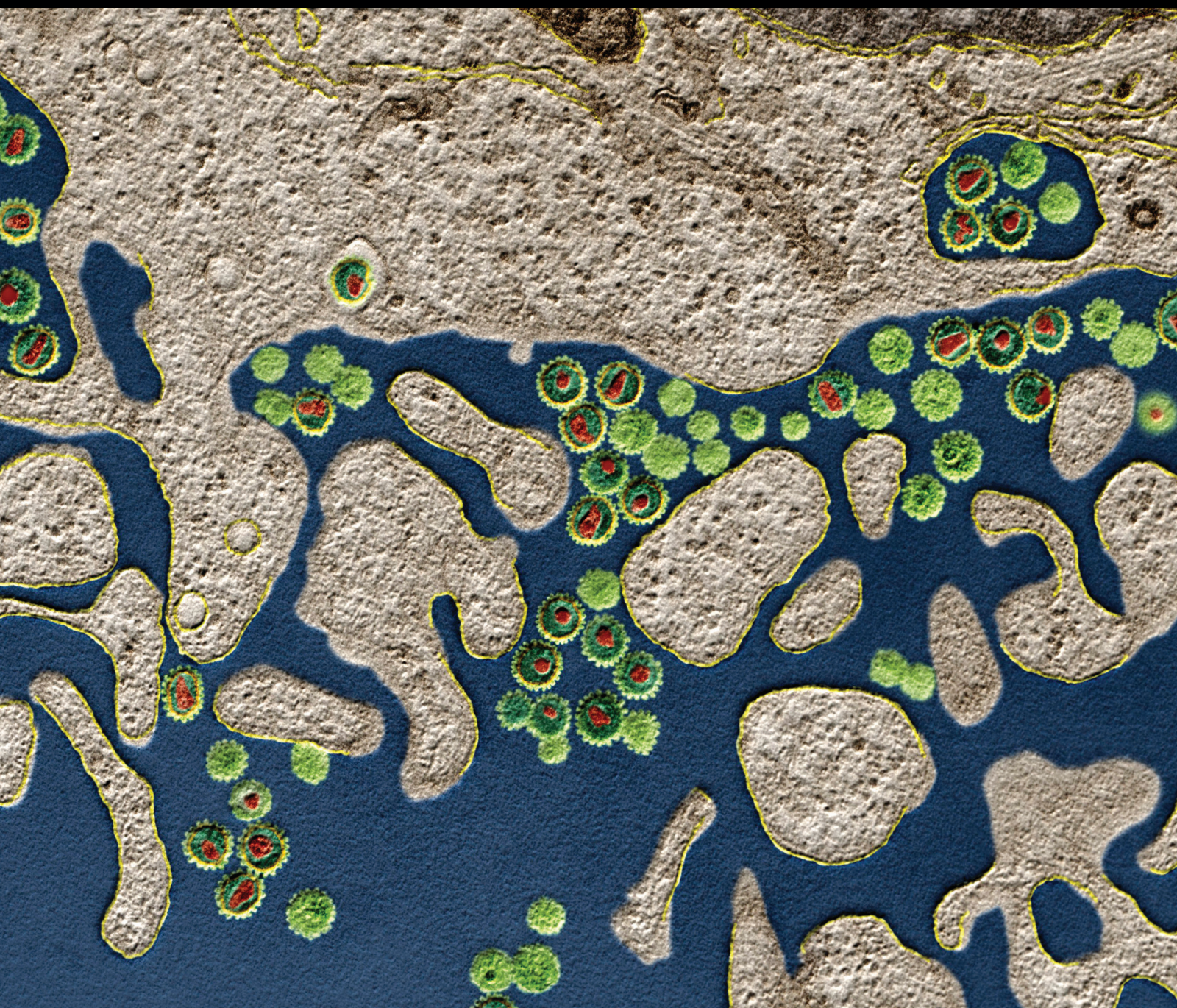
Journal of Immunology Research

Multifaceted Roles of Neutrophils in Autoimmune Diseases

Lead Guest Editor: Yi Zhao
Guest Editors: Tony N. Marion and Qian Wang



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Editorial Board

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Editorial

Multifaceted Roles of Neutrophils in Autoimmune Diseases

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Neutrophils, also known as polymorphonuclear leukocytes, are the most abundant type of granulocytes. As the most abundant type of white blood cells in most mammals, neutrophils are an essential barrier for host defense. Impaired development, maturation and function and death of neutrophils lead to abnormal conditions in the immune system, which can initiate autoimmune disorders. In response to infection or injury, neutrophils are recruited by chemokines and cytokines to areas of pathogen exposure and/or tissue damage where pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs), respectively, engage and stimulate the infiltrating neutrophils. Neutrophils acquire distinct and different phenotypic functions in response to stimulation that may include phagocytosis, degranulation, ROS production, and release of neutrophil extracellular traps (NETs) to impede pathogens or clear sterile inflammatory stimuli.

In recent years, neutrophils have emerged as players in the pathogenesis of various systemic autoimmune diseases and autoinflammatory syndromes, such as adult-onset Still's disease (AOSD) driven by innate immunity and rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and ANCA-associated vasculitis (AAV) driven by adaptive immunity. The precise role of neutrophils in inflammatory autoimmune disease is generally poorly understood. Research advances in neutrophil biology and elucidation of the role of neutrophils in systemic autoimmune disease will hopefully not only better define how neutrophils contribute to autoimmune disease but also identify potential novel therapeutic targets for treatment of inflammation and autoimmune disease.

The multifaceted role of neutrophils refers not only to how neutrophils contribute to pathogenesis in autoimmune disorders but also to how their functions are essential for the elimination of pathogens and sterile stimuli, such as nanoparticles, in health or disease conditions. In this regard, P. Yang et al. have reviewed the heterogeneous phenotypes and functions of neutrophils and discussed their flexibility and elasticity and their presentation of "different faces" in response to various disease states