiple molecules have been implicated in Treg-mediated suppression, including CTLA-4, LAG3, TIGIT, IL-10, and ectoenzymes CD39 and CD73 expressed on Treg (36). Cells of the monocyte-macrophage lineage on the other hand are characterized by enormous plasticity. Pro-inflammatory M1 macrophages are induced under conditions of IFN-γ and TLR signaling, whereas IL-4 and IL-13 signaling skews polarization *via* STAT6 toward M2 macrophages (37). MDSC are yet another differentiation status of regulatory/suppressive myeloid cells. They comprise a heterogeneous group of cells where at least two groups (monocytic MDSC, granulocytic MDSC) can be differentiated on the basis of morphology and functional properties (38, 39). Overall, MDSC present in the tumor micromilieu contribute significantly to the immune escape in certain types of cancer by preventing efficient activation of tumor-infiltrating T cells. MDSC can inhibit IFN-γ production by T cells and degranulation of phosphoantigen-activated Vδ2 T cells (40, 41). Treatment of pancreatic ductal adenocarcinoma cells (PDAC)-patients with gemcitabine, the standard therapy for PDAC, can inhibit MDSC, while enhancing cross-presentation of tumor-associated antigens by DC (42). There is still a need to more fully characterize the influence of gemcitabine on the interaction of MDSC and γδ T cells. Treatment with other chemotherapeutic agents in combination with n-BP has shown to increase γδ T-cell cytotoxicity against tumor cells (43, 44).

In addition to MDSC, mesenchymal stromal cells (MSC) can enhance MDSC-mediated immunosuppression by inhibiting T-cell proliferation and IFN-γ production (45). MSCs have a broad functional repertoire and are crucial for tissue regeneration and homeostasis. As such, these stem cells are considered to hold significant therapeutic potential to reverse tissue damage in

conditions with unrestrained neutrophil activation (46). Given their anti-inflammatory properties, MSCs are being investigated as a means to treat autoimmune diseases, graft vs. host disease (GvHD) and allograft rejection following transplantation (47). The property of MSCs to inhibit T cell proliferation is thought to act as a double-edged sword in the context of malignancy. One of the mechanisms driving this inhibition is the cytoplasmic tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), that is produced by human MSCs in response to inflammation and acts to deplete the essential amino acid tryptophan in the local environment, which results in the inhibition of the growth and survival of T cells (48–50). IDO is also produced by MDSCs and is considered as an important checkpoint molecule as it functions to enable cancer cells to subvert immune targeting (50, 51). Other immune suppressive mechanisms of MSCs involve their ability to program neutrophils into an immunosuppressive and tumor-promoting phenotype. $CD11b^+$ $Ly6G^+$ neutrophils isolated from bone marrow of normal mice or spleen of tumor-bearing mice inhibited T cell proliferation *in vitro* after coculture with TNF-α-primed MSC with and enhanced 4T1 tumor progression *in vivo*. These TNF-α-primed MSC conditioned neutrophils had upregulated arginase activity and the expression of iNOS, saa3, some cytokines and chemokines and their receptors. iNOS inhibition attenuated some of the suppressive effect of TNF-α-primed MSC pre-cocultured neutrophils on T cell proliferation (52).

How do neutrophils modulate T cell activation? Although classically considered as effector cells of innate immunity, it is obvious that neutrophils can exert both positive and negative effects on T cell activation. Under inflammatory conditions (e.g., *in vitro* culture with GM-CSF, IFN-γ, TNF-α, or *ex vivo* in patients with inflammatory diseases), neutrophils can acquire DC-like properties with upregulation of MHC class II and costimulatory molecules such as CD86 and CD83, thus being able to present antigen to T lymphocytes (53, 54). Moreover, neutrophils can support T-cell responses by secreting chemokines that are important for the recruitment of DCs or T cells, for example in the context of infection or contact hypersensitivity (55, 56). Depending on the specific cellular environment, neutrophils can thus positively modulate adaptive T-cell responses. It is clear, however, that neutrophils are armed with various strategies to effectively inhibit T-cell activation as well (9). Through production of ROS, suppressive granulocytic MDSC inhibit T-cell activation at the level of reduction of TCRζ expression, inhibition of NF-κB activation, as well as induction of apoptosis. The degranulation of serine proteases, such as elastase, proteinase-3, cathepsin-G, by primary granules inhibits T-cell activation by the inactivation of cytokines and their receptors. In addition, ariginase-1 released by tertiary granules cleaves the amino acid arginine, which is essential for T-cell activation. Furthermore, granulocytic MDSC also deplete the cellular environment of cystine through the X_C^- transporter. In consequence, APC cannot reduce cystine into cysteine which is required for T-cell activation. Last but not least, neutrophils can upregulate PD-L1 and thereby deliver a negative signal to T cells *via* the PD-1 receptor (9, 35, 57). Approaches to investigate the modulation of T-cell activation *in vitro* using freshly isolated neutrophils showed that activated neutrophils can inhibit the polyclonal T-cell activation by CD3/CD28 antibodies, which was partially reversed by the ROS inhibitor catalase but not by NOS or myeloperoxidase (MPO) inhibitors. Suppression of T-cell activation by activated neutrophils was accompanied by significant, ROS-induced cell death (58). Other strategies which neutrophils use to inhibit T-cell activation include the production of suppressive cytokines, such as IL-10 and the upregulation of PD-L1. In a murine model of infection using *Mycobacterium bovis*, Doz et al. observed that neutrophils recruited by infected DCs produced a large amount of IL-10. It was further demonstrated in an OVA TCR transgenic model that IL-10 producing neutrophils specifically suppressed IL-17 but not IFN-γ production in OVA-specific T cells (59). In a different system, it was found that LPS-stimulated Treg induced IL-10 production in neutrophils in a cell contact-dependent manner. It was shown that LPS-activated Treg (but also exogenous IL-10) promoted specific histone modifications that activated the IL-10 genomic locus in neutrophils (60). Upregulation of PD-L1 on neutrophils as a means of T-cell suppression has been identified in various systems (9, 35). As an example, increased expression of PD-L1 was observed on neutrophils in the peripheral blood of patients infected with *Burkholderia pseudomallei*, and it was found that such neutrophils inhibited polyclonal T-cell activation in a PD-1/PD-L1 dependent manner (61). Furthermore, increased neutrophil expression of PD-L1 is also found in HIV-1 infected patients, again correlating with a PD-1/PD-L1 dependent inhibition of T-cell activation. In this study, IFN-α, the TLR7/8 agonist Resiquimod, and HIV-1 virions were identified as potent inducers of PD-L1 expression on neutrophils (62). Indeed, immune activation using TLR ligands or microbial products may be an effective therapeutic strategy to overcome cancer-associated immune suppression and increase the efficacy of anti-cancer cytotoxic T cell activity (63, 64).

Neutrophil Interactions With γδ T Cells

γδ T cells comprise a small subset of CD3-positive T cells in the peripheral blood but account for a major population of intraepithelial lymphocytes in mucosal tissue such as the small intestine. The dominant population of γδ T cells in human peripheral blood expresses a TCR composed of the Vγ9 chain paired with Vδ2. With substantial interindividual variability, such Vγ9Vδ2 T cells (termed Vδ2 in the following sections) make up anywhere between 50 and 95% of peripheral blood γδ T cells in adult healthy donors (65). The TCR repertoire of intestinal γδ T cells is different; non-Vδ2 (i.e., Vδ1 or Vδ3) T cells co-expressing any of the available Vγ elements are predominant (66). γδ T cells play a major role in (local) immune surveillance as they sense stressed and transformed cells by their TCR and additional activating receptors like NKG2D (66, 67). In line with this, γδ T cells are potent cytotoxic cells and are known to kill a broad range of tumor cells in a MHC non-restricted but TCR and/or NKG2D-dependent manner (68, 69). The NKG2D receptor present on virtually all human γδ T cells (in addition to NK cells, CD8 T cells and a small subset of CD4 T cells) binds to corresponding ligands, such as MHC class I chain-related gene A or B (MICA/B) and members of the ULBP family expressed on

tumor cells, thereby triggering PI3-kinase dependent signaling pathways leading to cytokine production and cytotoxic effector activity (70). The TCR of Vδ2 T cells recognizes pyrophosphate molecules which are intermediates of both the non-mevalonate and the dysregulated mevalonate pathways of isoprenoid synthesis in prokaryotic and eukaryotic cells, respectively. The prototypic microbial "phosphoantigen" (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) exclusively activates human Vδ2 T cells at pico- to nanomolar concentrations (71, 72). The transient increase of γδ T cells in the peripheral blood during the acute phase of many bacterial and parasitic infections is due to the release of microbial phosphoantigens (73, 74). The intermediate isopentenyl pyrophosphate (IPP) of the eukaryotic mevalonate pathway of cholesterol synthesis is similarly recognized by Vδ2 T cells but requires much higher concentrations (in the micromolar range). While normal resting cells, including neutrophils (72), do not generate enough IPP to activate γδ T cells, many tumor cells have a dysregulated mevalonate pathway with increased IPP production and concomitant sensitivity to γδ T cell recognition and killing (75). The selective activation of human Vδ2 T cells by microbial or eukaryotic phosphoantigens requires the presence of the butyrophilin molecule 3A (BTN3A/CD277); in the absence of CD277, Vδ2 T cells are not activated by pyrophosphate molecules (76, 77). It appears that pyrophosphates bind to the intracellular B30.2 signaling domain of BTN3A1 resulting in a conformational change of the extracellular part of CD277, which is then recognized by the Vγ9Vδ2 TCR (78). Importantly, the intracellular production of IPP can be pharmacologically manipulated. Nitrogen-containing bisphosphonates such as zoledronic acid (ZOL) (which are in clinical use for the treatment of bone diseases) block an enzyme downstream of IPP synthesis in the cholesterol synthesis pathway, leading to the upstream accumulation of the γδ T cell-stimulating IPP (75). As a consequence, the uptake of nitrogen-bisphosphonates by monocytes but not by neutrophils within peripheral blood mononuclear cells (PBMC) induces a strong, selective expansion of Vδ2 T cells in the presence of recombinant IL-2 (rIL-2) (72, 79). Exposure of tumor cells to nitrogen-bisphosphonates drastically increases their sensitivity to γδ T cell-mediated killing (80). Transient activation and expansion of Vδ2 T cells is also observed *in vivo* upon application of ZOL and low-dose rIL-2 (81). Given that the abundance of γδ T cells among tumor-associated immune cells is a favorable prognostic marker (82) and in view of the developing strategies to apply γδ T cells for immunotherapy (83), it is important to consider the possible reciprocal interactions between γδ T cells and neutrophils (84). In mice, γδ T cells are an early source of IL-17 required for neutrophil migration in bacterial and fungal infections (85, 86). However, the same activity of murine γδ T cells might be detrimental in cancer as IL-17 producing γδ T cells were shown to promote metastasis formation in murine models of breast cancer, due to the mobilization of neutrophils which suppressed efficient CD8 T-cell responses (87). IL-17 producing γδ T cells can also recruit MDSC of monocytic and granulocytic origin, thereby again promoting tumor progression (88, 89). Overall, these results have raised the notion that γδ T cells play an ambiguous role in tumor immunity. While their potent cytotoxic activity against many cancers offers the promise for immunotherapy, the potential pro-tumorigenic activities of γδ T cells need to be targeted as well (90). In this context, it is of interest that tumor-associated neutrophils were recently shown to suppress IL-17 producing γδ T cells in the tumor microenvironment through the induction of oxidative stress (91). This finding corroborates previous data indicating that neutrophils can inhibit the *in vitro* activation of human γδ T cells (92). Here, the suppressive mechanism at play was deduced to be ROS production as it was abrogated in the presence of catalase (91). Using ZOL to activate and expand human Vδ2 T cells *in vitro* in the presence of exogenous rIL-2, we found that neutrophils inhibit γδ T-cell activation and proliferation. In addition to ROS, our results also pointed to a role of serine proteases and arginase-I in the γδ T-cell inhibition, based on the partial reversion by corresponding individual inhibitors and the complete reversion by the combination of inhibitors for ROS, serine proteases and arginase-1 (93). Even though neutrophils express CD277 and take up ZOL efficiently, they do not support Vδ2 T-cell activation, likely due to their strongly impaired production of IPP (72). Instead, they appear to suppress the activation of resting γδ T cells through the release of inhibitory molecules. Our observation that neutrophil serine proteases inhibit γδ T-cell activation (94) extends to their role in inhibiting conventional αβ T cells by membrane-associated proteinase 3 expressed by granulocytes (95).

In view of (i) the potent anti-tumor activity of γδ T cells, (ii) the complex interplay between neutrophils and tumor cells, and (iii) the reported reciprocal interactions between neutrophils and γδ T cells, we studied the modulation of anti-tumor cytotoxicity of short-term expanded human γδ T-cell lines by freshly isolated neutrophils and the effects of ZOL treatment.

RESULTS AND DISCUSSION

Zoledronic Acid-Stimulated Neutrophils Diminish γδ T-Cell Cytotoxicity Against Pancreatic Ductal Adenocarcinoma Cells

Zoledronic acid (ZOL) is an approved drug in clinical use for bone fragility disorders and cancer-associated bone disease. In addition to its role as an anti-resorptive agent, ZOL selectively activates human Vγ9Vδ2 T cells and induces their expansion when used in combination with rIL-2. A partial success of tumor reduction after application of ZOL together with rIL-2 was observed in several pilot studies, and this benefit can be further improved by combining adoptive transfer of activated γδ T cells together with ZOL and rIL-2 administration (68, 81, 96–101). ZOL is taken up *via* endocytosis by monocytes or tumor cells, a process which results in a strong selective expansion of γδ T cells and potentiation of their cytotoxic activity (75, 79, 80). Our previous reports demonstrated that neutrophils can also take up ZOL. This uptake, however, resulted in the release of neutrophil-derived hydrogen peroxide, serine proteases and arginase, which collectively inhibited proliferation and cytokine production of resting γδ T cells within purified

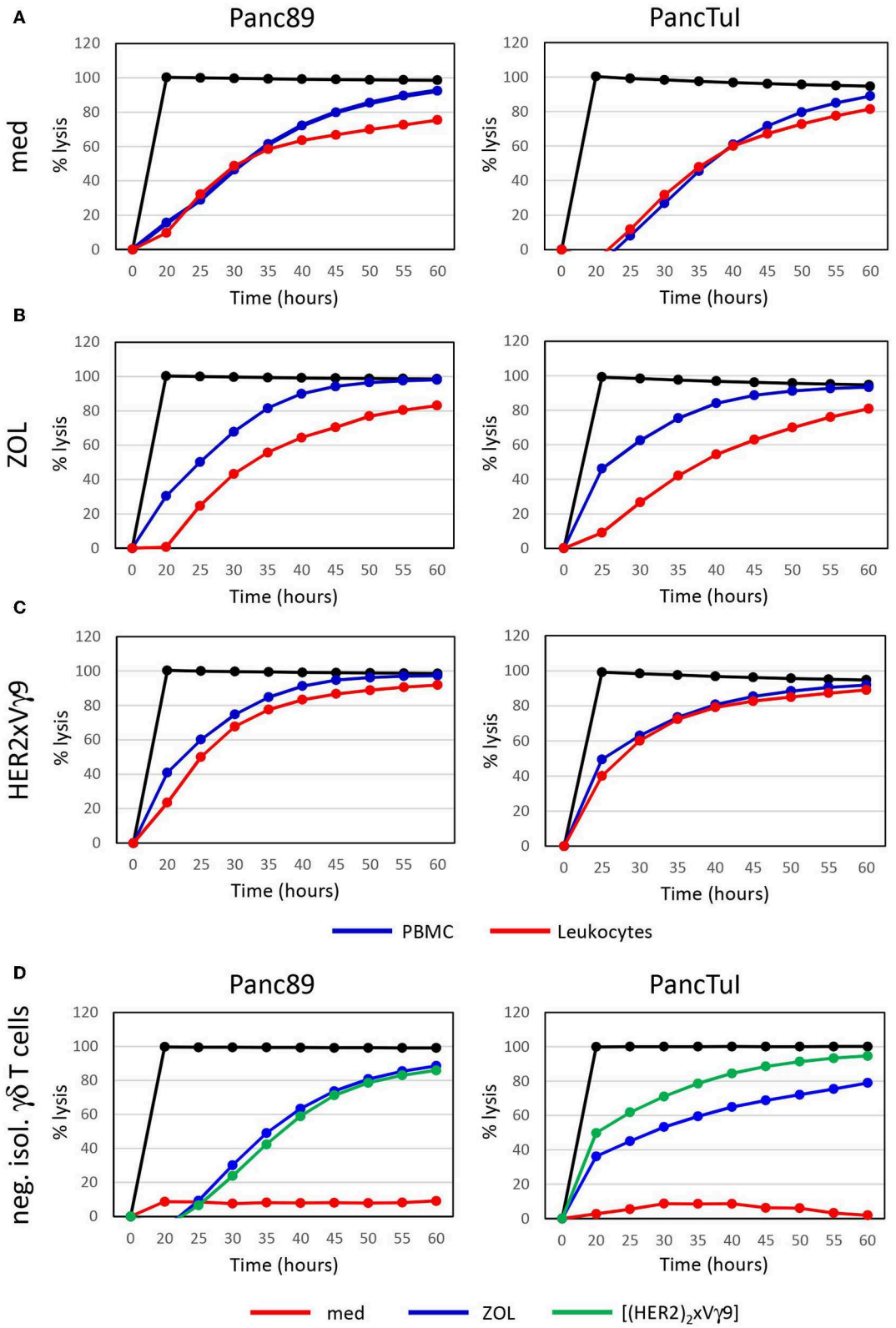

FIGURE 1 | Zoledronic acid-stimulated leukocytes diminish γδ T cell-cytotoxicity against PDAC cells. **(A–C)** Cytotoxicity of 250 × 10^3 PBMC (blue line) or 500 × 10^3 leukocytes (red line), which each comprised ~7,500 γδ T cells or **(D)** 125,000 negatively isolated, resting γδ T cells (neg. isol. γδ T cells) in co-cultures with

(Continued)

FIGURE 1 | Panc89- (left panel) or PancTul cells (right panel). Co-cultures were set up in rIL-2 medium only **(A)**, or were additionally stimulated with **(B)** zoledronic acid (ZOL, 2.5 μM) or **(C)** with bispecific Ab (bsAb) ([(HER2)$_2$ ×Vγ9], 1 μg/mL). Cytotoxictiy was analyzed by RTCA. 5 × 10^3 PDAC cells were seeded 24 h before addition of effector cells. Percentage of lysis ("% lysis") was analyzed from RTCA data by calculating the normalized impedance of spontaneous lysis (cell growth of tumor cells in medium alone) in relation to the maximal lysis induced by 1% Triton-X-100 (black line) at indicated time points. Shown is one representative experiment with the same donor out of three independent experiments with different donors.

leukocytes (93, 94). Here, we have performed additional studies to dissect the interaction between neutrophils and resting γδ T cells regarding their cytotoxicity against cancer cells. Using PDAC as target cells, we initially compared two experimental conditions: (i) Ficoll-Hypaque gradient separated peripheral blood mononuclear cells (PBMC) which contain resting γδ T cells and monocytes, but no neutrophils; (ii) red blood cell-lysed leukocytes, which contain all cells present in PBMC plus neutrophils. All co-cultures contained medium supplemented with rIL-2, and were additionally stimulated or not (**Figure 1A**) with ZOL (**Figure 1B**) or the bispecific antibody [(HER2)$_2$xVγ9] (**Figure 1C**), both of which induce selective Vγ9Vδ2 γδ T-cell activation and cytotoxic effector functions. While γδ T cells within PBMC were able to exert their full cytotoxic activity against PDAC target cells with both ZOL or bispecific antibody treatments, γδ T cells within leukocytes showed an impaired cytotoxicity after activation with ZOL (**Figure 1B**) compared to the bispecific antibody (**Figure 1C**). These results suggest that the uptake of ZOL by neutrophils can inhibit cytotoxic γδ T-cell function, as previously shown for proliferative activation of γδ T cells (93). Whereas, the bispecific antibody, which specifically targets human epidermal growth receptor 2 (HER2)-expressing tumor cells and Vγ9-bearing γδ T cells, does not induce the same inhibitory activity of neutrophils (102–104). Since PBMC and leukocytes contain Natural Killer (NK) cells which could respond to rIL-2 alone (**Figure 1A**, medium control), we additionally applied negatively isolated resting γδ T cells in our studies. As shown in **Figure 1D**, negatively isolated, resting γδ T cells did not exert cytotoxic activity cultured in medium containing rIL-2. In contrast, the stimulation of the γδ T cells with ZOL or bispecific antibody drastically enhanced γδ T-cell cytotoxicity against PDAC cells in the absence of NK cells and other accessory cells (**Figure 1D**). Similar to the treatment with bispecific antibodies, stimulation with phosphoantigens that specifically activate γδ T cells, such as bromohydrin pyrophosphate (BrHPP), resulted in fully cytotoxic effector functions of γδ T cells in the presence of neutrophils (data not shown). In contrast to ZOL, phosphoantigens like BrHPP or HMBPP directly activate γδ T cells and do not appear to induce neutrophil burst or release of ROS and proteases that would inhibit γδ T cell-functions (105, 106). Recently, we reported that serine proteases released by neutrophils, such as proteinase 3, elastase and cathepsin G, decreased the cytotoxicity of freshly isolated, resting γδ T cells after their activation with BrHPP (94). The inhibition of IFN-γ and TNF-α production by resting γδ T cells in the presence of neutrophil-derived serine proteases (94) may play a key role in the reduced γδ T-cell cytotoxicity in the presence of neutrophils. ZOL, but not BrHPP, can trigger the release of ROS and serine proteases in neutrophils, which likely accounts for the observed differences in resting γδ T-cell activation within leukocytes in the presence of ZOL- vs. bispecific antibody- or BrHPP-stimulation (**Figures 1B,C**). To overcome ZOL-induced neutrophil-mediated suppression on the release of IFN-γ and TFN-α from resting γδ T cells, we cultured neutrophils with short-term activated γδ T cells, which are already continuously producing TNF-α and IFN-γ, and measured their cytotoxicity against PDAC cells with and without ZOL treatment.

Interestingly, clinical trials have shown that repetitive *in vivo* stimulation of γδ T cells with ZOL and rIL-2 can result in partially reduced tumor growth for a number of different types of cancers, including prostate cancer, advanced breast cancer and multiple myeloma. However, this repeated activation protocol was associated with exhaustion, anergy, and depletion of γδ T cells (81, 107, 108). This may be due to the simultaneous ZOL-induced release of serine proteases and ROS by neutrophils. In contrast, clinical trials using the adoptive transfer of γδ T cells that were short-term expanded with ZOL and rIL-2 more consistently reduced tumor growth in the context of advanced renal carcinoma, non-small-cell lung cancer, and other solid tumors (97, 100, 101), which suggests that pre-activation of γδ T cells can be helpful. Interestingly, the adoptive transfer of short-term expanded γδ T cells together with bispecific antibodies and rIL-2 reduced the growth of pancreatic tumors grafted into immunocompromised mice more significantly in comparison to adoptively transferred γδ T cells in conjunction with ZOL and rIL-2 (102).

Sun and colleagues demonstrated that IFN-γ and TNF-α, which are abundantly produced by NK cells, can convert tumor-promoting neutrophils into tumor-suppressing ones (109). The observation that an enhanced number of neutrophils in relation to lymphocytes has been associated with poor clinical outcome and reduced overall survival of cancer patients (110, 111) encouraged Sun and coworkers to target neutrophil effector functions as means of improving patient outcomes (109). Having shown that the presence of neutrophils apparently inhibits the γδ T-cell cytotoxicity stimulated by ZOL of resting γδ T cells (i.e., contained within leukocytes vs. PBMC), we therefore asked how neutrophils would impact on cytotoxic effector function of short-term expanded γδ T cells, which also produce high amounts of IFN-γ and TNF-α. To this end we analyzed whether the suppressive effect of neutrophils on γδ T cell-cytotoxicity could be overcome, similar to what has been observed with NK cells (109).

Enhanced Anti-tumorigenic Effect of γδ T Cells Co-cultured With Autologous Neutrophils Against PDAC Cells

Neutrophils can act as a double-edged sword in cancer progression due to their remarkable heterogeneity and plasticity

(9, 112). For instance, neutrophils can be polarized toward distinct phenotypes, not only by NK cells, but also by tumor derived signals (113). For example, Panc89 cells can produce high levels of IL-6 (unpublished observation), which is reported to induce N2 polarization of neutrophils (6, 114).

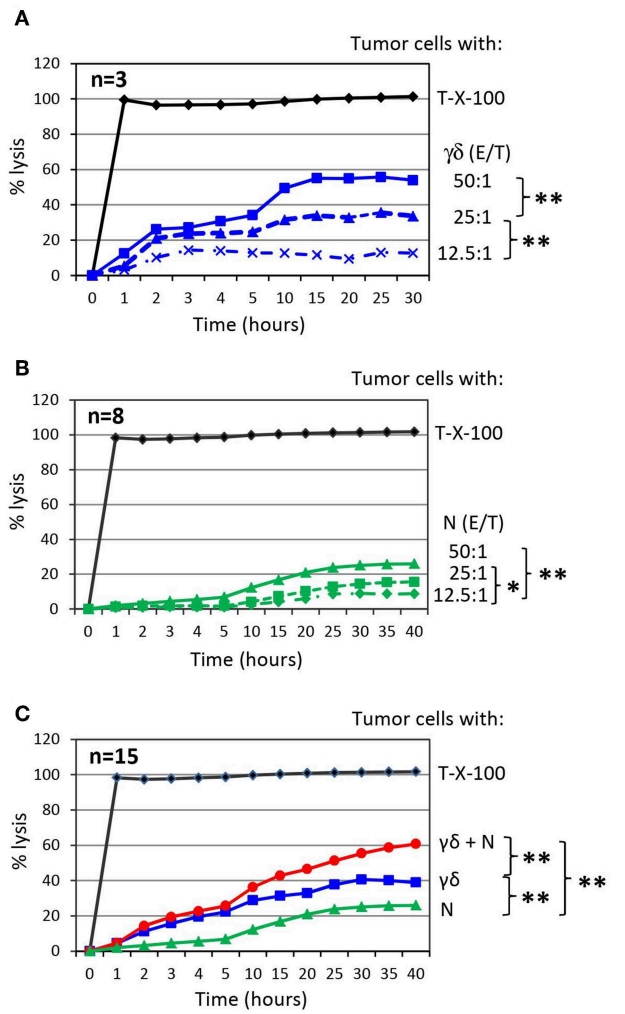

FIGURE 2 | Co-culture of γδ T cells with autologous neutrophils in the presence of PDAC enhances cytotoxicity. **(A)** Cytotoxicity induced by short-term activated γδ T cells (γδ) at the indicated Effector/Target ratio (E/T). The mean of three individual samples for the indicated time points are shown. **(B)** Cytotoxicity induced by neutrophils (N) at the indicated Effector/Target ratio (E/T). E/T ratio was 12.5:1 (dashed line with x, 62.500 effector cells), 25:1 (dashed line with triangle, 125,000 effector cells) and 50:1 (solid line with square, 250,000 effector cells). The mean of eight individual samples for the indicated time points are presented. **(C)** Cytotoxicity of 250 x 10^3 neutrophils (N, green line) or 125 × 10^3 γδ T cells (γδ, blue line, E/T 25:1) alone or in combination (γδ + N, red line) against Panc89 cells was measured by RTCA. The mean of 15 individual samples for the indicated time points are shown. Percentage of lysis was analyzed from RTCA data by calculating the normalized impedance of spontaneous lysis (cell growth of tumor cells in medium alone) in relation to the maximal lysis induced by 1% Triton-X-100 (T-X-100, black line) at indicated time points. **(A–C)** Statistical analysis was performed by *t*-test. Significances are presented as *P*-Value; $^{**}P < 0.01$ and $^{*}P < 0.05$.

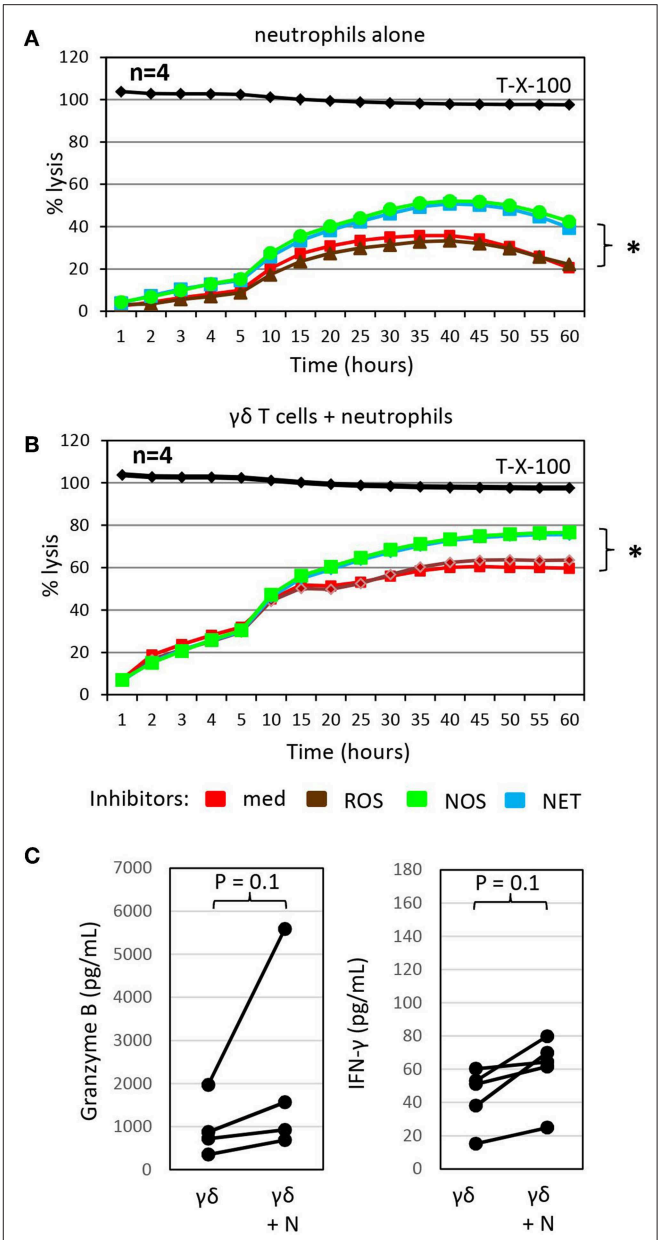

FIGURE 3 | Inhibitors of NETosis and NO production slightly enhance γδ T-cell mediated cytotoxicity in the presence of autologous neutrophils and granzyme B and IFN-γ release. **(A)** Cytotoxicity of 250 × 10^3 neutrophils alone or **(B)** in combination with 125 × 10^3 γδ T cells (γδ + N, E/T 25:1) against Panc89 cells was determined by RTCA. Three hours before the addition of **(A)** medium or **(B)** γδ T cells, tumor cells and neutrophils were pre-incubated with inhibitors against radical oxide synthase (ROS, brown line; catalase, 4,500 U/mL), nitric oxid synthase (NOS, green line; N^G,-N^G-dimethyl-L-Arginine, 100 nM), NETosis (NET, blue line; GSK484, 10 μM) or no inhibitor (med, red line). The mean of four individual samples for the indicated time points are shown. Percentage lysis was analyzed from RTCA data by calculating the normalized impedance of spontaneous lysis (cell growth of tumor cells in medium alone) in relation to the maximal lysis induced by 1% Triton-X-100 (T-X-100, black line) at indicated time points. **(C)** In parallel, granzyme B and IFN-γ release was determined by ELISA from supernatants generated under the same conditions as the RTCA data and collected after 24 h. **(A–C)** Statistical analysis was performed by *t*-test. Significances are presented as *P*-Value; $^{*}P < 0.05$ or indicated significances.

IL-6, however, does not influence γδ T-cell cytotoxicity against tumor cells (115). In general, tumor-suppressing (N1 polarized neutrophils) are short-lived cells with mature phenotype and high cytotoxicity; whereas, tumor-promoting (N2 polarized neutrophils) are long-lived cells with immature phenotype and low cytotoxicity (6, 113).

To investigate the impact of purified neutrophils on the cytotoxic activity of short-term expanded γδ T cells against PDAC cells, we co-cultured target cells with γδ T-cells or neutrophils as effector cells with varying effector/target (E/T) ratios and analyzed cytotoxicity. We observed an E/T ratio-dependent lysis of PDAC cell line Panc89 by γδ T cells or neutrophils (**Figures 2A,B**). The highest E/T ratio showed very moderate lysis of Panc89 cells by neutrophils compared to that of γδ T cells with the same E/T ratio. More interestingly, when Panc89 cells were co-cultured with γδ T cells and neutrophils, lysis of tumor cells was significantly increased; up to 60 % in comparison to γδ T-cell effector cells alone with the 15 different blood donors tested (**Figure 2C**, red line). For the graphical presentation, we selected an E/T ratio at which both effector cells alone (γδ T cells: 25:1; neutrophils: 50:1) lysed the tumor cells to a similar extent. Although Panc89 cells were not completely lysed by either γδ T cells or neutrophils alone, a striking synergistic effect against Panc89 cells was observed. Lysis of Panc89 cells by γδ T cell occurs primarily through the release of granzymes and perforin (102, 116); whereas, neutrophils did not release granzymes, even after treatment with ZOL (unpublished observation). The inhibitory effects of neutrophils on tumor growth and tumor progression are mediated by different mechanisms (9, 117). After interaction with tumor cells, neutrophils can release ROS or NOS to trigger oxidative damage followed by cell death (15, 118), induce NETosis (117), or antibody-mediated trogocytosis (19). None of the direct cytotoxic mechanisms appear to play a major role for the observations made in our study since none of the applied inhibitors, which are described under section Synergism of neutrophils and γδ T cells toward PDAC cell lysis in more detail, inhibited the neutrophil-mediated cytotoxicity against PDAC cells (**Figure 3A**). Alternatively, activated neutrophils can release a wide array of cytokines, chemokines and proteases that influence the effector functions of other immune cells including T cells and NK cells (6, 23, 119, 120).

Synergism of Neutrophils and γδ T Cells Toward PDAC Cell Lysis

To probe the potential indirect mechanism(s) by which neutrophils influence γδ T-cell cytotoxicity against tumor cells, we treated neutrophils co-cultured with Panc89 cells with different inhibitors of antimicrobial mediators that are known to be released by neutrophils for 3 h prior to the addition of γδ T cells. While catalase, an enzyme that degrades hydrogen peroxide, did not influence γδ T cell-mediated lysis of PDAC cells co-cultured with neutrophils, the NOS inhibitor, N^G, N^G dimethyl L-arginine, and the PDA4 inhibitor, GSK484, which attenuates NETosis, both modestly enhanced lysis of Panc89 target cells (**Figure 3B**). Similar effects were seen with these inhibitors when neutrophils were cultured alone with Panc89 cells (**Figure 3A**). The observation that catalase did not influence PDAC lysis in these experiments suggests that neutrophil-derived superoxide anion ($O2^-$) or hydrogen peroxide (H_2O_2) are not major contributors in this setting. $O2^-$ can, however, also react with nitric oxide (NO) to form reactive nitrogen species (RNS), such as peroxynitrite (19). NO can be formed from arginine by the enzyme inducible nitric oxide synthase (iNOS) expressed by activated macrophages, which were not present in our cultures. The NOS inhibitor, N^G, N^G dimethyl L-arginine, is an endogenous iNOS inhibitor, and it competes with endogenous L-arginine as a substrate for iNOS. L-arginine is an essential amino acid for T cells (19), and the addition of N^G, N^G dimethyl L-arginine in our experiments showed some benefit to PDAC lysis. This suggests that L-arginine may be limiting for γδ T cells in our experimental set-up, and its addition could help support their anti-cancer effector functions. In addition, treatment with the NETosis inhibitor moderately enhanced γδ T cell-mediated lysis of PDAC cells, but did not reduce cytotoxicity of neutrophils (**Figures 3A,B**). This observation suggests that neutrophils may be producing some additional factor(s) (potentially cytokines or AMP) that indirectly influence granzyme B release from γδ T cells. In this context, it is of considerable interest that the release of granzyme B and IFN-γ by γδ T cells co-cultured with Panc89 cells was clearly enhanced in the presence of neutrophils compared to the cultures without neutrophils (**Figure 3C**). The increased release of cytotoxic mediators by γδ T cells can explain the enhanced cytotoxic activity against Panc89 cells (**Figures 2C**, **3C**). Taken together, the results argue for a synergistic rather than an additive effect of γδ T cells and neutrophils in killing Panc89 cells. Riise and colleagues recently reported that the activation of neutrophils induced an increase in IFN-γ production in T cells (119). In line with these results, we observed that the presence of neutrophils served to potentiate γδ T-cell mediated tumor cytotoxicity, at least partly *via* enhanced degranulation and augmented Th1 cytokine release (**Figures 2C**, **3C**). While IL-17 producing γδ T cells reportedly contribute to the expansion of granulocytic MDSC (89, 121), our study indicates that Th1-type γδ T cells do not induce immunosuppressive neutrophils.

Zoledronic Acid Enhanced Cytotoxicity of Activated γδ T Cells Against PDAC Cells in the Presence of Neutrophils

We previously reported that the PDAC cell line Pan89 cannot be completely lysed by γδ T cells unless they were additionally stimulated with selective γδ T cell agonists, such as ZOL or bispecific antibodies (69, 102). ZOL is taken up by several tumor cells as well as by neutrophils; however, unlike the case with neutrophils, tumor uptake of ZOL results in the stimulation of γδ T cells and an unleashing of their cytotoxic effector functions (69, 122). As shown in **Figure 4A**, treatment with ZOL in the presence of short-term expanded γδ T cells induced complete lysis of Panc89 cells by activated γδ T cells, which could not be further potentiated by the addition of neutrophils. ZOL further increased the release of granzyme B and IFN-γ by γδ T cells compared to the cultures without ZOL (**Figures 4B**, **3C**). Notably, the presence of ZOL-activated neutrophils additionally enhanced the release of the mediators secreted by γδ T cells in these co-cultures. These results further underline synergistic

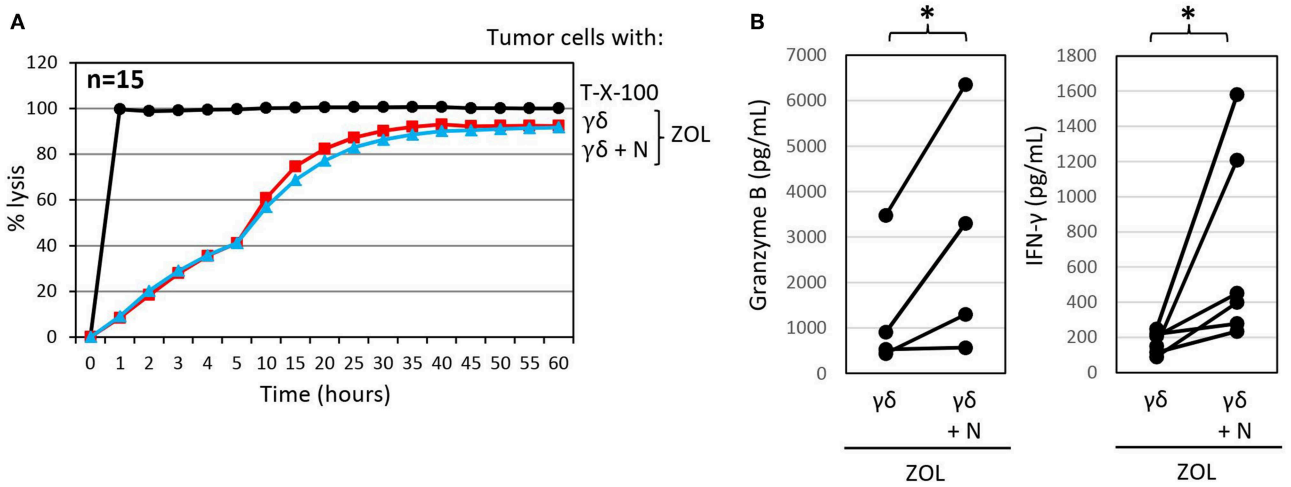

FIGURE 4 | Zoledronic acid pre-treated autologous neutrophils do not influence γδ T-cell mediated cytotoxicity but further enhance granzyme B and IFN-γ release. **(A)** Cytotoxicity of 125 × 10^3 γδ T cells alone or 250 × 10^3 neutrophils in combination with 125 × 10^3 γδ T cells (γδ + N, E/T 25:1, blue line) toward Panc89 cells was measured by RTCA. Tumor cells and neutrophils were pretreated with ZOL (2.5 μM) 3 h before addition of γδ T cells. Percentage lysis was analyzed from RTCA data by calculating the normalized impedance of spontaneous lysis (cell growth of tumor cells in medium alone) in relation to the maximal lysis induced by 1% Triton-X-100 (T-X-100, black line) at indicated time points. The mean of 15 individual samples for the indicated time points are shown. **(B)** Granzyme B and IFN-γ release was measured after 24 h by ELISA from supernatants generated under the same conditions as the RTCA experiments. The mean of four to six individual samples for the indicated time points are shown. Statistical analysis was performed by *t*-test. Significances are presented as *P*-Value; $^*P < 0.05$.

effects of neutrophils and short-term-activated γδ T cells in the lysis of PDAC cells, and suggest that cytotoxicity of short-term expanded γδ T cells is less susceptible to inhibition by ZOL-activated neutrophils.