including autoimmunity and autoinflammation as well as cancer. H. Yang et al. have reviewed how neutrophils engage and respond to different types of nanoparticles. This topic is timely and germane to recent research and development of nanoparticles as drug delivery vehicles, each with varying potential to initiate sterile inflammation. Depending upon structure and size, nanoparticles can easily penetrate extracellular matrix barriers and engage the innate immune system. This review also summarizes NETs formation induced by various types of nanoparticles, for example, gold, silver, polystyrene, and nanodiamond. The authors also provide insight about how NETosis triggered by different nanoparticles may facilitate initiation and resolution of inflammatory responses.

RA is a chronic inflammatory autoimmune disease characterized by autoantibodies and systemic inflammation manifested as chronic synovitis, bone erosion, and articular deformity. The presence of autoantibodies against citrullinated protein antigens is thought to be a major cause of disease. It has been speculated that citrullinated proteins in RA were generated from neutrophils during NETosis. C. L. Holmes et al. determined that depending upon the stimulus,

human or mouse neutrophils produced citrullinated, uncitrullinated, or mixed citrullinated and uncitrullinated NETs *in vitro*. Interestingly, monosodium urate (MSU) crystals and *Candida albicans* induced mostly citrullinated NETs in human-isolated neutrophils. In addition, PAD4 but not PAD2 was indispensable for citrullinated proteins in NETs. W. Chen et al. reviewed neutrophil functions in RA by discussing updated concepts and regulators of neutrophil migration in RA inflammatory conditions. As a supposed initiating factor of RA, potential role of NETosis in autoantibody production was also reviewed as decision-making for therapeutic strategies for RA.

Evidence indicates that neutrophil infiltration is involved in the pathophysiology of various autoimmune diseases. Inhibition of neutrophil migration may be essential as a target for treatment of autoimmune disorders. M. V. Jones and M. Levy identified a CXCR2 antagonist as a potential inhibitor of neutrophil migration in neuromyelitis optica animal models. The potential for CXCR2 inhibitors to reduce inflammation in experimental animal models, including for neuromyelitis optica, merits further investigation. K. Orczyk and E. Smolewska tested S100A12 levels in patients with juvenile idiopathic arthritis and defined S100A12 as a potential diagnostic biomarker and prognostic indicator for juvenile idiopathic arthritis.