CONCLUDING REMARKS

Taken together, this study adds to our understanding of how neutrophils can influence γδ T cell-cytotoxicity depending on the situational factors present, such as the activation status of the cells, the cytokine (and chemokine) milieu and the contribution of cytotoxic mediators by other immune cells (e.g., macrophages). Certainly, other important factors that modulate the interaction between neutrophils and γδ T cells in the context of malignancy are also at play—and these include the type or entity of the tumor as well as tumor-derived-signals (e.g., cytokines or damage-associated molecular patterns). The multi-faceted interactions between tumor cells, neutrophils and γδ T cells are graphically summarized in **Figure 5**. As human γδ T cells infiltrate in many tumors and have attracted much attention for their potential application for cancer immunotherapy (82, 104), partly due to their HLA-independent recognition of antigens, understanding the relationship between neutrophils and γδ T cells is very important. Our study demonstrated that neutrophils can under certain circumstances enhance the killing capacity of short-term expanded γδ T-cell lines by increasing their release of cytotoxic mediators. In on-going studies, we aim to explore whether expansion of γδ T cells by their selective γδ T-cell agonists and enhancing their cytotoxicity by bispecific antibodies can really overcome an immunosuppressive tumor microenvironment, as we have postulated (102, 116). Neutrophils or neutrophil-like cells can infiltrate in tumors, and—given their high heterogeneity and plasticity—are subject to polarization to distinct phenotypes, either promoting tumor development/progression or killing tumor cells (6, 19, 112). The conditions for neutrophils may be different in the tumor microenvironment compared to peripheral blood. It appears that neutrophils can bind to tumor cells in the bloodstream and transport them to potentially new metastatic sites and conditioning them to support pro-tumorigenic functions (123, 124). In line, an increased number of neutrophils in the blood of cancer patients has been associated with poor clinical outcome (110, 111). Pro-tumorigenic tumor-associated neutrophils (TAN) are described to enhance tumor cell growth and metastasis, support tumor angiogenesis and mediate immunosuppression (19, 112). In contrast, anti-tumorigenic TANs can lyse tumor cells by the release of noxious substances or exert antibody-dependent cellular cytotoxicity (ADCC) by their expression of Fc receptors (19). Neutrophil-mediated ADCC is described by Matlung and colleagues to occur through trogocytosis-related necrosis of tumor cells opsonized by therapeutic monoclonal antibodies like trastuzumab. These observations support the concept that neutrophils can be therapeutically targeted to enhance their cytotoxic activity (19). We recently reported that the bispecific antibody, [$(HER2)_2$xCD16], has the potential to enhance cytotoxicity of CD16 (FcRγIII)-expressing γδ T cells as well as NK cells to target HER2-expressing solid tumors (116). The fact that CD16 is also expressed on neutrophils suggests that neutrophils may also be a good target for [$(HER2)_2$xCD16] to modulate their anti- tumorigenic properties or to overcome their pro- tumorigenic function.

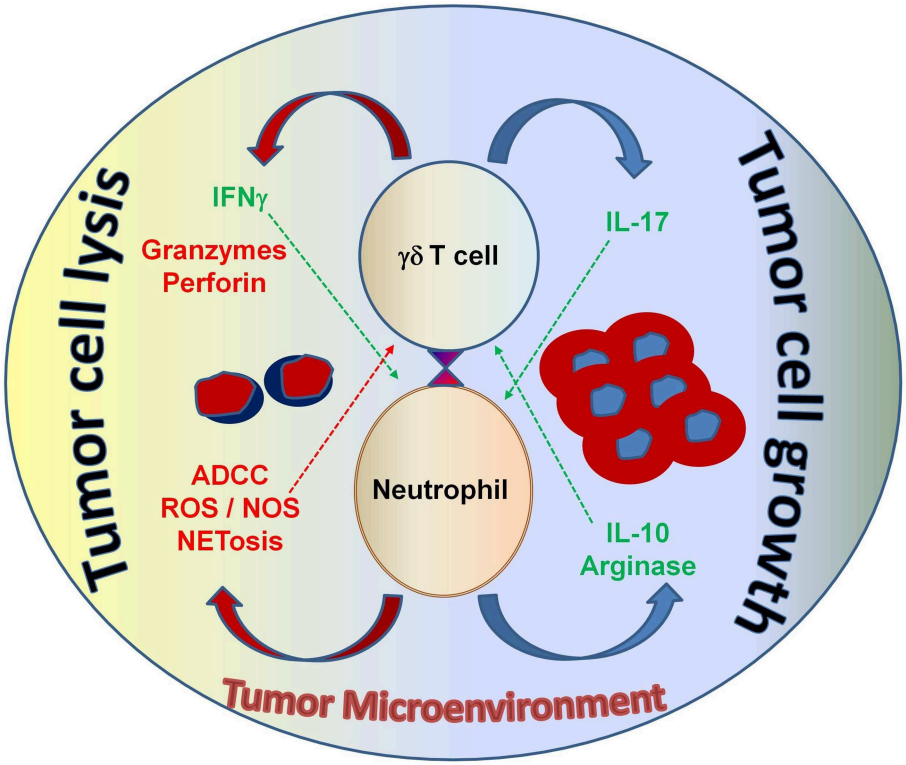

FIGURE 5 | The interaction between neutrophils and γδ T cells can be both pro- and anti-tumorigenic, depending on the tumor microenvironment. Neutrophils can function as myelosuppressive cells and release factors, such as arginase and IL-10 that work to suppress anti-tumor immune effector functions. Alternatively, neutrophils can directly take on anti-tumor effector functions by releasing noxious substances that kill cancer cells. Both tumor-derived factors and accessory immune cells play a role in modulating neutrophil function in the context of malignancy. γδ T cells that secrete IL-17 promote myelosuppressive cells; whereas, γδ T cells that secrete IFN-γ augment anti-tumor effector functions and display potent cytotoxicity, which is important for tumor cell eradication. Understanding this interplay will help develop strategic therapeutic agents that shape the immune environment to elicit strong anti-cancer effector functions from both neutrophils and γδ T cells—with the goal of improving immunotherapy outcomes for patients with cancer. ADCC, antibody-dependent cellular cytotoxicity; ROS, reactive oxygen species; NOS, nitric oxide species; NETosis, the process of cell death induced by the release of chromatin and granular contents into the extracellular space.

In conclusion, neutrophil- and neutrophil-like cell subsets play an important role in cancer, and the nature of the function of these cells can influence patient outcomes. Our increasing knowledge of how the behavior of these different neutrophil subsets can be modulated opens the door to exploring new promising strategies that aim to optimize the interaction between neutrophils and γδ T cells to overcome malignancy.

MATERIALS AND METHODS

Tumor Cell Lines

Pancreatic ductal adenocarcinoma cell lines (PDAC) Panc89 and PancTuI were kindly provided by Dr. Christian Röder, Institute for Experimental Cancer Research UKSH/CAU, Kiel. Panc89 cells as well as PancTuI cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM Hepes, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FCS (complete medium). For removing adherent tumor cells from flasks, cells were treated with 0.05% trypsin/0.02% EDTA. Mycoplasma negativity was routinely analyzed once per month by RT-PCR and the genotype of PDAC cells was recently confirmed by short tandem repeats analysis.

Isolation of PBMC, Leukocytes, Neutrophils and Establishment of γδ T-Cell Lines

PMBC as well as leukocytes were isolated from heparinized- or EDTA blood from adult healthy blood donors of the Institute of Immunology. In accordance with the Declaration of Helsinki, all blood donors provided written informed consent, and the study was approved by the relevant institutional review board of Kiel University Medical Faculty (D406/14, D445/18). PBMC were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation and leukocytes from EDTA blood of the same donors by lysis of red blood cells using RBC lysing solution (BioLegend; Koblenz, Germany). To separate freshly isolated γδ T cells out of PBMC, a negative selection kit [T cell receptor (TCR) $\gamma\delta^+$ T Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach] was used, according to the manufacturer's instructions. PBMC, leukocytes or negatively isolated γδ T cells were co-cultured with PDAC cells (24 h after their adherence) in

medium with 50 U/mL rIL-2 (Novartis, Basel, Switzerland) or stimulated with 2.5 μM zoledronic acid (ZOL, Novartis, Basel, Switzerland) or 1 μg/mL [$(HER2)_2$xVγ9] bispecific antibody and rIL-2. Generation and binding capacity of the bsAb [$(HER2)_2$xVγ9] is described elsewhere (102). To establish short-term γδ T-cell lines, PBMC were cultured in complete medium, and stimulated with 2.5 μM of ZOL with 50 U/mL rIL-2. 50 U/mL rIL-2 was added every 2 days over a culture period of 14 days. The majority of the γδ T-cell lines had a purity of >97% Vγ9Vδ2 γδ T cells determined by staining the cells with anti-CD3 (clone SK7, BD Biosciences), anti-TCR γδ (clone 11F2, BD Biosciences), anti-Vδ2 (clone Immu389, Beckman Coulter) and anti-Vγ9 [clone 7A5; (125)] mAbs followed by flow cytometry analysis. After two weeks, neutrophils were isolated from EDTA blood samples of the same donors. EDTA blood samples of these donors were treated with RBC lysing solution to eliminate red blood cells. Thereafter, neutrophils were isolated by a negative separation using the EasySep Human Neutrophil Enrichment Kit (#19257; Stem Cell Technologies, Grenoble, France). Isolated cells were routinely stained with anti-CD66b mAb (G0F5, BioLegend, San Diego, CA) and analyzed by flow cytometry. All stained samples were measured on a LRS Fortessa flow cytometer (BD Biosciences) using DIVA 8.0 software.

Real-Time Cell Analyzer

Cytotoxicity of PBMC, leukocytes, γδ T cells, neutrophils or the combination of the latter two against adherent PDAC cells was measured by a Real Time Cell Analyzer (RTCA, X-Celligence, ACEA, San Diego, CA, USA) in triplicates as described elsewhere (102–104, 116). By using RTCA, the impedance of the cells is monitored *via* electronic sensors located on the bottom of 96-well micro-E-plate every 5 min for up to 24 hrs. To this end, 50 μL medium followed by 50 μL of 5×10^3 adherent PDAC cells/well in complete medium were added to the plates. Impedance of the cells reflects changes in cellular parameters such as cell proliferation, morphological changes (e.g., spreading, adherence) and cell death, and is expressed as an arbitrary unit called cell index (CI). Since the initial adherence in different wells can differ slightly, the CI was normalized to one after having reached the linear growth phase. After 24 h, medium, 2.5 μM ZOL or 1 μg/mL bsAb [$(HER2)_2$xVγ9] as indicated were added together with PBMC or leukocytes. Alternatively, previously titrated optimal concentrations of inhibitors were added together with neutrophils and medium or ZOL as indicated in the appropriate figures, 3 h before addition of autologous γδ T-cell lines at the indicated effector/target (E/T) ratio together with 12.5 U/mL rIL-2. A final concentration of 4500 U/mL ROS inhibitor Catalase (Sigma-Aldrich, C3556), 10 nM NOS inhibitor N^G, N^G dimethyl L-arginine (Santa Cruz Biotechnology, Santa Cruz, CA) or 10 μM Peptidyl arginine deiminase (PAD) 4 inhibitor GSK484, which prevents NETosis (Cayman Chemical, Ann Arbor, MI) were added in several experiments. When γδ T-cell lines, neutrophils or both together induced lysis of the PDAC cells, the loss of impedance of PDAC cells is shown as decrease of the normalized CI. PDAC cells were treated with 1 % Triton X-100 (final concentration) as a positive control for killing. All cells were monitored every minute for the indicated time points for analysis of cytotoxicity. The experiments were repeated several times as indicated in the Figure Legends under equal conditions using different donors in independent experiments. By using the RTCA software (version 2.0.0.1301, Copyright © 2004 −2012, ACEA) the raw data files were exported to Microsoft Excel [version 14.0.7128.5000, (32-bit)] for further calculation and described as follows. The mean of Triton-X-100 samples was calculated and defined as 100 % lysis after addition of effector cells. The percentage of lysis of each sample was calculated compared to control sample without effector cells or maximal lysis with Triton-X-100.

Enzyme-Linked Immunosorbent Assay

Five thousand Panc89 cells were seeded in 96-well flat bottom microtiter plates (Nunc, Wiesbaden, Germany) overnight. After 24 h, medium or a final concentration of 2.5 μM ZOL were added together or not with 250,000 neutrophils/well in complete medium 3 hrs before addition of 125,000 γδ T-cell lines (E/T ratio: 25:1) supplemented with 12.5 U/mL rIL-2 for further 24 hrs. To quantify IFN-γ as well as granzyme B released by γδ T-cell lines co-cultured with PDAC cells in the absence or presence of neutrophils, supernatants were collected after incubation time and stored at −20°C until use. IFN-γ was measured by human IFN-γ DuoSet® ELISA and granzyme B by a human granzyme B sensitive sandwich ELISA (both from R&D System) in duplicates following the procedures outlined by the manufacturer.

Statistics

Data from at least fifteen donors in independent experiments with three biological replicates were used to test for normal distribution with the Shapiro-Wilk test (Graph pad Prism) followed by a parametrical *t*-test using Microsoft Excel. All statistical tests were two-sided and the level of significance was set at 5%.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

H-HO and DW performed experiments, helped to design the study, and wrote parts of the manuscript. SK contributed to the discussion and wrote parts of the manuscript. DK designed the project and wrote and finalized the manuscript.

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Platelets Aggregate With Neutrophils and Promote Skin Pathology in Psoriasis

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Psoriasis is a frequent systemic inflammatory autoimmune disease characterized primarily by skin lesions with massive infiltration of leukocytes, but frequently also presents with cardiovascular comorbidities. Especially polymorphonuclear neutrophils (PMNs) abundantly infiltrate psoriatic skin but the cues that prompt PMNs to home to the skin are not well-defined. To identify PMN surface receptors that may explain PMN skin homing in psoriasis patients, we screened 332 surface antigens on primary human blood PMNs from healthy donors and psoriasis patients. We identified platelet surface antigens as a defining feature of psoriasis PMNs, due to a significantly increased aggregation of neutrophils and platelets in the blood of psoriasis patients. Similarly, in the imiquimod-induced experimental *in vivo* mouse model of psoriasis, disease induction promoted PMN-platelet aggregate formation. In psoriasis patients, disease incidence directly correlated with blood platelet counts and platelets were detected in direct contact with PMNs in psoriatic but not healthy skin. Importantly, depletion of circulating platelets in mice *in vivo* ameliorated disease severity significantly, indicating that both PMNs and platelets may be relevant for psoriasis pathology and disease severity.

Keywords: psoriasis, neutrophil, platelet, platelet-neutrophil complexes (PNCs), imiquimod

KEY POINTS

- Neutrophils in the blood of psoriasis patients show a distinct "platelet signature" of surface antigens.
- Platelets congregate with neutrophils in psoriatic skin lesions.
- Circulating platelets contribute to psoriasis skin pathology.

INTRODUCTION

Psoriasis is a frequent, chronic, immune-mediated inflammatory skin disease of unknown etiology (1). Its most common form, plaque psoriasis, shows epidermal hyperplasia, increased endothelial proliferation, and a prominent infiltrate of polymorphonuclear neutrophils (PMNs) (1, 2). The accumulation of PMNs in psoriatic plaques and micro-abscesses is accompanied by an increase of PMNs in the circulation of psoriasis patients but their precise role in the disease remains enigmatic (2, 3). Furthermore, it remains unclear which factors prompt PMNs, plasmacytoid dendritic cells and T cells to accumulate in psoriatic skin. The latter cells drive a chronic phase of disease, dominated by IL-17 cytokines (1, 2, 4). Apart from its strong manifestation in the skin, psoriasis is now considered a systemic disease, and besides frequent joint involvement (psoriasis arthritis), alterations in circulating immune cell subsets have been reported: for example, the frequency of $CD16^+$ monocytes is altered in psoriasis patients and these cells were observed to aggregate increasingly with other monocytes or lymphocytes in patient blood (5). Changes within the T cell compartment have also been reported (6). Additionally, a strong link between psoriasis and cardiovascular comorbidities has been noted (7–9), for which IL-17A may be an important factor (10). Regarding cardiovascular events, psoriasis severity correlates with the incidence of cerebrovascular, peripheral vascular and heart structural disorders [reviewed in (7)]. Here a potential etiology involving platelets has been proposed (11) but was not experimentally proven, especially regarding a direct link between PMNs and platelets in humans.

Recent research has uncovered an intimate relationship between different leukocyte populations, including PMNs, and platelets. Interestingly, the existence of direct leukocyte-platelet aggregates *in vitro* and in human blood has been known for some time (12, 13). Ludwig et al. reported that P-selectin Glycoprotein Ligand-1 (PSGL-1)-P-selectin-mediated interactions between platelets and leukocytes promoted rolling in murine skin micro vessels and the same receptors were responsible for activated platelets to interact with murine PBMCs, when co-cultured *in vitro* (14). When these co-cultured PBMCs were infused in mice, rolling in the murine skin microvasculature was observed. The authors speculated whether platelets might also prompt leukocyte invasion into the inflamed skin and noted that P-selectin expression correlated with psoriasis score. However, their studies were largely based on transfusion experiments and did not focus on PMNs. More recently, the capacity of platelets to direct PMN extravasation experimentally was extensively studied by *in vivo* models (15), and (16) proposed that initially CD40-CD40L interactions mediate leukocyte capture at the vessel wall for PSGL-1-P-selectin interactions to guide subsequent extravasation. Whilst this is required for peripheral defense, in rheumatoid arthritis it was also observed that platelets attract neutrophils into the synovium where they become trapped and contribute to disease severity (17). Whether Platelet-Neutrophil complexes (PNCs) occur in human psoriasis patients and whether they contribute to psoriasis skin inflammation was not experimentally studied.

In order to identify surface antigens on human peripheral blood immune cells that might be involved in their skin-homing in psoriasis, we screened 322 surface antigens in PMNs, monocytes and T- and B-lymphocytes from psoriasis patients and healthy controls. This unbiased approach identified surface antigen signatures specific for different blood immune cell populations in psoriasis. For PMNs platelet markers were significantly increased and this surface antigen signature was attributable to direct PNCs. Such PNCs were also observed upon psoriasis induction in mice and in the skin of psoriasis patients. Interestingly, depletion of platelets in the imiquimod (IMQ)-induced experimental *in vivo* setting of psoriasis drastically decreased ear and epidermal thickness of the skin. Collectively, our results establish a functional link between circulating PMN-platelet aggregates and disease activity in the psoriatic skin.

Abbreviations: MFI, mean fluorescence intensity; IMQ, imiquimod; IL, interleukin; PSGL1, P-selectin glycoprotein ligand-1; PLT, platelet; PNCs, Platelet-Neutrophil complexes; PMNs, polymorphonuclear neutrophils; PCA, principal component analysis; PASI, psoriasis area severity index.

MATERIALS AND METHODS

Reagents

All chemicals were from Sigma or Invitrogen, respectively, unless otherwise stated in **Supplementary Materials**. Antibodies and recombinant cytokines are listed in **Table S1**.

Mice

Sex- and age-matched C57Bl/6J SPF mice (Jackson Laboratories) bred and maintained according to local animal welfare guidelines and applicable regulations (see **Supplementary Information**) were used at the age of 6–8 weeks. Mice were bred and maintained under the same specific pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at Johns Hopkins University and handled according to procedures described in the Guide for the Care and Use of Laboratory Animals as well as Johns Hopkins University's policies and procedures as set forth in the Johns Hopkins University Animal Care and Use Training Manual, and all animal experiments were approved by the Johns Hopkins University Animal Care and Use Committee.

Study Participants and Sample Acquisition

All patients and healthy blood donors included in this study provided their written informed consent before study participation. Approval for use of their biomaterials was obtained by the local ethics committees at the University Hospitals of Tübingen and Heidelberg, in accordance with the principles laid down in the Declaration of Helsinki as well as applicable laws and regulations. All blood or skin samples obtained from psoriasis patients (median age 41.8 years, psoriasis area severity index (PASI) $\geq$10 (except for two donors in **Figures 2A,B**, **Figures S3B,C** where PASI was >4.5) no systemic treatments at the time of blood/skin sampling) were obtained at the University Hospitals Tübingen or Heidelberg, Departments of Dermatology,

and were processed simultaneously with samples from at least one healthy donor matched for age and sex (recruited at the University of Tübingen, Department of Immunology). Skin sections were obtained from 11 patients with plaque psoriasis and one patient with psoriasis guttata. Platelet counts were determined in the course of established clinical routines at the time of study blood sampling.