A consequence of immune evolution, especially for innate immune function, has been a selective emphasis for speed in recognizing and responding to potential pathogens. The replicative vigor of pathogens presents the evolutionary rationale for this selection. The emergence of PAMPs and DAMPs as the recognition signals for pathogen presence is further evidence for emphasis on speed to detect pathogens. PAMP and DAMP recognition is inherent. Neither PAMPs nor DAMPs need to be modified to be detected by innate immune cells or proteins. This system for pathogen recognition is inherently dangerous since the same system for pathogen recognition can mistakenly identify necessary self-molecules and tissues as having potential pathogenic origin. The outcome of that "mistake," of course, can be damaging inflammation and/or autoimmunity. Neutrophils are critical early in the pathogen recognition process and, consequently, critical to immune protection and potential immune damage. The reports included in this special issue document the latter and illustrate the critical role of neutrophils in autoimmune disease.

Conflicts of Interest

The authors declare no conflicts of interest with this special issue.

Yi Zhao
Tony N. Marion
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Research Article

Insight into Neutrophil Extracellular Traps through Systematic Evaluation of Citrullination and Peptidylarginine Deiminases

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In rheumatoid arthritis, an autoimmune inflammatory arthritis, citrullinated proteins are targeted by autoantibodies and thus thought to drive disease. Neutrophil extracellular traps (NETs) are a source of citrullinated proteins and are increased in rheumatoid arthritis and therefore also implicated in disease pathogenesis. However, not all NETs are citrullinated. One theory aiming to clarify the intersection of citrullination, NETs, and rheumatoid arthritis suggests that specific stimuli induce different types of NETs defined by citrullination status. However, most studies do not evaluate uncitrullinated NETs, only citrullinated or total NETs. Further, the requirement for peptidylarginine deiminase (PAD) 2 and 4, two important citrullinating enzymes in neutrophils and rheumatoid arthritis, in the formation of different NETs has not been clearly defined. To determine if specific stimulants induce citrullinated or uncitrullinated NETs and if those structures require PAD2 or PAD4, human and murine neutrophils, including from $PAD4^{-/-}$ and $PAD2^{-/-}$ mice, were stimulated *in vitro* and NETs imaged and quantified. In humans, phorbol myristate acetate (PMA), ionomycin, monosodium urate (MSU), and *Candida albicans* induced NETs with MSU and *C. albicans* inducing primarily citrullinated, PMA primarily uncitrullinated, and ionomycin a mix of NETs. Only ionomycin and *C. albicans* were strong inducers of NETs in mice with ionomycin-induced NETs mostly citrullinated and *C. albicans*-induced NETs a mix of citrullinated and uncitrullinated. Interestingly, no stimulus induced exclusively citrullinated or uncitrullinated NETs. Further, PAD4 was required for citrullinated NETs only, whereas PAD2 was not required for either NET in mice. Therefore, specific stimuli induce varying proportions of both citrullinated and uncitrullinated NETs with different requirements for PAD4. These findings highlight the complexity of NET formation and the need to further define the mechanisms by which different NETs form and their implications for autoimmune disease.

1. Introduction

Neutrophil extracellular traps (NETs) are complex webs of chromatin and proteins extruded from neutrophils during the programmed cell death process of NETosis [1]. NETs can be antimicrobial [1–4] and aid in the resolution of inflammation [5]. However, NETs also appear to be pathologic in multiple autoimmune diseases including rheumatoid arthritis [6, 7], systemic lupus erythematosus [8, 9], antiphospholipid antibody syndrome [10, 11], and small vessel vasculitis [12]. In rheumatoid arthritis, the pathology is thought to hinge on the presence of citrullinated proteins on NETs. Citrullination is the posttranslational deimination of arginine residues to citrullines, catalyzed by the peptidylarginine deiminases (PADs). Most patients with rheumatoid arthritis generate autoantibodies that bind citrullinated

proteins [13]. Since NETs are increased in rheumatoid arthritis [6, 7] and contain citrullinated proteins targeted by anti-citrullinated protein antibodies [6, 14, 15], NETs are hypothesized to be a significant source of citrullinated proteins in rheumatoid arthritis, thus driving inflammation.