Cell Surface Marker Expression Screening in Whole Blood

A cell surface antigen screening was performed using the LegendScreen from Biolegend (**Figure 1A**). Whole blood (EDTA-anticoagulated) was drawn from five psoriasis patients and five sex- and age-matched controls. Erythrocyte lysis was performed for 5 min at 4°C on a roller shaker using 154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 8 (10× buffer), pH of buffer adjusted to 7.3 and sterile filtered (0.22 μm). After a short spin, FC block was performed and the cells were stained with anti-CD3, -CD15, and -CD19, excluding dead cells using Zombie Yellow. Subsequently, the stained cells were aliquoted into 96 well-plates, each containing a PE-labeled antibody directed against one of 332 surface antigens, and 10 isotype controls in PE. The following washing and further steps were performed using the manufacturer's instructions, except that one kit was divided for the measurement of four donors. FACS measurements were performed using a MACSQuant analyzer (Miltenyi) and subsequently FlowJo V10 was used to analyze the data. The gating strategy is depicted in **Figure S1** and T cells, PMNs, and B cells gated according to the Abs in the master mix. Monocytes were gated by granularity and size but not additionally verified with CD14 staining. However, in the well-containing anti-CD14-PE Abs, all gated events were CD14-positive.

Differential Expression Analysis of Surface Marker Screening Data

Conceptually, the goal was to identify surface antigens, which are significantly different between patients and healthy donors within the targeted cell types. This relationship was formulated as MFI ∼ health_status + cell_type, where the mean fluorescence intensity (MFI) depends on the two main factors: health_status with levels {patient, healthy donor} and cell_type with levels {B cells, Monocytes, Neutrophils, T cells} [(16) T cells], gated as indicated in **Figure S1**. All cell types were measured simultaneously in one FACS screen per subject (patient or healthy donor) and the resulting intrinsic within-subject variance (across cell types) was accounted for by extending MFI ∼ health_status + cell_type + subject/cell_type. In this notation, cell_type is "nested" within "subject," accommodating the above. The implementation of the analysis was done in R [version 3.4.4, with linear mixed models using the R package nlme (18, 19) (version 3.1-131.1, details and code upon request). The fitted models were subject to *post-hoc* analysis with Tukey's "Honest Significant Difference" test to compute adjusted pair-wise differences among the cell types (20). The lsmeans (version 2.27-61) (21) R package implementation was used in order to compute the adjustments. With lsmeans' pairs all pair-wise contrasts of patient vs. healthy donor by the given cell_type were calculated. From all contrasts of the different cell_type levels the p-values and the fold changes of all surface antigens were extracted. This analysis provides nominal, multiple-comparison-adjusted p-values and fold changes between patients vs. healthy donors for each cell type (20). In this exploratory setting, surface antigens with $p < 0.1$ were included into the combined analysis. For the platelet analysis an unpaired t-test method was used as only one cell type had to be considered. Receptors with MFI values missing for more than one donor per group were filtered out. For the generation of the PCA plots, the R package ggplot2 (version 2.2.1) was used.

Flow Cytometry

Two hundred microliter of the cell suspension was transferred into a 96 well-round bottom plate and spun down for 5 min at 448 × g, 4°C. FcR block was performed using pooled human serum diluted 1:10 in FACS buffer (PBS, 1 mM EDTA, 2% FBS heat inactivated) for 15 min at 4°C. After washing, the samples were stained for 20–30 min at 4°C in the dark. Thereafter, fixation buffer (4% PFA in PBS) was added to the cell pellets and incubated for 10 min at RT in the dark. After an additional washing step, the cell pellets were resuspended in 100 μl FACS buffer. Measurements were performed on a MACSQuant analyzer (Miltenyi). Analysis was performed using FlowJo V10 analysis software.

Fluorescence Microscopy of Fixed Whole Blood Cells

The cells were seeded in a 96 well-plate, 200 μl cell suspension per well. FcR block, staining, fixation, and permeabilization were performed as for flow cytometry. After 0.05% Saponin permeabilization, nuclear DNA was stained using Hoechst 33342 (1 μg/ml, Thermo Fisher). The cell pellets were resuspended in 50–100 μl FACS buffer. Forty microliter of the cell suspension was pipetted on a Poly-L-Lysine coated coverslip (734–1005, Corning) and the cells were left to attach for 1 h in the dark. ProLong Diamond Antifade (P36965, Thermo Fisher) was used to mount the coverslips on uncoated microscopy slides. The slides were left to dry overnight at RT in the dark and were then stored at 4°C before microscopy. The measurements were conducted with a Nikon Ti_2 eclipse (100× magnification) and the analysis was performed using ImageJ/Fiji analysis software (22).

Fluorescence Microscopy of Tissue Samples (Human and Mouse)

Skin samples from psoriasis patients or healthy skin samples and mouse ear samples were paraffin-embedded according to standard procedures and were then deparaffinized and rehydrated using Roti Histol (Roth, 6640.1) and decreasing concentrations of ethanol (100 (2×), 95, 80, and 70%). After rinsing in ddH_2O, antigen retrieval was performed by boiling for 10–20 min in citrate buffer (0.1 M, pH = 6). The skin tissue was then washed three times for 5 min with PBS. Blocking was performed using pooled human serum (1:10 in PBS) for 30 min at RT. The primary antibody was added either overnight at 4°C or for 1 h at RT. After three washes, the secondary

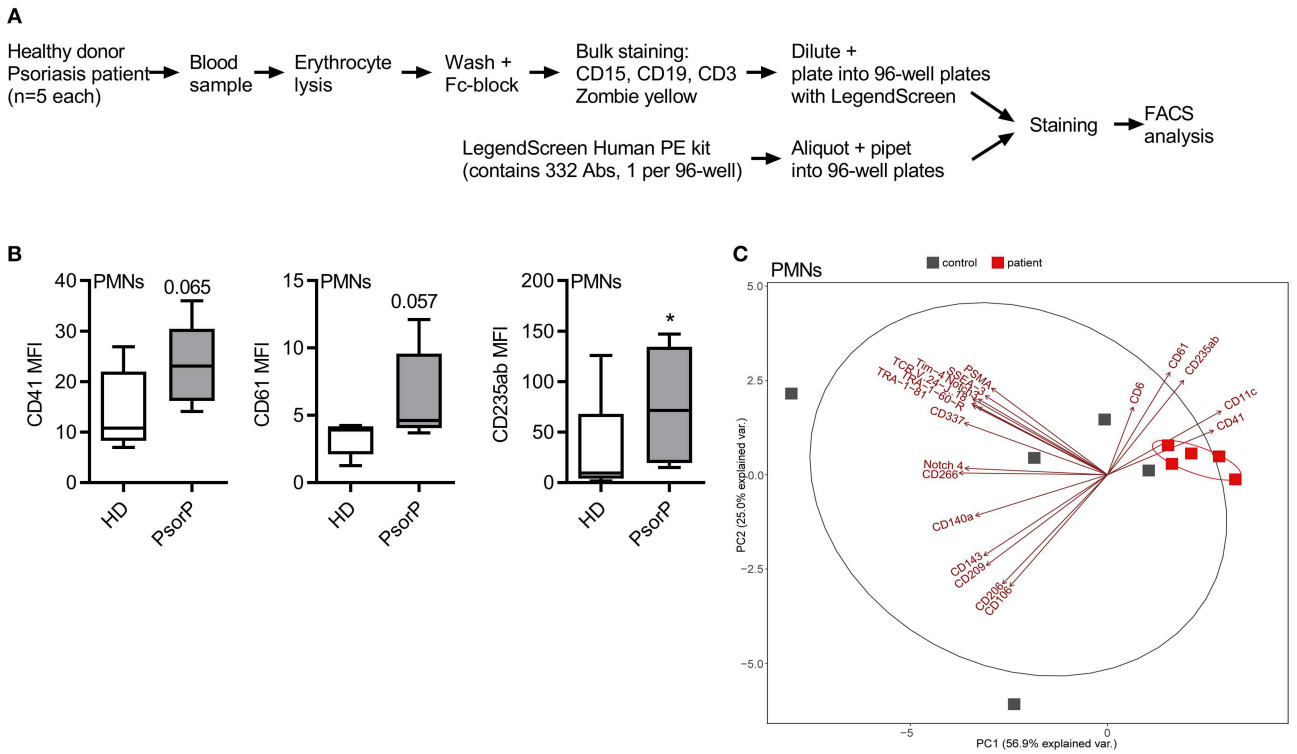

FIGURE 1 | Psoriasis PMNs show a distinct "platelet signature." **(A)** Schematic overview of surface antigen screen procedure. **(B)** Selected surface antigens with the most pronounced differences in MFI between healthy blood donors (HDs) and psoriasis patients (PsorP) PMNs, $n = 5$ each. **(C)** Principal component analyses of PMN surface antigens for healthy donors (red) and psoriasis patients (gray) with top significant antigens (based on nominal $p < 0.1$, $n = 5$ each) contributing to a separation of patients and healthy controls. **(B,C)** represents combined data (mean + SD) from "*n*" biological replicates (each dot represents one donor). *$p < 0.1$ nominal by two-way ANOVA followed by Tukey's multiple comparisons correction.

antibody was incubated for 30 min at RT in the dark. Thereafter, the samples were washed again and Hoechst 33342 (1 μg/ml, ThermoFisher) was added for 5 min. Then three last washes were performed before using ProLong Diamond Antifade (P36965, Thermo Fisher) for mounting. The samples were left to dry overnight at RT in the dark before being used for microscopy or stored at 4°C. The specimens were analyzed on a Nikon Ti2 eclipse microscope (40–60× magnification) and the analysis was performed using ImageJ/Fiji analysis software.

Imiquimod Model of Psoriatic Skin Inflammation

Prior to and after daily topical application of 5% imiquimod cream to the ears of anesthetized (2% isoflurane) mice ear thickness was measured with a manual caliper (Peacock) on d0 to d5. For flow cytometry analysis, retro-orbital blood samples were collected on d0 and d5, diluted in TBS containing 5 U/ml Heparin and subsequently PBS and an aliquot stained using appropriate antibodies (see **Table S1**). Murine blood PMNs were detected by CD11b + Ly6C^{int} cells, thereby avoiding issues with antibody masking by anti-Ly6G (mAb clone 1A8). The same applied to murine blood platelets, which were depleted with anti-CD42b mAb, but detected by flow cytometry with anti-CD41 mAbs. Full thickness ear skin was excised on d5, fixed in 10% formalin and paraffin-embedded. Skin cross-sections (4 μm) were stained by H & E according to standard procedures. At least 10 epidermal thickness measurements per mouse were averaged.

PMN or Platelet Depletion and PSGL-1 Blockade

For PMN depletion, 500 μg of anti-Ly6G mAb (clone 1A8) or isotype control were diluted in sterile PBS and injected i.p. on days−2, 0, 2, and 4 of IMQ treatment. For platelet depletion in mice, 4 μg/g of anti-CD42b (clone R300, Emfret Analytics) or rat IgG isotype control (clone R301, Emfret Analytics) in sterile PBS was administered i.v. one day before, and 2 μg/g administered i.p. 3 days after the first IMQ treatment. For PSGL-1 blockade, 50 μg anti-PSGL-1 or Rat IgG1 isotype control (both BioXcell) were diluted in sterile PBS and injected i.v. on day−1, and again i.p. on d1 and d3 of IMQ treatment.

Statistics

Statistics regarding differential surface antigen expression analysis are described above. All other experimental data were analyzed using Excel 2010 (Microsoft) and/or GraphPad Prism 6, 7, or 8, microscopy data with ImageJ/Fiji, flow cytometry data with FlowJo V10. In case of extreme values, outliers were statistically identified using the ROUT method at a stringency

of 2% and normality tested using the Shapiro-Wilk test for the subsequent choice of a parametric or non-parametric test. *p*-values ($\alpha = 0.05$, $\beta = 0.8$) were then calculated and multiple testing was corrected for in Prism, as indicated in the figure legends. Values < 0.05 were considered as statistically significant and denoted by * throughout. Comparisons were made to unstimulated controls unless indicated otherwise by brackets.

RESULTS

Circulating Neutrophils in Psoriasis Show a Distinct Platelet Signature of Surface Antigens

To phenotype immune cells in psoriasis patients with a view to identifying surface antigens disparately regulated in psoriasis PMNs, we combined the so-called LegendScreen, a screening format consisting of antibodies directed against $n = 332$ different surface antigens and 10 corresponding isotype controls, with a whole blood staining assay using anti-CD3, CD15, CD19 Abs and a live/dead marker (**Figure 1A**), based on previously published work (23). Five psoriasis patients with PASI $\geq$ 10 and without systemic therapy (at the time of blood drawing) and 5 healthy donors were analyzed, the major cell populations gated as described in **Figure S1A** and mean fluorescence intensities (MFIs) acquired for each of the 322 surface antigens (**Table S2**). Differential expression analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons correction (see section Methods and Supplemental R code). Conceptually, the goal of the differential expression analysis was to identify surface antigens, which are significantly different between PMNs from patients and healthy donors. **Figure 1B** shows selected surface antigens with the most prominent MFIs differences between patient and control PMNs. Evidently, monocytes also showed multiple differentially regulated surface antigens (**Table S1** and **Figure S2**). Although individual surface markers were of primary interest, we next considered whether a certain combination ("signature") of molecules was suitable to discriminate patients and healthy donors. In order to check whether the most differently expressed surface antigens (based on a nominal $p < 0.1$) were able to separate the two groups a principal component analysis (PCA) was performed (**Figure 1C**). This showed that for PMNs from psoriasis patient samples clustered together tightly (**Figure 1C**). Antigens contributing significantly to the separation of both groups were CD6, CD11c, CD41, CD61, and CD235ab. As CD6 expression was not detectable on PMNs from psoriasis patients or from healthy controls in follow-up flow cytometry, CD6 was considered as false positive and not pursued any further. CD11c is a known marker on DCs, monocytes but also granulocytes (24) and is considered to be important for phagocytosis and for DC antigen presentation (25, 26). It is conceivable that CD11c is also found on PMNs and may displays similar functions there. CD235ab expression is usually found on terminally differentiated, anucleated erythrocytes and considered to be erythrocyte-specific (27). We detected higher CD235ab expression on PMNs from psoriasis patients. As PMNs can become more "sticky" under certain conditions, e.g., in cancer (28), we interpreted CD235ab positivity to likely result from membrane-fragments of erythrocytes stuck to PMNs. Notably, two of the five combination markers, namely CD41 (also known as GPIIb/IIIa) and CD61 (also termed Integrin β3), are known platelet antigens, which usually act in concert and are required for platelet adhesion and aggregation (29, 30). As little is known regarding the role of these molecules in psoriasis, we focused on the surface antigens CD41 and CD61 in the following. Collectively, our analysis in untreated whole blood samples shows significant differences in surface antigen expression across circulating immune cell populations and that selected groups of surface antigens are able to discriminate patients and healthy donors on the basis of PMN surface antigens.

Circulating Neutrophils in Psoriasis Directly Interact With Platelets

Since PMN infiltration is a hallmark of psoriasis, we focused on PMN surface antigens and noted that several of the aforementioned psoriasis-associated surface antigens, e.g., CD41 and CD61, are typically associated with platelets. These data indicated a "platelet-like" signature on psoriasis PMNs, and, upon re-inspection of the screen data, CD41 and CD61 showed pronounced differences for PMNs that barely missed statistical significance (*cf.* **Figure 1B**). As mentioned before, platelet association with PMNs in the form of PNCs is not uncommon, especially in the context of inflammatory disorders. Whereas, PNCs would persist in unmanipulated whole blood, Ficoll density gradient purification was shown to separate PMNs and platelets due to their different densities and hence sedimentation behavior (31). In order to check whether increased platelet marker expression on PMNs was due to *de-novo* surface expression on PMNs or due to platelet association to PMNs, we analyzed whole blood and Ficoll-purified PMNs together with PBMCs from the same patients and healthy controls that were processed identically. Indeed, compared to whole blood staining, where psoriasis patient samples showed higher CD41 and CD61 expression than healthy donors (**Figure 2A**), Ficoll purification reduced these differences in CD41 and CD61 staining between psoriasis patients and healthy donors: Ficoll-purified PMNs showed more similar levels of CD41 and CD61 positivity between the two groups (**Figure 2B**). As Ficoll is unlikely to lead to a shedding of CD41 or CD61 (for example, CD62L was unaffected), we concluded that the platelet surface markers seen in psoriasis PMNs in whole blood analysis were due to formation of PNCs. Similar results were obtained for monocytes, which also formed complexes with platelets (**Figures S3A–C**). The existence of PNCs was confirmed by bright-field fluorescence microscopy, which showed small $CD41^+$ $CD62P^+$ entities, clearly interpretable as platelets, attached to the outer surface of the $CD66b^+$ PMNs (**Figure 2C**). Including anti-CD62P (P-Selectin) Abs in the analysis, we noted that the platelets were $CD62P^+$ (**Figure 2D**) i.e., activated, in line with recent analyses (15). Thus, the expression of platelet markers on psoriasis PMNs is attributable to an increased formation of PNCs.

To gain deeper insight into whether the formation of PNCs and monocyte-platelet complexes may be a result of skin inflammation, we analyzed complex formation in the IMQ-induced *in vivo* mouse model of experimental psoriasis. In

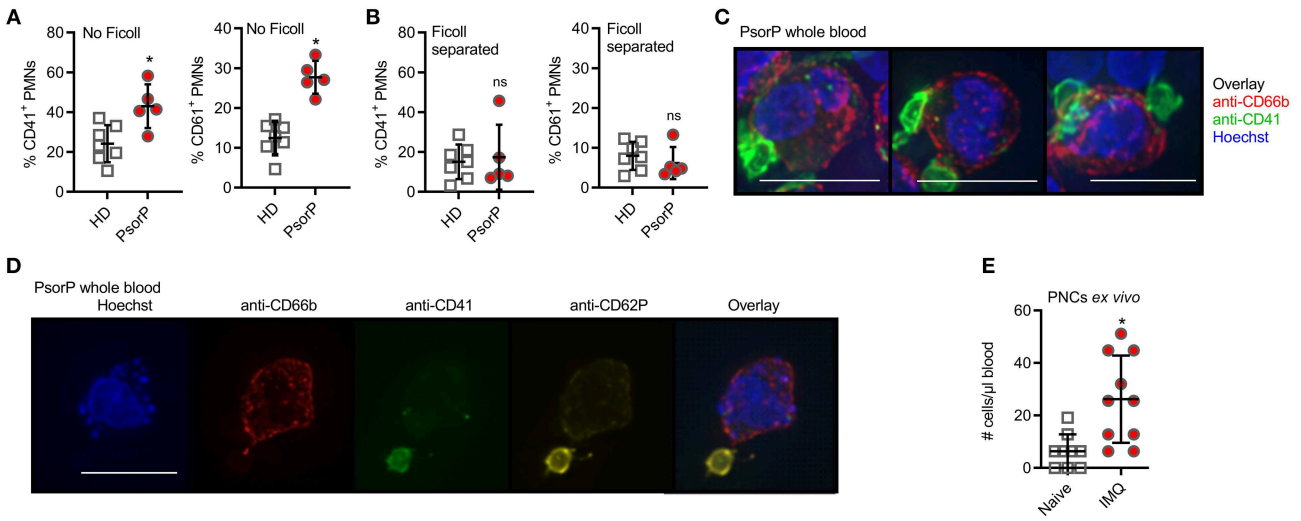

FIGURE 2 | Psoriasis PMNs directly interact with platelets more prominent in psoriasis blood samples and upon *in vivo* psoriasis induction. **(A,B)** Flow cytometric analysis of CD41- **(A)** or CD61-positive **(B)** PMNs (defined as $CD15^+CD66b^+$ and $CD41^+$ or $CD61^+$) analyzed in HD or PsorP (HD $n = 7$, PsorP $n = 5$) in whole blood samples (no Ficoll centrifugation, left panels) or Ficoll density gradient centrifugation (right panels). **(C)** Fluorescence microscopy of PMNs in a PsorP whole blood stained as indicated (scale bar = 10 µm). **(D)** as in **(C)**. **(E)** Mean number of PMN-platelet aggregates comparing naïve (day 0) and IMQ-treated (day 5) isotype mice ($n = 10$ each). **(A,B,E)** represent combined data (mean + SD) from "*n*" biological replicates (in **A,B,E** each dot represents one donor or mouse). In **(C,D)** one representative of "*n*" biological replicates (donors) is shown (mean + SD). *$p < 0.05$ according to Mann-Whitney test **(A,B)**, unpaired Student's *t*-test **(E)**.

this model the application of the TLR7 agonist IMQ leads to a fulminant local and psoriasiform inflammation accompanied by PMN influx (32). **Figure 2E** shows that IMQ-treated mice exhibited significantly higher levels of PNCs compared to control mice, in agreement with the data obtained from humans with psoriasis. Monocyte-platelet aggregates were also affected **(Figure S3D)**. Collectively, these results indicate that PNCs and monocyte-platelet complexes are increased in psoriasis and coincide with skin inflammation.

Platelets Are Detectable in Psoriatic Skin and Show Distinct Surface Antigens in the Circulation of Psoriasis Patients

Although platelet activation is known to promote leukocyte association, we wondered whether a mere increase in total platelet counts would correlate with psoriasis, as an increased platelet count might favor platelet-PMN association. As expected, platelet counts in the circulation of psoriasis patients were 62% higher than in healthy blood donors ($p < 0.0001$, **Figure 3A**). To investigate if this higher abundance might also pertain to the skin of patients where PMNs are frequently infiltrating, we stained for platelet markers in psoriasis skin, which according to our knowledge has not been done before. Whereas, neither PMNs (identified by anti-neutrophil elastase, NE, Abs) nor platelets (stained using anti-CD41 and –CD42b) were readily detectable in healthy skin or isotype-stained samples, in psoriasis skin samples both cell types were present (**Figures 3B,C** and quantified in **Figure 3D**). Certain samples even showed co-localization of PMNs and platelets within infiltrated areas (**Figures 3E,F** and quantified in **Figure 3G**). This indicates that platelets are only infiltrating lesional skin, and may preferentially do so in tandem with PMNs.

Interference With Platelets *in vivo* Ameliorates Skin Pathology

Consistent with previous results (33), PMNs are relevant for disease severity in the IMQ-induced psoriasis model (**Figure 4A**, significant depletion verified in **Figure S4A**), as shown using anti-Ly6G Abs that were shown to both deplete and block the recruitment of PMNs (34). To check whether platelets are also causally involved in PMN skin accumulation and aggravate skin pathology in the context of psoriasis, we investigated the effects of platelet depletion in the IMQ model. Importantly, *in vivo* depletion of circulating platelets on d1 and d3 of IMQ-treatment using anti-CD42 antibodies (35), but not control IgG, showed a significant decrease in ear skin thickness (**Figure 4B**) and epidermal dysplasia (**Figure 4C**, quantified in **Figure 4D**), concomitant with the verified depletion of total platelets in circulation (**Figure 4E**). In accordance with the decrease in PMN-platelet aggregates (**Figure 4F**), the number of free PMN was significantly increased in the blood (**Figure 4G**). Moreover, platelets, often in close proximity to PMNs, were frequently found in IMQ-isotype treated mouse skin (**Figure 4H**), positive for anti-Histone H3 extracellular staining, indicating the possibility for platelets to activate PMNs and assist in NET formation (36) (**Figure S4C**). Conversely, platelet-depleted mice showed no PMN or platelet infiltration (**Figures 4H,I** and quantified in **Figures 4J,K**). When PSGL-1 (PMNs)-P-selectin (platelets) interactions were probed using i.v. infusion of anti-PSGL-1 Abs (14, 15) in a preliminary experiment, we observed an intermittent and intermediate effect

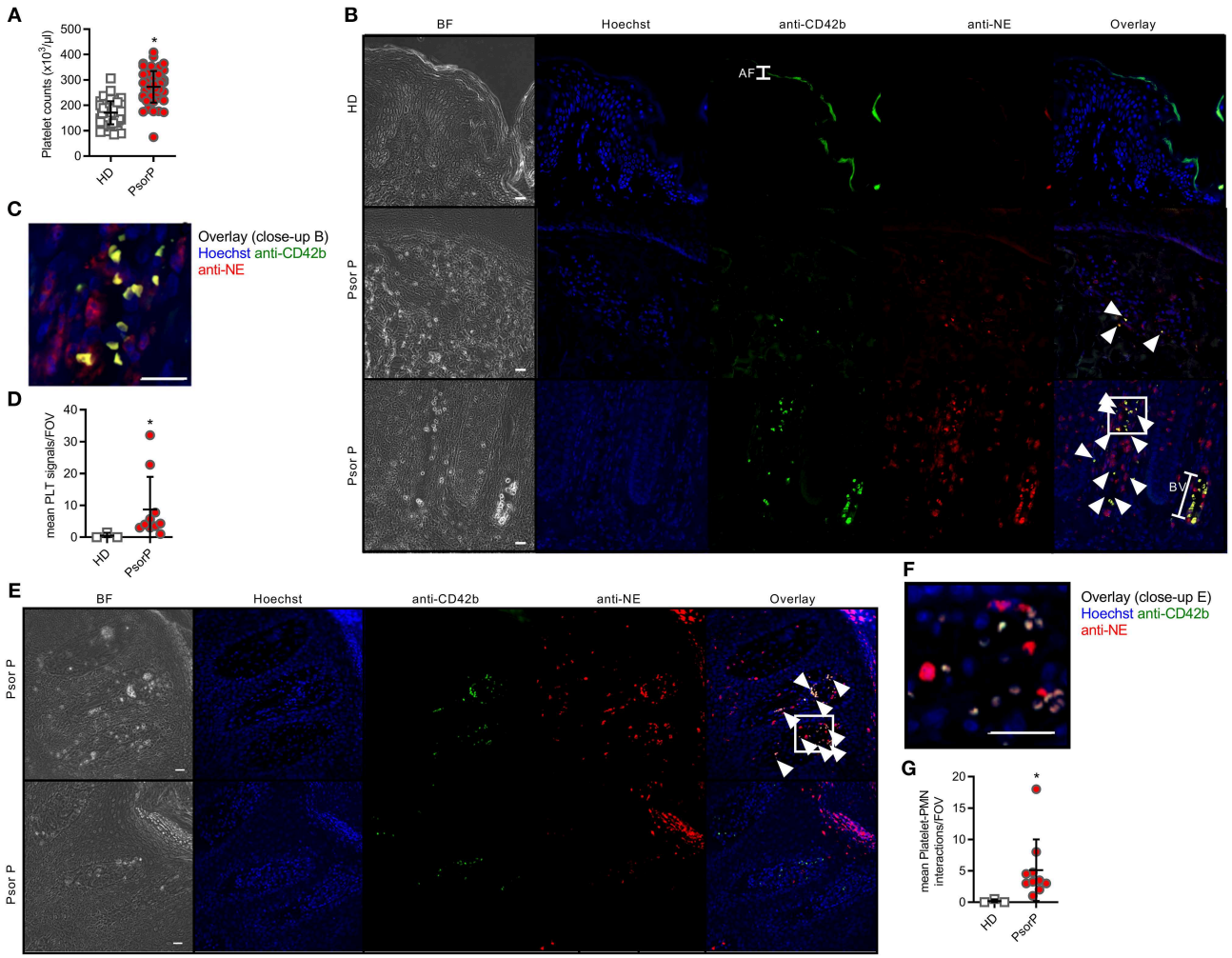

FIGURE 3 | Platelets are more abundant in the blood and skin of psoriasis patients. Total platelet counts in HDs and PsorP (**A**, $n = 52$ vs. 53). **(B,C)** Fluorescence microscopy analysis of skin sections stained as indicated from healthy donor or psoriasis-affected skin ($n = 12$ patients and three healthy controls, scale bar = 20 µm, close-up shown in **C**) and quantified in **(D)**. **(E,F)** as in **(B)** but showing platelet-PMN aggregates in certain skin samples (close-up shown in **F**), quantified in **(G)**. AF, autofluorescence; BV, blood vessel. **(A,D,G)** represent combined data (mean + SD) from "*n*" biological replicates (each dot represents one donor or mouse). In **(B,C,E,F)** representatives of "*n*" biological replicates (donors) are shown (mean + SD). *$p < 0.05$ according to an unpaired Student's *t*-test **(A)** or Mann-Whitney test **(C,E)**.

on ear thickness compared to control IgG (**Figure S4B**). Taken together, these data indicate that circulating platelets significantly contribute to IMQ-induced skin inflammation and pathology.

DISCUSSION

In this present study, we screened the surface antigens of different immune cells in the blood of psoriasis patients and identified respective alterations compared to healthy controls, most notably an association of PMNs with platelets. Although the power of our analysis is limited in terms of sample size and by only nominal *p*-values calculated for individual surface antigens, PCA analysis showed different combinations of surface receptors to distinguish patients from healthy donors. It will be informative to analyze the suggested surface antigen combinations in larger cohorts and in the course of different topical or systemic psoriasis treatments. The whole blood-based protocol developed here may be useful for such a future application.

Psoriasis has long been viewed as an immunologically-driven disease, in which different leukocytes and T cells play important roles. Although platelet interactions with leukocytes have been reported for psoriasis mouse models and are typically found in many other inflammatory diseases (11, 14, 37), a specific role for platelets in the skin lesions of psoriasis patients has not been noted. In agreement with the systemic inflammatory nature of psoriasis, we found platelet-PMN (and –monocyte) aggregates in the circulation to correlate with skin disease. Of note, the depletion of circulating platelets—and consequently aggregates thereof–unexpectedly and drastically ameliorated skin

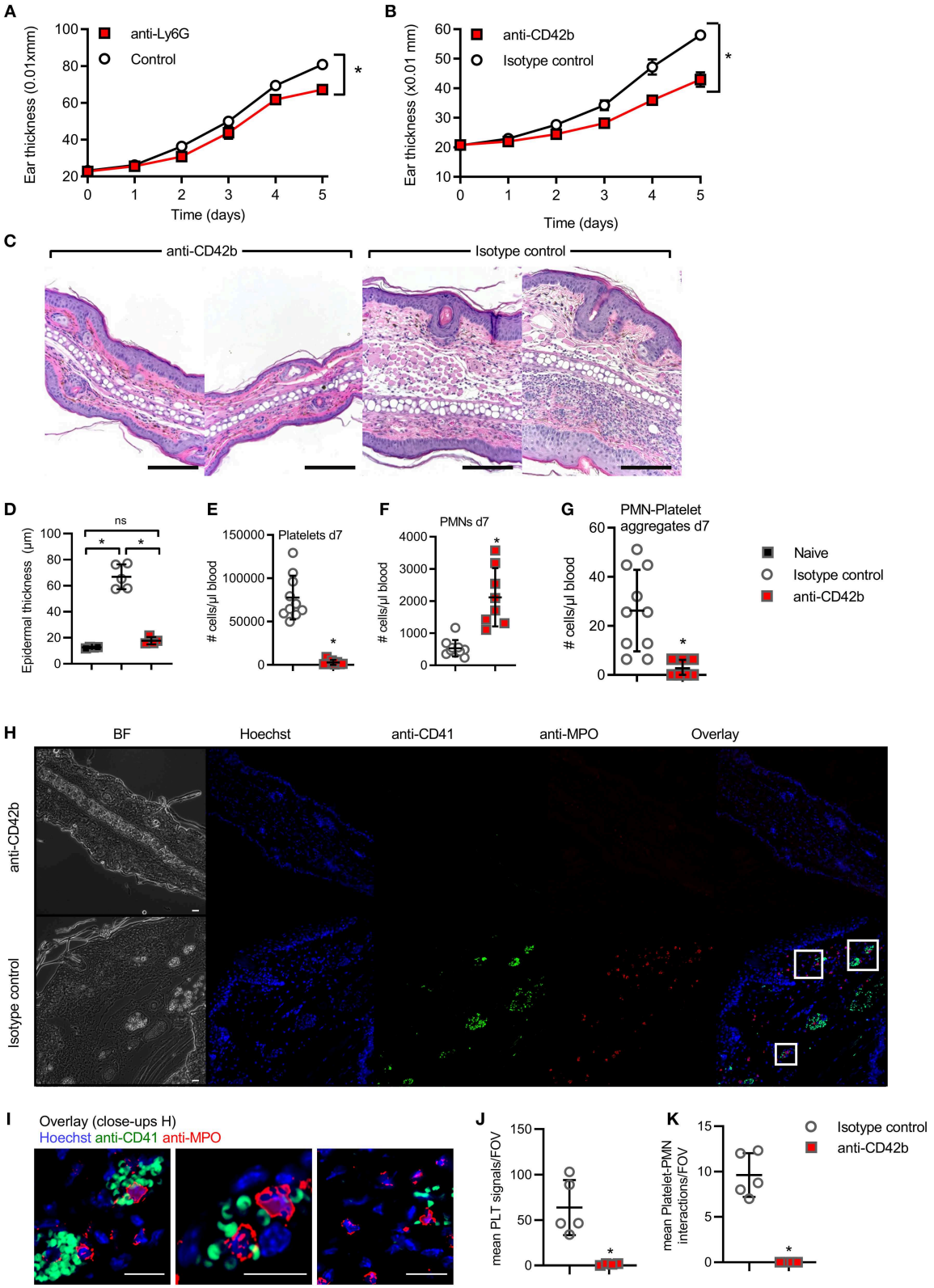

FIGURE 4 | *In vivo* interference with platelets in experimental psoriasis model. **(A,B)** IMQ-induced experimental psoriasis model in BL/6 mice with ear thickness (mm × 0.01) measured upon PMN depletion by anti-Ly6G **(A)**, platelet depletion by anti-CD42b **(B)**, **(C)** representative H&E staining from anti-CD42b Ab and isotype-treated skin (n = 4–5, scale bar = 180 µm), quantified in **(D)**. **(E,G)** Flow cytometry analysis of total platelet counts on d7 **(E)**, PMN-platelet **(F)** and free PMNs **(G)** on d7 upon

(Continued)

FIGURE 4 | infusion of IMQ-treated animals with anti-CD42b Ab or isotype control ($n = 7$–8 each). **(H,I)** representative IF staining from anti-CD41 and isotype-treated skin ($n = 4$–5 for each group, scale bar = 20 μm, close-ups shown in **I**) and the quantification of platelet aggregates in the skin **(J)** and platelet-PMN aggregates **(K)** is shown. **(A,B,D–G,J,K)** represent combined data (mean + SD) from "*n*" biological replicates (each dot represents one mouse). In **(C,H,I)** representatives of "*n*" biological replicates (mouse biopsies) is shown $^*p < 0.05$ according to two-way ANOVA **(A,B)**, unpaired Student's *t*-test **(D,I,J)**, Mann-Whitney test **(E–G)**.

disease. Our data are in line with platelet depletion experiments conducted in an experimental model of atopic dermatitis, another inflammatory skin disease characterized by severe itching of the skin (38). For both atopic dermatitis and psoriasis it could be envisaged that extravasation of leukocytes from skin-proximal blood vessels is facilitated by platelets and platelets then are transported along into the skin. There are several possible underlying mechanisms that await exploration: for example, serotonin-mediated increased vascular permeability induced by platelets (39) may contribute to the effect on PMN infiltration, a notion that fits with the observation that serotonin uptake inhibitors reduce the need for systemic therapies in psoriasis (40). Alternatively, platelets could directly steer the infiltration of their PMN complex partners. The small and intermittent effect of anti-PSGL1 treatment (*cf.* **Figure S4B**) would be consistent with such a notion: Ludwig and colleagues indeed showed that PSGL-1-P-selectin-mediated direct interactions between platelets and leukocytes promoted rolling in murine skin micro vessels (14). Alternative mediators, such as peripheral node addressin (PNAd) and neutrophil L-selectin (41) may explain the only partial effects of PSGL-1 blockade. Whether a scenario of joint PNC "piggyback homing" applies only to the skin or whether other organs apart from the skin, e.g., aortic tissue, are also affected remains to be investigated but may unearth a possible link to the cardiovascular comorbidities observed in psoriasis patients (10). Whichever the mechanism, it is conceivable that platelets that end up in the skin could directly contribute to disease severity—either in concert with leukocytes (42, 43) or acting separately. The responsiveness of platelets to microbe- and danger-associated molecular patterns, the ability for active locomotion and for secretion of inflammatory mediators described for platelets may be properties that could be involved (11, 44). Although a feedback to myelopoiesis cannot be excluded, the increase of free PMNs in the blood of platelet-depleted mice may suggest that the absence of circulating platelets traps PMNs in circulation and prevents them from entering and fueling local inflammation in the skin. If so, this would add psoriasis to the list of pathologies in which therapeutics preventing platelet-PMN interactions may prove beneficial but possibly at the expense of reducing peripheral innate defenses, which rely on PMNs being able to enter the periphery (15).

Platelets have been credited with the ability to promote peripheral homing of innate immune cells. However, the observation that platelets themselves enter psoriatic skin was unexpected. Further exploration of the role of skin-infiltrating platelets and their mechanisms of skin-homing or even active migration (45) in psoriasis and other inflammatory conditions may thus prove informative. On the other hand, the role of platelets in the cardiovascular comorbidities often associated with psoriasis is intriguing. Given the sensitivity of platelets to IL-17 (46), a key psoriasis-associated cytokine, investigation of the IL-17-axis and effects of anti-IL-17 biologicals on isolated platelets and platelet-leukocyte aggregates may prove insightful in this context.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethics committees at the University Hospitals of Tübingen and Heidelberg, as well as the principles laid down in the Declaration of Helsinki and applicable laws and regulations. Approval for biomaterial use was obtained from the local ethics committees. All included subjects provided their written informed consent.

AUTHOR CONTRIBUTIONS

FH, ZB, and NA performed experiments. AW, FH, MC, NA, and SH analyzed data. MH, ML, JW, RB, MS, DS, KS, SG, KG, and LM were involved in sample and reagent acquisition. FH and AW were involved in the conceptual development of the study and wrote the manuscript. AW co-ordinated and supervised the entire study. All authors commented on or revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01867/full#supplementary-material

Figure S1 | Gating strategy used in the surface antigen screen in human whole blood.

Figure S2 | Selected surface antigens with significant differences in MFI between the monocytes from healthy donors (HDs) and psoriasis patients (PsorP), $n = 5$

each. Graphs represent combined data (mean+SD) from 'n' biological replicates. *$p < 0.1$ nominal by two-way ANOVA followed by Tukey's multiple comparisons correction.

Figure S3 | (A) Expression analysis screening results for CD41 (upper) and CD61 (lower panel) MFIs on gated monocytes between healthy blood donors (HDs) and psoriasis patients (PsorP), $n = 5$ each. **(B,C)** Flow cytometric analysis of CD41- or CD61-positive gated monocytes in HD or PsorP (HD $n = 7$, PsorP $n = 5$) in whole blood samples **(B)** or Ficoll density gradient centrifugation **(C)**. **(D)** Mean number of monocyte-platelet aggregates comparing naïve (d0) and IMQ-treated (d5) isotype mice ($n = 10$ each). **(A–D)** Represent combined data (mean+SD) from "n" biological replicates (each dot represents one donor). *$p < 0.1$ nominal by two-way ANOVA in **(A–D)** *$p < 0.05$ by unpaired Student's t-tests.

Figure S4 | (A) Flow cytometry analysis of PMN depletion on d5 upon infusion of IMQ-treated animals with anti-Ly6G Abs or isotype control ($n = 9$–10 each). The data represent cells first gated on live cells with PI, then gated on CD11b+ cells, and the data represent Ly6Cint cells as percentage of the CD11b+ population. Cells were isolated from blood at the end of the experiment for both isotype control and anti-Ly6G (clone 1A8) treated mice. **(B)** Ear thickness (mm × 0.01) measured upon anti-PSGL-1 or control antibody infusion ($n = 4$–5 each). **(C)** Representative IF staining from IMQ-treated mouse ear skin ($n = 5$, scale bar = 20 μm). P, platelet; N, extracellular Histone H3 adjacent to MPO staining indicative of NETs; BV, blood vessel. **(A,B)** Represent combined data (mean+SD) from "n" biological replicates, **(C)** is representative for "n" biological replicates. *$p < 0.05$ by Mann-Whitney U-test **(A)** or one- way ANOVA followed by Sidak's multiple comparisons correction **(B)**.