However, different stimuli can produce NETs with different composition and cargo [6, 16, 17] as well as potentially different types of NETs with different roles for citrullination [18]. For example, leukotoxic hypercitrullination (LTH) generates NETs characterized by hypercitrullination and can be induced by the membrane attack complex [19] or pore-forming bacterial proteins [20]. In contrast, phorbol myristate acetate (PMA) stimulates NETosis without citrullination [16, 17]. Based on the literature, a categorization of NETs has been hypothesized with NETosis induced by several stimuli including PMA, fungi, and monosodium urate (MSU) without citrullination and LTH induced by pore-forming molecules with citrullination [18]. Such a categorization is helpful for understanding different types of NETs, their mechanisms of formation, their functions, and their potentially different roles in autoimmune disease. For example, if LTH induced by the membrane attack complex leads to hypercitrullination and NETosis induced by *Candida albicans* does not involve citrullination, then membrane attack complex-induced LTH might drive rheumatoid arthritis and *C. albicans*-induced NETosis might not. However, there is variation among reports regarding which stimuli induce NETs. For example, some studies show that ionomycin and *C. albicans* induce extensive NETs and others report that these stimuli induce few to no NETs [3, 16, 21–25]. Further, most studies evaluate either total or citrullinated NETs, so much less is known about uncitrullinated NETs. Given the gaps in the literature and the importance of understanding different types of NETs in autoimmune disease, it would be of benefit to determine which stimuli induce citrullinated and uncitrullinated NETs.

There are also questions regarding the roles of PAD2 and PAD4 in the formation of different types of NETs. These two PADs are found in neutrophils [26] and the rheumatoid joint [27] and each independently contributes to murine rheumatoid arthritis [28, 29]. Further, specific inhibitors of each of these PAD enzymes are being developed with consideration for treatment in rheumatoid arthritis [30, 31]. Many NET studies have focused on PAD4, which citrullinates histones enhancing chromatin decondensation during NETosis [32, 33]. Further, PAD4 was shown to be required for the production of NETs induced by various stimuli [2, 28, 34–38]. However, PMA inhibits PAD4 while inducing NET formation [16] and PAD4 is not required for NETs formed in response to *Klebsiella pneumoniae* [39] or *C. albicans* [25], suggesting that PAD4 may not be required for the formation of all NETs. Much less is known about the role of PAD2 in NETosis. PAD2 is present on NETs [40], but is not required for the formation of NETs in response to TNFα and LPS [28]. No other studies have investigated a requirement for PAD2 in NET formation, a problematic gap in knowledge since PAD2 appears to be required for the bulk of citrullination in a murine model of rheumatoid arthritis [28].

In this report, we systematically quantify murine and human NETs formed in response to ionomycin, PMA, MSU, and *C. albicans* and determine if they are citrullinated or uncitrullinated. We also evaluate if PAD2 or PAD4 is required for the NETs induced by these stimuli.

2. Materials and Methods

2.1. Human Subjects. This study was carried out in accordance with the recommendations of the Association for the Accreditation of Human Research Protection Program. The protocol was approved by the Institutional Review Board at the University of Wisconsin-Madison. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Human subjects 18 years or older were recruited and provided a blood sample.

2.2. Animals. Age- and sex-matched wild-type, PAD2$^{-/-}$ [41], and PAD4$^{-/-}$ [2] mice back-crossed to a DBA/1J background (Jackson Laboratories, Bar Harbor, USA) were used. Animals were housed in a pathogen-free facility. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the ARRIVE guidelines, the National Centre for the Replacement, Refinement and Reduction of Animals in Research. The protocol was approved by the University of Wisconsin Animal Care and Use Committee.