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Disruption of Neutrophil Extracellular Traps (NETs) Links Mechanical Strain to Post-traumatic Inflammation

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Inflammation after trauma is both critical to normal wound healing and may be highly detrimental when prolonged or unchecked with the potential to impair physiologic healing and promote *de novo* pathology. Mechanical strain after trauma is associated with impaired wound healing and increased inflammation. The exact mechanisms behind this are not fully elucidated. Neutrophil extracellular traps (NETs), a component of the neutrophil response to trauma, are implicated in a range of pro-inflammatory conditions. In the current study, we evaluated their role in linking movement and inflammation. We found that a link exists between the disruption and amplification of NETs which harbors the potential to regulate the wound's response to mechanical strain, while leaving the initial inflammatory signal necessary for physiologic wound healing intact.

Keywords: neutrophils, NET, trauma, movement, inflammation

INTRODUCTION

Musculoskeletal trauma poses a unique challenge due to the need for early mobilization to facilitate rehabilitation. However, movement also up-regulates local inflammation which portends a poor wound healing prognosis (1–6). Therefore, immobilization or "resting" of an injured joint is often implemented to reduce local inflammation and edema (7–9). The mechanism for this local effect of immobilization remains poorly characterized. Although anti-inflammatory therapeutics such as corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) reduce systemic inflammation (10), they do not target pathways specific to the pro-inflammatory effects of mechanical strain; additionally, these drugs cause well-recognized adverse effects including poor wound healing, diabetes, hyperlipidemia, and gastrointestinal toxicity (10–12). Identification of specific pathways through which mechanical strain propagates the inflammatory response would expand therapeutic strategies to mitigate acute inflammation, prevent pathologic wound healing, reduce time to rehabilitation, and improve patient recovery. In addition to musculoskeletal trauma, these findings may influence treatment for a broad spectrum of pathologies mediated by mechanical strain (13–15).

Neutrophil extracellular traps (NETs), discrete structural "webs" composed of DNA and citrullinated histones released by neutrophils, have been studied for their role in both septic and aseptic inflammation (16–26). Several models of infection have demonstrated that NETs provide a protective effect, likely through bacterial trapping (27).

Other studies have suggested that NETs are causative factors for inflammatory conditions including lupus (16–19), rheumatoid arthritis (20, 21), non-healing diabetic wounds (28), and vascular thromboses (22). However, the role of NETs as a link between mechanical strain and exacerbated inflammation remains unclear. Because NETs are composed of macromolecules with expansive structural features, we hypothesize that movement mediates inflammation through physical disruption of these structures and subsequent expansion of the NET superstructure. This leads to a phenomenon we describe as "NET-induced NETosis," a type of secondary NET formation by which mechanical stress is capable of independently propagating the inflammatory process following the inciting inflammatory event.

RESULTS

To study how mechanical disruption mediates acute inflammation, we used a model of musculoskeletal trauma with Achilles tendon transection (tenotomy) with a device that provides extremity immobilization. In this model, when mice were allowed to ambulate *ad lib* absent the cast-immobilizer, they develop pathologic ectopic cartilage at the injury site within 3 weeks after injury followed by ectopic bone at the injury site—a condition called heterotopic ossification (29, 30). Mice underwent tenotomy with or without cast-immobilization of the hindlimb (**Figure 1A**). Histologic examination of the tendon transection site at 48 h and 1 week after injury showed a significant reduction in cellular infiltrates in the immobile hindlimbs (**Figures 1B,C**, **Supplemental Data Figures 1A,B**). These findings were confirmed using flow cytometry to quantify neutrophils (CD11b+Ly6G+) and macrophages (CD11b+Ly6G-F4/80+) at the injury site of mobile or immobile hindlimbs (**Figures 1D,E**, **Supplemental Data Figures 3A,B**). These findings suggest that immobilization of the tendon transection site reduces acute inflammation. Single cell analysis of the neutrophil population attracted to the injury site 7 days after injury demonstrated early elevation in mRNA encoding several cytokines previously demonstrated to be associated with neutrophil and monocyte attraction and specifically with NETosis (e.g. *Ccl3, Ccl4, Ccr1, Cxcl16, Gpi1, Il1b, Il18, Osm, Tnf*), when compared with non-inflammatory mesenchymal cell populations (**Supplemental Data Figures 2A–K**). Chemotactic agents derived from non-inflammatory cells, like *Cxcl12*, were found to be relatively down-regulated in infiltrating neutrophils (31–38). Given these data, we examined the presence of NETs in the immobile and mobile hindlimbs 48 h after injury. Citrullinated histones represent one way to visualize NETs (20). Histologic examination showed expansive NETs (H3Cit+DAPI+) in the mobile hindlimb; NETs were present in the immobile hindlimb, but they were substantially reduced in number and expansive character (**Figures 1F,G**).

To control for potential confounding variables associated with movement in the mobile hindlimb, we examined whether chemical destabilization of NETs in the immobile hindlimb is capable of propagating the inflammatory response. Mice received tenotomy and cast immobilization with intravenous DNase I to enzymatically disrupt the DNA scaffold of NETs (26) (**Figure 1H**). DNase I has previously been described for transient chemical disruption of NETs with *in vivo* efficacy through systemic administration (28, 39). DNase I significantly increased the number of neutrophils and macrophages at the tendon transection site 48 h after injury in the immobile hindlimb (**Figure 1I**, **Supplemental Data Figure 4**). This effect persisted when assessed 1 week after injury as well (**Supplemental Data Figures 5A,B**). These findings suggest that chemically destabilized NETs augment inflammation. When mobile mice were treated with DNase I, however, treatment did not alter levels of infiltrations of macrophages and neutrophils 48 h after injury suggesting that DNase I and movement may have redundant effects on NETs (**Figure 1J**, **Supplemental Data Figure 6**). Immobile hindlimbs in mice treated with DNase I had more expansive NETs when compared with immobile hindlimbs in control-treated mice (**Figure 1K**).

We next employed a series of *in vitro* experiments to determine whether mechanically disrupted NETs augment inflammation by inducing NETosis of other neutrophils (**Figure 2A**). An initial set of mouse-derived neutrophils (1° neutrophils) was exposed to phorbol 12-myristate 13-actetate (PMA) to induce NETosis (1° NETs). Subsequently, the medium was gently exchanged for fresh media *without* PMA. In this new medium, 1° NETs were gently pipetted to induce mechanical disruption ("mobile"), or left intact without pipetting ("immobile"), and a second wave of neutrophils (2° neutrophils) was introduced (**Figure 2A**). NET-induced NETosis was evaluated using PMA-induced NETs as a guide for quantification and cell trapping (**Figure 2B**). When 1° NETs were gently disrupted after media change, 2° neutrophils underwent NETosis with expansive 2° NETs (**Figure 2C**); however, 2° neutrophils did not form similarly expansive structures when 1° NETs were left undisturbed after medium exchange (**Figure 2D**). These observations were confirmed based on metrics including increased mean number of NETs per high-powered field (hpf) (**Figure 2E**), increased NET complexity with mechanical disruption (**Figure 2F**), and increased number of trapped cells per NET (**Figure 2G**). As expected, in the absence of PMA, 1° neutrophils did not form NETs (**Supplemental Data Figure 7A**).

To assess that residual PMA present after the media change was not responsible for causing NETosis among 2° neutrophils, a control experiment was performed in which medium with PMA (without 1° neutrophils) was changed and replaced with media followed by addition of 2° neutrophils (**Supplemental Data Figure 7B**). In this control experiment, 2° neutrophils did not undergo PMA-induced NETosis verifying adequate removal of PMA in this experimental setup (**Supplemental Data Figure 7C**). Although this allows us to assess the removal of a majority of PMA from the chamber and associated media, it does not account for PMA that may be captured within the 1° NETs themselves. There remains the possibility of residual PMA presence even after media washing,

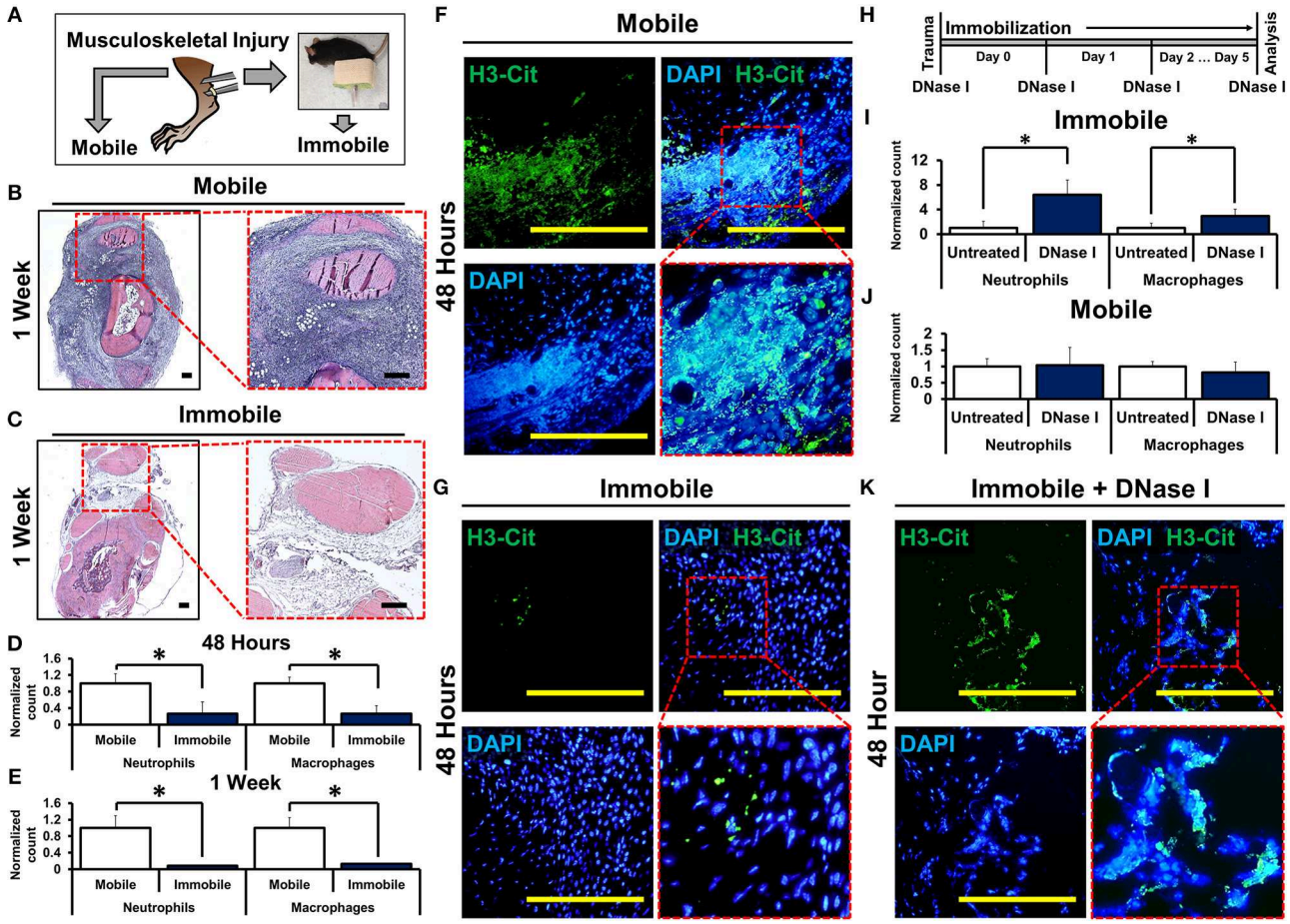

FIGURE 1 | Mechanical or chemical disruption of NETs augments inflammation and induces pathologic wound healing. **(A)** Mice undergo tendon transection and either remain mobile or are immobilized via splint; **(B)** H&E staining of mobile hindlimb 1 week post-injury intense blue staining represent infiltrated cells casting (4x *left*; 10x *right*); **(C)** H&E staining of immobile hindlimb 1 week post-injury; **(D)** Immobile hindlimbs ($n = 6$) have significantly reduced normalized neutrophil (1.0 vs. 0.27, $p < 0.05$) and macrophage (1.0 vs. 0.26, $p < 0.05$) counts compared with mobile hindlimbs ($n = 6$) 48 h after injury; **(E)** Immobile hindlimbs ($n = 3$) have significantly reduced normalized neutrophil (1.0 vs. 0.08, $p < 0.05$) and macrophage counts (1.0 vs. 0.13, $p < 0.05$) compared with mobile hindlimbs ($n = 3$) 1 week after injury; **(F)** H3-Cit immunostaining and DAPI showing NETs in the mobile hindlimb 48 h after injury (40x); **(G)** H3-Cit immunostaining and DAPI showing NETs in the immobile hindlimb 48 h after injury (40x); **(H)** Experimental strategy with DNase I; **(I)** DNase I significantly increases normalized neutrophil (1.0 vs. 6.39, $p < 0.05$) and macrophage (1.0 vs. 3.0, $p < 0.05$) counts in the immobile hindlimb ($n = 8$) 48 h after injury; **(J)** DNase I does not increase normalized neutrophil (1.0 vs. 1.04, $p = 0.87$) and macrophage (1.0 vs. 0.81, $p = 0.23$) counts in the mobile hindlimb ($n = 5$) 48 h after injury; **(K)** H3-Cit immunostaining and DAPI showing NETs in the DNase I-treated immobile hindlimb (40x). All *in vivo* studies had $n \geq 3$/group. Scale bars are 200 μm. *$p < 0.05$.

which is subsequently released from the disrupted NETs. Finally, NETs were not observed after disruption of 1° NETs without the addition of 2° neutrophils, indicating that (1) mechanical disruption effectively disaggregates NETs, and (2) NETs observed after the addition of 2° neutrophils were indeed 2° NETs and not re-settled 1° NETs (**Supplemental Data Figures 8A,B**). These results indicate that mechanically disrupted NETs induce NETosis, a phenomenon which can be described as "NET-induced NETosis."

We next examined whether NET-induced NETosis is responsible for the inflammatory response observed in DNase I-treated hindlimbs using Cl-Amidine, an inhibitor of peptidyl arginine deiminase 4 (PAD4) and consequently citrullination which has previously been demonstrated to diminish NETosis (16, 17, 40–45). Mice received tenotomy, cast immobilization, and DNase I with or without immediate Cl-Amidine (**Figure 3A**). Immediate Cl-Amidine significantly reduced neutrophil and macrophage infiltration in DNase I-treated mice with hindlimb immobilization (**Figure 3B**, **Supplemental Data Figure 9A**). These findings demonstrate that the inflammatory effect of DNase I is dependent on the ability of neutrophils to undergo NETosis. A similar reduction in both neutrophil and macrophage infiltration was demonstrated in the mobile hindlimbs of Cl-Amidine-treated mice (**Figure 3C**, **Supplemental Data Figure 9B**). Finally, Cl-Amidine did not reduce macrophage or neutrophil recruitment in the immobile hindlimb of mice which did not receive DNase I (**Figure 3D**, **Supplemental Data Figure 9C**).

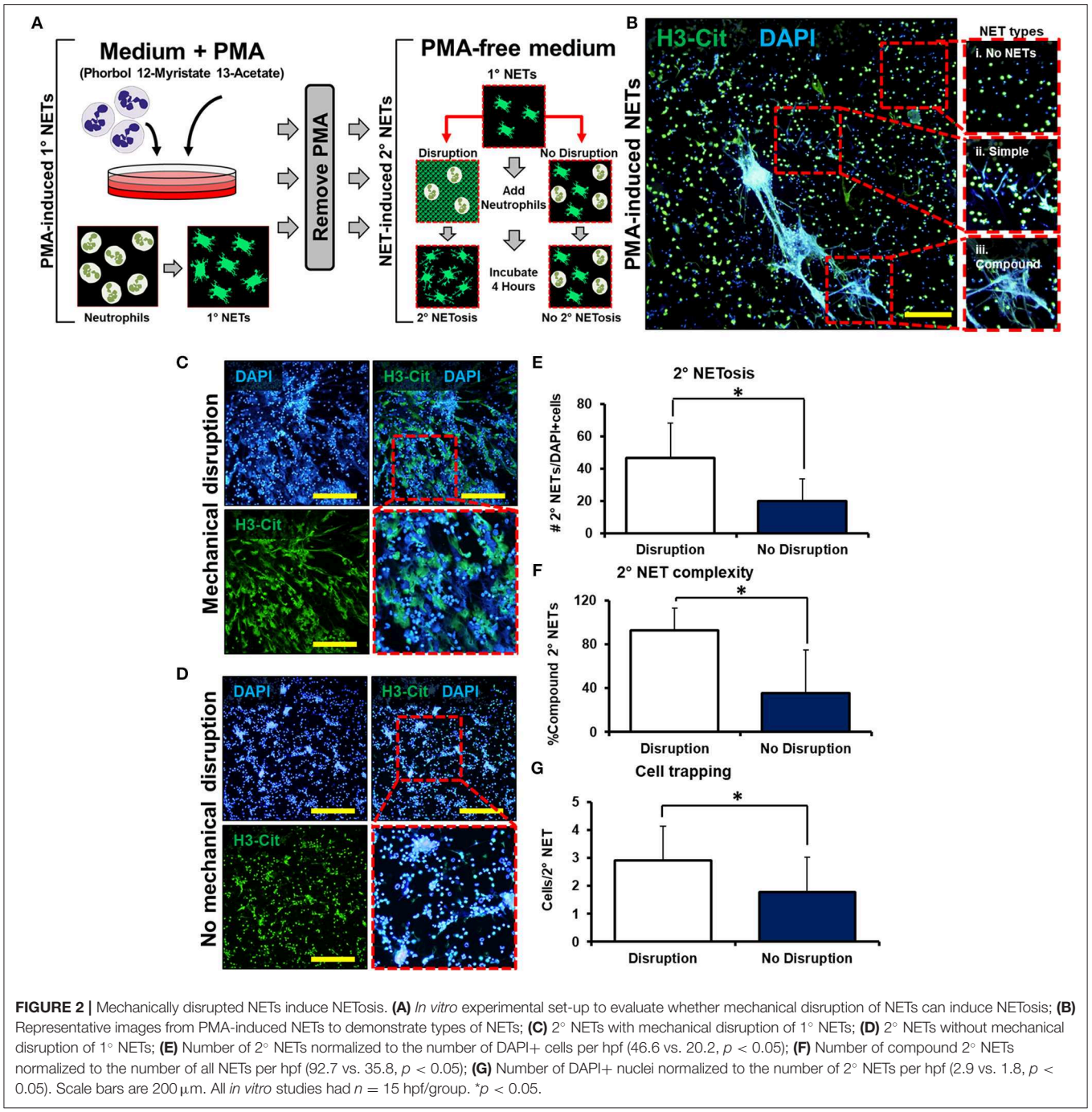

FIGURE 2 | Mechanically disrupted NETs induce NETosis. **(A)** *In vitro* experimental set-up to evaluate whether mechanical disruption of NETs can induce NETosis; **(B)** Representative images from PMA-induced NETs to demonstrate types of NETs; **(C)** 2° NETs with mechanical disruption of 1° NETs; **(D)** 2° NETs without mechanical disruption of 1° NETs; **(E)** Number of 2° NETs normalized to the number of DAPI+ cells per hpf (46.6 vs. 20.2, $p < 0.05$); **(F)** Number of compound 2° NETs normalized to the number of all NETs per hpf (92.7 vs. 35.8, $p < 0.05$); **(G)** Number of DAPI+ nuclei normalized to the number of 2° NETs per hpf (2.9 vs. 1.8, $p < 0.05$). Scale bars are 200 μm. All *in vitro* studies had $n = 15$ hpf/group. *$p < 0.05$.

Histologic sections of the mobile hindlimb from Cl-Amidine-treated mice confirmed a reduction in NETs based on immunostaining, when compared with mobile hindlimbs from untreated mice (**Figures 3E,F**). These findings suggest that NETosis links post-injury inflammation with mechanical strain. However, these findings do not establish that disrupted NETs augment inflammation, as Cl-Amidine administered immediately after injury prevented initial NET formation (**Figures 3E,F**). Given the reduction in citrullination (H3-Cit) associated with PAD inhibition *in vivo* we next sought to identify whether other markers of neutrophil activity, specifically myeloperoxidase (MPO) would demonstrate the same results. We found that MPO appropriately costained with H3-Cit and DAPI in NETs formed *in vivo* (**Supplemental Data Figure 10**). Under conditions of Cl-Amidine treatment there was a gross reduction in formation of MPO (+) NETs (**Supplemental Data Figure 10**).

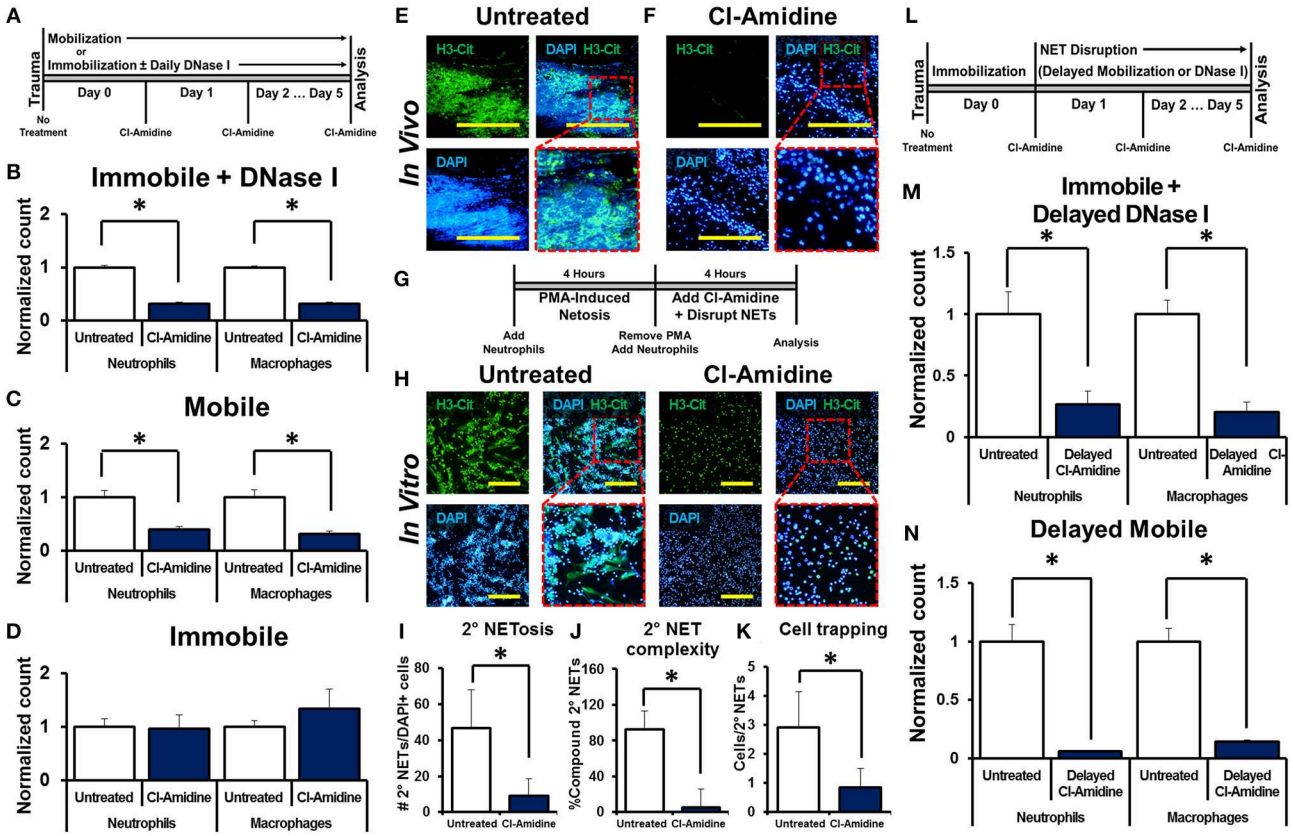

FIGURE 3 | NET-induced NETosis augments inflammation. **(A)** *In vivo* experimental design with Cl-Amidine; **(B)** Cl-Amidine significantly reduces normalized neutrophil (1.0 vs. 0.31, $p < 0.05$) and macrophage (1.0 vs. 0.32, $p < 0.05$) counts in the immobile hindlimb of DNase I-treated mice ($n = 4$) 48 h after injury vs. untreated immobile plus DNase I-treated controls ($n = 3$); **(C)** Cl-Amidine significantly reduces normalized neutrophil (1.0 vs. 0.40, $p < 0.05$) and macrophage (1.0 vs. 0.31, $p < 0.05$) counts in the mobile hindlimb ($n = 5$) 48 h after injury vs. untreated mobile controls ($n = 5$); **(D)** Cl-Amidine does not reduce normalized neutrophil (1.0 vs. 0.96, $p = 0.79$) or macrophage (1.0 vs. 1.35, $p = 0.08$) counts in the immobile hindlimb without DNase I ($n = 5$) 48 h after injury vs. untreated immobilized controls ($n = 5$); **(E)** H3-Cit immunostaining and DAPI showing NETs in the mobile hindlimb 48 h after injury; **(F)** H3-Cit immunostaining and DAPI showing NETs in the mobile hindlimb with Cl-Amidine 48 h after injury; **(G)** *In vitro* experimental set-up with Cl-Amidine; **(H)** H3-Cit and DAPI showing 2° NETs with or without Cl-Amidine after mechanical disruption of 1° NETs; **(I)** Number of 2° NETs normalized to the number of DAPI+ cells per hpf with Cl-Amidine (46.6 vs. 9.1, $p < 0.05$); **(J)** Number of compound 2° NETs normalized to the number of all NETs per hpf with Cl-Amidine (92.7 vs. 5.4, $p < 0.05$); **(K)** Number of DAPI+ nuclei normalized to the number of 2° NETs per hpf with Cl-Amidine (2.9 vs. 0.9, $p < 0.05$); **(L)** *In vivo* experimental-set up with delayed Cl-Amidine; **(M)** Cl-Amidine significantly reduces normalized neutrophil (1.0 vs. 0.27, $p < 0.05$) and macrophage (1.0 vs. 0.20, $p < 0.05$) counts in the immobile hindlimb of DNase I-treated mice 48 h after injury vs. immobile DNase I-treated controls; **(N)** Cl-Amidine significantly reduces normalized neutrophil (1.0 vs. 0.06, $p < 0.05$) and macrophage (1.0 vs. 0.14, $p < 0.05$) counts in the mobile hindlimb 48 h after injury vs. untreated mobile controls. Scale bars are 200 μm. All *in vivo* studies had $n \geq 3$/group. All *in vitro* studies had $n = 15$ hpf/group. *$p < 0.05$.

Therefore, we first confirmed that Cl-Amidine inhibits NET-induced NETosis using a series of *in vitro* experiments. The addition of Cl-Amidine to 2° neutrophils prevented 2° NETosis caused by mechanically disrupted 1° NETs (**Figures 3G,H**). These observations were confirmed based on metrics including increased mean number of NETs per hpf (**Figure 3I**), increased NET complexity with mechanical disruption (**Figure 3J**), and increased number of trapped cells per NET (**Figure 3K**). To demonstrate that Cl-Amidine prevents NET-induced NETosis *in vivo*, mice received tenotomy and were initially immobilized to allow NETosis to proceed, followed by chemical or mechanical disruption and simultaneous Cl-Amidine 24 h after injury (**Figure 3L**). Histologic sections from immobile hindlimbs taken 24 h after injury confirmed the presence of NETs (**Supplemental Data Figure 11**). Immobile hindlimbs from mice treated with delayed DNase I and Cl-Amidine 24 h after injury had significantly reduced neutrophil and macrophage counts when compared with mice that received DNase I, but not Cl-Amidine 24 h after injury (**Figure 3M**, **Supplemental Data Figure 12A**). Similarly, hindlimbs which were allowed to be mobile and treated with Cl-Amidine after 24 h of post-injury immobilization had significantly reduced neutrophil and macrophage counts when compared with hindlimbs that were allowed to be mobile 24 h after injury without Cl-Amidine (**Figure 3N**, **Supplemental Data Figure 12B**).

Neutrophils in mice have several physiologic differences from those found in humans and can have significantly different responses to stress, infection, and the inflammatory response. To determine whether this was a model specific phenomenon

we next isolated human neutrophils which were evaluated *in vitro* utilizing the same techniques previously applied to murine cells. Human-derived neutrophils demonstrated similar NET-forming behavior to murine cells (**Figure 4A**). Human-derived neutrophils demonstrated evidence of NET-induced NETosis *in vitro (***Figures 4A,C**).

We next sought to determine *the mechanism through which* disrupted NETs induce NETosis. Because DNA is a substantial component of NETs, we hypothesized that toll-like receptors (TLRs) 7/8/9, known receptors for oligonucleotides, are mediators of NET-induced NETosis (46–52). In addition, NETs have been shown to activate dendritic cells and monocytes through TLR activity. *in vitro* treatment with ODN-2088, an oligonucleotide inhibitor of TLR 7/8/9, significantly reduced 2° NETosis after mechanical disruption of 1° NETs (**Figures 5A,B**). These observations were confirmed quantitatively (**Figures 5C–E**). However, ODN-2088 did not inhibit PMA-induced NETosis, indicating that non-TLR-mediated NETosis remained intact despite ODN-2088 (**Supplemental Data Figures 13A,B**). The mobile hindlimbs of mice treated with ODN-2088 showed significantly reduced macrophage and neutrophil infiltration (**Figures 5F,G**, **Supplemental Data Figure 14**). Likewise, the response of human neutrophils to ODN2088 during NETosis matched what we had previously identified in murine cells (**Figures 4B,C**).

DISCUSSION

The interaction between movement or mechanical strain and inflammation after trauma carries implications for the care of the tens of millions of patients with orthopedic and soft tissue injuries annually. NET formation, or NETosis, represents an emerging and critically important field of study in the modulation and amplification of the inflammatory cascade after injury and infection.

Our findings suggest a model in which NETosis is propagated and augmented by the physical or chemical disruption of formed NETs both *in vivo* and *in vitro*. The model of musculoskeletal injury we employed in this study allowed for interrogation of the relationship between mechanical strain and inflammation because the hindlimb can be immobilized. Other models in which mechanical strain can induce inflammatory responses such as ventilator-associated lung injury or reperfusion injury do not provide a similar opportunity for "immobilization." If a link were to exist between mechanical fragmentation of NETs and propagation of NETosis then we would expected that animals which had been immobilized would demonstrate a decrease in both neutrophil migration to the site of injury and in the size, number, and complexity of NET structures *in vivo* when compared with those which had been allowed to ambulate post-trauma.

While our data supported this hypothesis, the degree of confounding factors associated with mobile vs. immobilized extremities rendered it difficult to draw conclusions from these experiments in isolation. Thus, we sought to identify a non-mechanical surrogate mechanism to confirm our findings. DNase I, when provided exogenously, acts to facilitate the enzymatic breakdown of extracellular DNA thus chemically disrupting a critical part of the backbone of a NET and leading to the breakdown of the NET structure. As DNase I should cleave NETs by a fundamentally different mechanism when compared with mechanical strain, we hypothesized that an immobilized mouse should show some evidence of NET disruption and consequently enhanced inflammation with DNase I administration. Furthermore, as the half-life of DNase I *in vivo* is short, greatest effect should occur upon the primary NETs, while, at least partially, sparing secondary NETs which form later. Again, our data supported evidence of NET disruption followed by enhanced inflammation with DNase I administration, so we next asked how the combination of mechanical and chemical disruption would affect the inflammatory cascade after injury. Interestingly, we found that addition of DNase I to mobilized animals did not augment the inflammatory response to the degree found in immobilized animals. While unexpected, this would be consistent with a model in which both mobility and DNase I are acting by a similar downstream mediator without affecting separate or confounding parts of the inflammatory cascade.

Our *in vitro* findings suggest that NETs are susceptible to mechanical disruption, leading to gross disintegration of their superstructure. Furthermore, when NETs are mechanically disrupted there is a more robust NET-forming response in new neutrophils added to the system compared to when these 1° NETs are kept intact. These data suggest a link between the presence of mechanically disrupted NETs and NETosis in otherwise inactive neutrophils—a process we refer to as NET-induced or 2° NETosis. Given the link implied by our *in vitro* findings between mechanical strain and the propagation of NETosis, we sought to identify whether movement may be sufficient to physical disrupt the fragile structure of NETs after injury and induce the NET-induced NETosis response.

Based upon our *in vitro* data, we hypothesized that disruption of the NET superstructure and the presence of NET fragments may represent the central signaling agent mediating this enhanced inflammatory response. However, there remained the possibility that, while DNase I and mechanical strain shared a single mechanism in their effect upon neutrophil infiltration, that mechanism could still be something other than NETosis. To address this we utilized Cl-Amidine, a PAD4 inhibitor which had previously been demonstrated to prevent to formation of NETs *in vivo*. We hypothesized that introduction of Cl-Amidine to the system would render it resistant to perturbations in inflammation secondary to any mechanism which specifically targeted NETosis. Cl-Amidine resulted in diminished inflammatory infiltrate in both the mobile untreated and immobile, DNase-treated animal, however, it did not further attenuate the inflammatory response present in the immobilized animal. This was consistent both with immediate administration of the PAD4 inhibitor and delayed treatment suggesting that the action of mechanical stress and exogenous DNase I is inhibited specifically by inhibition of NETosis even in a system where NETs had previously been formed.

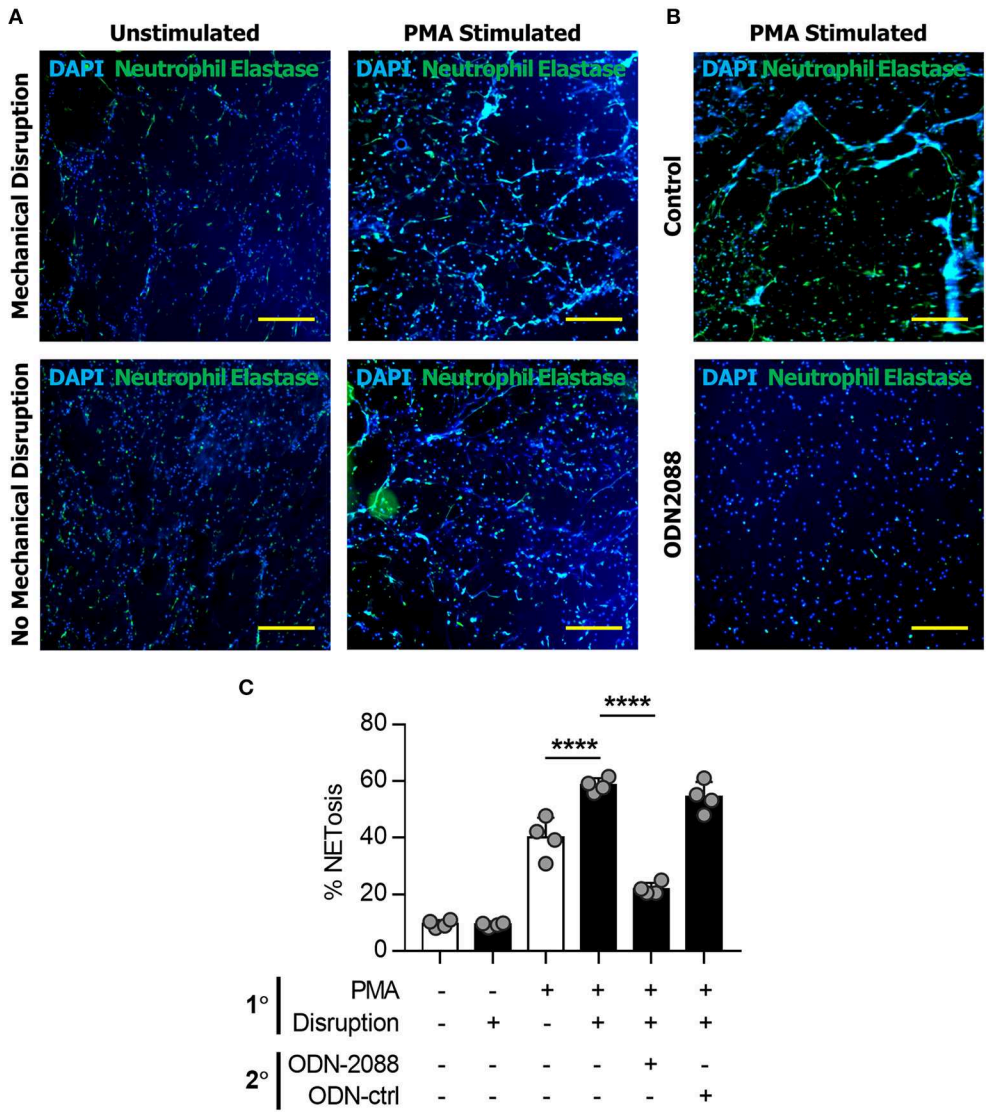

FIGURE 4 | NETs induce NETosis in human neutrophils. **(A)** Human neutrophils *in vitro* demonstrating the increase in NET formation after Mechanical Disruption of primary (PMA-induced) NETs. **(B)** Demonstration of the effect of ODN2088 on NET-induced NETosis of human neutrophils *in vitro*. **(C)** Quantification of increased NETosis after disruption of primary NETs; this effect is abrogated by ODN2088. Scale bars are 300 μm. ****$p < 0.0001$, by one-way ANOVA.

Given the above findings, we are led to believe in the following five points: (1) Addition of neutrophils to a system of previously disrupted NETs will result in more robust NETosis when compared with a system of in which the initial NETs were not disrupted; (2) Mechanical stress and exogenous DNase I both increase post-injury neutrophil infiltration; (3) Mechanical stress and exogenous DNase I share a common downstream signaling mediator; (4) The increase in post-injury neutrophil infiltration is dependent upon the presence of NETs; and (5) The action of mechanical stress and exogenous DNase I is inhibited by inhibition of NETosis even in a system where NETs have previously formed. Based upon the above, we propose a model of NET-induced NETosis whereby mechanically disrupted NETs augment NETosis, NETosis augments inflammation, and that this is a mechanism which links mechanical strain to inflammation (**Supplemental Data Figure 15**).

We then utilized this framework to try and better identify the pathway by which NET-induced NETosis modulated the inflammatory cascade. Previous studies have shown that NETs can activate monocytes and dendritic cells through TLRs. However, direct propagation of the inflammatory response through TLR-mediated, NET-induced NETosis would represent an important advance in our understanding of how movement and mechanical strain augment inflammation. We utilized both our *in vitro* and *in vivo* models to demonstrate that ODN-2088 inhibition of TLR 7/8/9 significantly reduced NET-induced

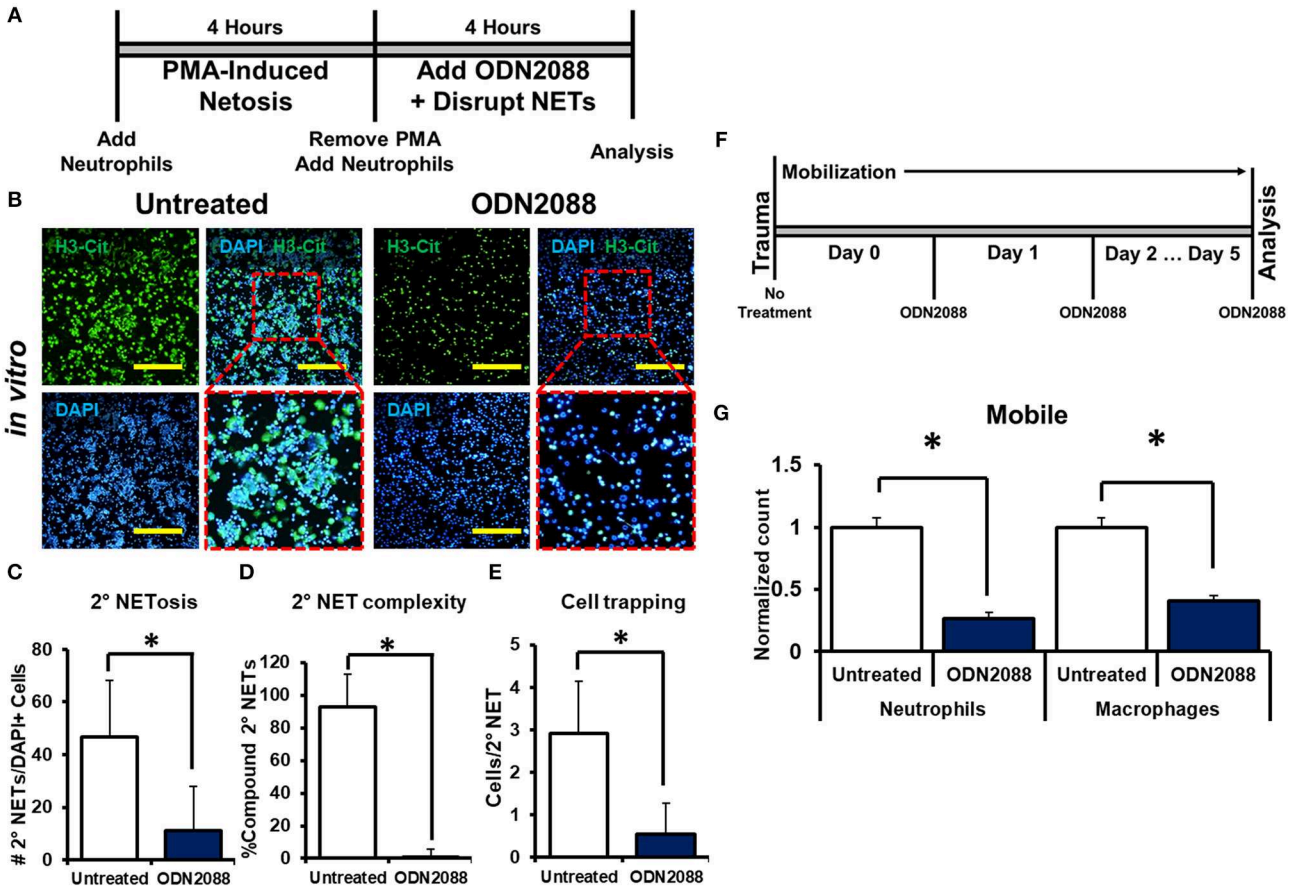

FIGURE 5 | NETs induce NETosis through TLR activity. **(A)** *In vitro* experimental design with ODN-2088; **(B)** H3-Cit and DAPI showing 2° NETs with or without ODN-2088 after mechanical disruption of 1° NETs; **(C)** Number of 2° NETs normalized to the number of DAPI+ cells per hpf with ODN-2088 (46.6 vs. 11.0, $p < 0.05$); **(D)** Number of compound 2° NETs normalized to the number of all NETs per hpf with ODN-2088 (92.7 vs. 0.90, $p < 0.05$); **(E)** Number of DAPI+ nuclei normalized to the number of 2° NETs per hpf with ODN-2088 (2.9 vs. 0.60, $p < 0.05$); **(F)** *In vivo* experimental set-up with ODN-2088; **(G)** ODN-2088 significantly reduces normalized neutrophil (1.0 vs. 0.27, $p < 0.05$) and macrophage (1.0 vs. 0.41, $p < 0.05$) counts in the mobile hindlimb 48 h after injury vs. untreated mobile controls; All *in vivo* studies had $n \geq 3$/group. All *in vitro* studies had $n = 15$ hpf/group. Scale bars are 200 μm. *$p < 0.05$.

but not PMA-induced NETosis. Consistent with our findings, previous studies have shown that NETs contain mitochondrial DNA (mtDNA) while others have shown that mtDNA released from damaged tissue after trauma induce NETosis (53). In the context of our findings, post-injury release of mtDNA from endogenous tissues may be the cause for *initial* NETosis as seen in the immobile hindlimb; however propagation of the inflammatory response caused by movement is likely due to disrupted NETs, which are not immediately cleared from the injury site. Our findings also identify a novel mechanism through TLR inhibitors may reduce inflammation, specifically by preventing NET-induced NETosis. Furthermore TLR 7/8/9, which are inhibited by ODN-2088, are oligonucleotide receptors suggesting that NET-induced NETosis is mediated through the DNA component of disrupted NETs. The specificity of these pathways remains an area for future study to identify which specific TLRs are associated with this phenomenon and whether this link might be utilized for therapeutic benefit moving forward.

Our findings shed light on a poorly understood clinical phenomenon—the exacerbation of inflammation with movement or mechanical strain. Neutrophil activity represents one of the earliest responses to injury. While movement and destabilized NETs may activate other cells including monocytes or dendritic cells, our findings suggest that the earliest propagation of inflammation is likely fueled by neutrophils activated by disrupted NETs. The identification of TLR-mediated NET-induced NETosis presents targets to mitigate inflammation, which will benefit patients who are unavoidably faced with mechanical strain involving sites of tissue damage such as those with musculoskeletal injury, joint arthropathy, ventilator-induced lung injury, and reperfusion injury (13–15).

MATERIALS AND METHODS

Ethics Statements

This study was carried out in accordance with the recommendations of the University of Michigan's Human Research Protection Program (HRPP). The protocol (HUM00044257) was approved by the Institutional Review Boards of the University of Michigan Medical School (IRBMED). All subjects gave written informed consent in accordance with the Declaration of Helsinki. Additionally, this study was carried out in accordance with the guidelines provided in the Guide for the Use and Care of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan (IACUC) under protocol (PRO0005909).

Animal Housing

All animals were housed in UCUCA-supervised facilities, not to exceed four mice housed per cage at 72 ± 4°F, receiving 12 h of light exposure each day, with no diet restrictions. For all *in vitro* and *in vivo* studies, 8–10 weeks old, mixed-gender, C57BL/6J mice from Charles River (Wilmington, MA) were defined as wild type.

Mouse Cell Isolation

Neutrophils were isolated from bone marrow collected from the femurs of wild type C57BL/6 mice using layered Histopaque gradient (Sigma-Aldrich) isolation as previously described (53). Neutrophils (1° neutrophils) were seeded at 2×10^4 cells in 500 μl RPMI 1640 (Corning) + 2% fetal bovine serum (FBS, Corning) per well to an 8-well chamber slide and incubated for 1 h at 37°C under 5% CO_2.

Human Cell Isolation

Human neutrophils were isolated as we have done previously (54). Blood from healthy volunteers was collected by venipuncture into heparin tubes, and fractionated by Ficoll/Hypaque gradient (GE Healthcare) to separate peripheral-blood mononuclear cells from neutrophils. Neutrophils were then further purified by dextran sedimentation of the red blood cell layer, before lysing residual red blood cells with 0.2% sodium chloride.

Generation of 1° Mouse NETs *in vitro*

Seeded 1° neutrophils were stimulated by the addition of 100 μL of 600 nM Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in RPMI (55–58). Unstimulated control well-received 100 μL RPMI without PMA. Treatment control groups additionally received either ODN-2088 (10 μm, Invivogen) or Cl-Amidine (200 μm, EMD-Millipore). All samples were then incubated for 4 h at 37°C under 5% CO_2. Control samples with terminal endpoints at 4 h were quenched via removal of PMA-containing medium and fixed with 10% buffered formalin for 15 min at 4°C in preparation for immunocytochemistry. All experiments were performed in triplicate.

Generation of 2° Mouse NETs *in vitro*

After incubation PMA-containing medium was removed and fresh PMA-free RPMI + 2% FBS was added. To simulate mechanical disruption of PMA-induced NETs, gentle pipette disruption was performed for 15 s in each well at a rate of ∼1 complete plunge and release of the pipette per second for an average of 15 plunges per cycle. A second group of 2×10^4 neutrophils (2° neutrophils) obtained from WT mice were added to wells with or without mechanical disruption and incubated for 4 h. Primary neutrophil only controls in the presence or absence of mechanical disruption were similarly maintained for an additional 4 h in PMA-free media without the addition of 2° neutrophils. At the time of 2° neutrophil addition treatment groups additionally received either ODN-2088 (10 μm) or Cl-Amidine (200 μm). After 4 h all samples were quenched via removal of medium and fixed with 10% buffered formalin for 15 min at 4°C in preparation for immunocytochemistry. All experiments were performed in triplicate.

Generation of 1° Human NETs *in vitro*

Purified neutrophils were resuspended in RPMI media (Gibco) supplemented with L-glutamine and 2% fetal bovine serum (Gibco). 1×10^5 neutrophils were seeded into each well of poly-L-lysine (Sigma)-coated four-chamber slides (Lab-Tek). To induce NET formation, neutrophils were incubated in the presence of 100 nM PMA (Sigma) for 3 h at 37°C and 5% CO_2. Following incubation, PMA-containing media was replaced with fresh culture media. Cells were then either left undisrupted or gently pipetted for 15 s per well to simulate mechanical disruption.

Generation of 2° Human NETs *in vitro*

An additional round of 1 × 105 neutrophils in culture media was added to each well containing primary NETs as described above. Neutrophils were further incubated in the presence of ODN-2088 or ODN-Control (final concentration of 10 μM) for an additional 3 h at 37°C and 5% CO_2. Following incubation, media was discarded, and cells were fixed with ice-cold 4% paraformaldehyde for 15 min at room temperature, followed by overnight blocking at 4°C in 10% fetal bovine serum diluted in PBS (blocking buffer).

Tenotomy Model

C57BL/6 mice underwent sterile dorsal hindlimb tendon transection at the midpoint of the Achilles tendon (Achilles' tenotomy) with placement of a single 5-0 vicryl suture to close the skin. Mice were then divided into the following groups for further intervention: mobile, delayed mobile, immobile. These groups were then further subdivided on the basis of treatment with chemical modulators of NETosis: untreated, DNase I treated, Cl-Amidine treated, or ODN-2088 treated. All subgroups had $n \geq 3$.

Immobilization

Immediately after injury, mice designated as immobile were fitted with a rigid cast immobilizer over their injured limb. Immobilizers were made from Eppendorf tubes with foam; the immobilizer was placed around the hindlimb and affixed with

glue. Rigid immobilization in this manner has previously been described (59). Mice designated as immobile were returned to their cage immediately after placement of the immobilizer. Mice designated as delayed mobile were returned to their cage for 24 h before being anesthetized for removal of their immobilizer. Mice designated as mobile were returned to their cages without placement of an immobilizer. After being returned to their cages groups were allowed to move *ad libitum* until euthanization.

DNase I Treatment

Mice treated with DNase I received 1,000 units in PBS delivered through intraperitoneal injection (1,000 U/500 μL). Within the DNase I treated groups subjects were divided on the basis of immediate or delayed DNase I injection. Mice receiving immediate DNase I injection were treated immediately after injury and/or the placement of the immobilizer. Mice receiving delayed DNase I injection were treated immediately after removal of their immobilizer 24 h post injury. All DNase I treatments occurred daily after initial injection lasting until terminal sacrifice of the animal. Groups acting as untreated controls for DNase I treatment groups received 500 μL of PBS vehicle only.

Cl-Amidine Treatment

Mice treated with Cl-Amidine received 0.2 mg in PBS delivered through intraperitoneal injection (0.2 mg/500 μL). Within the Cl-Amidine treated groups subjects were divided on the basis of immediate or delayed Cl-Amidine injection. Mice receiving immediate Cl-Amidine injection were treated immediately after injury and/or the placement of the immobilizer. Mice receiving delayed Cl-Amidine injection were treated immediately after removal of their immobilizer 24 h post injury or at the time of DNase I injection 24 h after injury. All Cl-Amidine treatments occurred daily after initial injection lasting until terminal sacrifice of the animal. Groups acting as untreated controls for Cl-Amidine treatment groups received 500 μL of PBS vehicle only.

ODN-2088 Treatment

Mice treated with ODN-2088 received 50 μg in PBS delivered through intraperitoneal injection (50 μg/500 μL). All ODN-2088 treatments occurred daily after initial injection lasting until terminal sacrifice of the animal. Groups acting as untreated controls for ODN-2088 treatment groups received 500 μL of PBS vehicle only.

Tissue Isolation and Digestion

Tissue was isolated from the tendon transection site using a standardized technique. Specifically tissue from the calcaneus (excluding bone) to the convergence of tendon with calf musculature was excised, minced at 4°C and digested. Tissue was digested for 120 min in 2 mg/ml Collagenase 3 (Worthington) in Hanks Balanced Salt Solution (HBSS) at 37°C under constant agitation. Samples were filtered using a 70-micron sterile strainer and centrifuged at 800 rpm for 5 min before removing the supernatant and washing in HBSS. This process was repeated three times before incubation with fluorescently labeled antibodies.

Flow Cytometry

Flow cytometry was performed to quantify the number of neutrophils (CD11b+Ly6G+) and macrophages (CD11b+F4/80+Ly6G–) in each sample. Antibodies used included: Ly6G-APC (Clone: 1A8, BD Bioscience; Cat. #: 560599), CD11b-V450 (Clone: M1/70, BD Bioscience; Cat. #: 560455), F4/80-PECy7 (Clone: BM8, eBioscience; Cat. #: 25-4801-82). No Fc block utilized. Following 1 h of incubation at 4°C, sample were washed and filtered through a 45-micron mesh filter before being run on a FACSAria II (BD Biosciences) Cell Sorter at the University of Michigan Flow Cytometry Core in the Biomedical Science Research Center. Samples were gated to separate debris and autofluorescent signals from the cell population. Data were then analyzed using the FlowJo software (TreeStar). Flow cytometric data was normalized to account for differences in aggregate number between cell types. For untreated group flow data was normalized to the mobile sample population. For all experiments with treated and untreated samples values were normalized to the untreated population.

Histologic Processing and Analyses

At 48 h and 1 week post-injury animals were euthanized for histology. The distal hindlimb was removed by sharp dissection at the hip. Skin was removed carefully to leave the injury site undisturbed and the toes were removed to facilitate rapid decalcification. Decalcification of the sample was completed with 19% ethylenediaminetetracetic acid (EDTA) solution for 28 days at 4°C. Decalcified tissues were dehydrated through graded ethanol, and paraffin embedded. Transverse sections from all sampled were completed with a width of 5 microns and mounted on charged microscope slides (Globe Scientific). Paraffin samples were dried overnight at 37°C. A representative subset of samples were stained for routine H&E to confirm anatomy and identify gross areas of inflammation.

Immunohistochemistry

Immunofluorescent staining was performed as previously described for Anti-Histone H3 (citrulline R2 + R8 + R17, Abcam; Cat. #: ab5103). Briefly, sections were deparaffinized and rehydrated in xylenes and graded ethanol. Antigen retrieval was performed with Citrate solution pH 6.0. Samples were then quenched for autofluorescence in 3% glycine before blocking and permeabilization. Primary antibodies were applied overnight at 4°C. Appropriate dilutions were determined prior to achieving final images. After washing, fluorescently conjugated secondary antibodies tagged with donkey anti-Rabbit IgG AlexaFluor 488 (Thermo Scientific; Cat. #: A-11008). Nuclear counterstain was DNA dye Hoechst 33342 (Thermo Scientific; Cat. #: 62249) and samples were mounted with aqueous mounting media (Sigma-Aldrich). Primary antibody, secondary antibody, and autofluorescent controls were run simultaneously with each tested sample.

Quantification of Mouse NETs by Immunocytochemistry

After fixation, *in vitro* samples were gently washed 3 times with phosphate buffered saline (PBS, Thermo Scientific).

Primary antibodies used for immunostaining included Anti-Histone H3 (citrulline R2 + R8 + R17, Abcam; Cat. #: ab5103). DNA was stained by the DNA dye Hoechst 33342 (Thermo Scientific; Cat. #: 62249). Secondary antibodies used included donkey anti-Rabbit IgG AlexaFluor 488 (Thermo Scientific; Cat. #: A-11008). After staining samples prepared with aqueous mounting media (Sigma-Aldrich) and cover-slipped for microscopy.

Quantification of Human NETs by Immunocytochemistry

NETs were detected by incubating with anti-human neutrophil elastase antibody (Abcam) for 1 h at 4°C, followed by incubation with FITC-conjugated secondary antibody (Southern Biotech) for an additional hour at 4°C. Nuclear DNA was stained with Hoechst 33342 (Invitrogen) for 10 min at room temperature. The chamber was gently removed from the attached slide, and ProLong Gold Antifade reagent (Invitrogen) was applied directly to the cells. The mount was secured in place by carefully lowering a coverslip onto the slide. Images were collected with a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek). The percentage of NETs (decondensed extracellular DNA co-localized with neutrophil elastase) was determined as the average of six to eight fields (20x). Experiments were performed at least four times (independent biological replicates).

Microscopy

All tissue sections and fluorescently stained samples were imaged using an Olympus BX-51 upright light microscope equipped with standard DAPI, 488, and TRITC reflector cubes attached to an Olympus DP-70 high resolution digital camera. H&E stained sections were imaged at 4 and 10x magnification. H3-Cit stained tissue sections were imaged at 40x magnification. All *in vitro* samples were imaged at 10 or 20x magnification. Scale bars were placed for all images with a standard 200 μm diameter. Images were visualized in Adobe Photoshop to perform to perform quantification of NET behavior and to overlay cells for co-staining.

Quantification of *in vitro* NETosis Assays

All *in vitro* experiments were performed in technical triplicates with 5 high-power fields evaluated per well (total $n = 15$ per tested group). High-power fields (hpfs) were selected using a random number generator and NETs quantified by two separate blinded observers with specific training for identification and categorization of NETs. For the purpose of *in vitro* quantification we defined a NET as: (1) a DAPI+/H3–Cit+ co-staining structure extending beyond a distinct nucleus with (2) loss of nuclear lobulation, and (3) one or more of the following: (a) ≥1 distinct branching structure (b) evidence of complete cytoplasmic disruption/rupture. Secondary NETosis is quantified as the average number of NETs as a fraction of distinct DAPI+ nuclei in each hpf. Secondary NET complexity is quantified as the fraction of NETs which exhibit one or more of the following properties: (a) >3 distinct branching structures, (b) evidence of complete cytoplasmic disruption/rupture. As a third metric we examined the ability of 2° NETs to trap other neutrophils. We defined cell trapping as the average number of distinct DAPI+ nuclei either (a) within or (b) immediately contiguous to a NET.

Single Cell Analysis

Sequencing data were first pre-processed using the 10X Genomics software Cell Ranger (10x Genomics Inc., Pleasanton, CA, USA) and aligned to mm10 genome. Cells were collected from 15 samples for four time points (4 samples for days 0, 7, and 21; 3 samples for day 3). Our time points of interest are days 3 and 7, corresponding to the peak of the inflammation. Downstream analysis steps were performed using Seurat. For each time point, cells with fewer than 500 genes per cell or more than 60,000 UMIs, and genes expressed in fewer than 10 cell were filtered out for quality control. The downstream analysis steps for each sample type include normalization, scaling, calculation of the variable genes, canonical correlation analysis, dimensionality reduction (t-SNE), unsupervised clustering, and the discovery of differentially expressed cell-type specific markers. Eight clusters were defined by unsupervised Louvain algorithm applied to the first 10 aligned canonical components. Violin plots of known markers were utilized to identify a mixed inflammatory/myeloid cluster (11,565 cells). Cells from other clusters were filtered out. We repeated the procedure, followed the method described above and found 9 sublcusters for the inflammatory/mieloyd cluster. To extract the neutrophils, firstly we characterize the subclusters we used Immgen [The Immunological Genome Project: network of gene expression in immune cells (60)]. Two subclusters (total 2,923 cells) were enriched for at two or three out of three granulocyte populations. Secondly, cells belonging to the two granulocyte-neriched subclusters were labeled as neutrophils if they were they showed a normalized expression of both Ccr1 and Csf3r $0.5 > 0$. This conditions retrieved 602 and 85 neutrophils at days for days 3 and 7.

Power Analysis and Allocation

Animal allocation was performed as described above for specific treatment groups. *A priori e*xclusion criteria for animals used in the experiments included only specimens requiring early termination of experiment for humane reasons—no animals necessitated exclusion from final statistical analysis. Animals were not formally randomized to treatment groups. Prior to animal allocation a power analyses were performed for each individual outcomes of interest. For flow cytometric analyses our outcome of interest was difference in aggregate number or neutrophils or macrophages as defined above. To confirm a 50% decrease in cell population with a power of 0.8 assuming a standard deviation of 8,000 cells and a mean of 40,000 cells in untreated mice, we required 3 mice per group. Consequently, all flow cytometric experiments were performed with $n \geq 3$ animal per group.

Statistical Analysis

All statistical analyses were performed using Student's *t*-test analysis with sample size $n \geq 3$ for all groups. Statistical analysis was performed using an appropriate analysis of

variance when more than two groups were compared, followed by a *post hoc* Student's *t*-test to directly compare two groups. Inequality of standard deviations was excluded by using the Levene's test. Outlier analysis for was performed using Grubbs' test for outliers with an alpha of 0.05. Flow cytometric data was normalized as described above. In figures, bar graphs represent means, whereas error bars represent one standard deviation. Asterisks are representative of statistical significance. Statistical significance was considered for $p < 0.05$.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Michigan's Human Research Protection Program (HRPP). The protocol (HUM00044257) was approved by the Institutional Review Boards of the University of Michigan Medical School (IRBMED). All subjects gave written informed consent in accordance with the Declaration of Helsinki. Additionally, this study was carried out in accordance with the guidelines provided in the Guide for the Use and Care of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan (IACUC) under protocol (PRO0005909).

AUTHOR CONTRIBUTIONS

SA, SJL, and BL contributed to the conception and design of the study. SA, SJL, DC, JL, GB, and SL acquired data. SA, SJL, SL, WC, MD, TS, SK, YM, PW, SM, and BL analyzed and provided critical interpretation of data. SA wrote the first draft of the manuscript. SA, SJL, SL, MD, YM, and BL wrote sections of the manuscript and/or designed figures. SA, SJL, SL, WC, MD, TS, SK, YM, PW, and BL provided critical review and edited the manuscript and/or figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02148/full#supplementary-material

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