2.3. Purification and Stimulation of Human Neutrophils. The human blood was collected into EDTA tubes, and neutrophils were purified using the EasySep Direct Neutrophil Isolation Kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. Neutrophil purity was at least 95% by flow cytometry. Neutrophils were plated onto acid-washed, poly-L-lysine (Sigma Diagnostics, Livonia, USA) coated 12 mm glass coverslips at a concentration of 50,000 cells per coverslip in media containing RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) with 2% fetal bovine serum (Atlanta Biologicals, Flowery Branch, USA) and 1% penicillin-streptomycin solution (Corning, Tewksbury, USA). Neutrophils were treated with the following and incubated for 4 hours at 37°C, 5% CO_2: 4 μM ionomycin (MilliporeSigma, Darmstadt, Germany), 560 μg/mL MSU crystals (InvivoGen, San Diego, USA), 25 nM PMA (Fisher BioReagents, Waltham, USA), or 1×10^6 *Candida albicans* strain SC5314 [42].

2.4. Purification and Stimulation of Murine Neutrophils. The mouse femurs and tibias were flushed with the media described above, and neutrophils were purified with the EasySep Mouse Neutrophil Enrichment Kit (StemCell Technologies) according to the manufacturer's protocol. Neutrophil purity was at least 91% by flow cytometry. Neutrophils were plated onto acid-washed, poly-L-lysine-coated 12 mm glass coverslips at a concentration of 70,000 cells per coverslip in the media described above. Neutrophils were incubated for 4 hours at 37°C, 5% CO_2 with the following stimuli: 5 μM ionomycin, 1200 μg/mL MSU crystals, 25 nM PMA, or 1×10^6 *C. albicans* strain SC5314.

2.5. Candida albicans. *C. albicans* was prepared as previously described [28]. Briefly, *C. albicans* was stored in 15% glycerol stock at -80°C with yeast extract peptone dextrose (YPD) medium supplemented with uridine (1% yeast extract, 2% peptone, 2% dextrose medium, and 0.08% uridine) prior to the experiments. Single *C. albicans* colonies were grown overnight in YPD with uridine at 30°C and orbital shaking at 200 RPM. Planktonic cells were used by diluting cultures 20-fold and incubating and shaking for an additional 2 hours. *C. albicans* was centrifuged and washed twice with the final concentration adjusted to 4×10^7 cells/mL in phosphate-buffered saline (PBS) before use.

2.6. Immunofluorescence. After stimulation, neutrophils were processed as previously [28] for immunofluorescence. Cells were incubated for 30 minutes at 4°C with 4% paraformaldehyde, 1% NP-40, and 0.5% Triton X-100 in PBS and then washed with PBS. Coverslips were then blocked overnight with 2.5% bovine serum albumin (BSA), 5% goat serum, and 0.5% Tween-20 in PBS followed by staining for 1 hour with anti-citrulline IgM (F95, MilliporeSigma) diluted 1 : 200 in blocking solution, washing with PBS, and then incubating for 1 hour with anti-mouse IgM-TRITC (SouthernBiotech, Birmingham, USA) diluted 1 : 200 and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, USA) diluted 1 : 1000 in blocking solution, and washing with PBS. Coverslips were mounted on glass microscope slides with Aquamount (Thermo Fisher Scientific). All staining was performed at room temperature. A Leica Fluorescence Microscope with Image Pro-Plus v.6.3 (Media Cybernetics, Rockville, USA) was used to image five predetermined fields on the coverslip at 400x. For Supplementary Figures, processing, staining, and imaging were identical as above, but F95 was replaced by anti-histone H4, citrulline 3 (MilliporeSigma) and anti-mouse IgM-TRITC was exchanged for anti-rabbit IgG-TRITC (Jackson Laboratories).

2.7. Quantification of NETs. Neutrophils and NETs present in the five predetermined fields were counted by eye in a blinded manner. NETs were defined as neutrophils with significant enlargement of the DNA area beyond the size of a condensed nucleus (evident in unstimulated samples) with spread morphology and diffuse DNA structure [43]. Citrullinated NETs also stained positively with F95.

2.8. Statistics. A t-test was used to compare the percentage of NETs between untreated neutrophils and each stimulant as well as between wild-type and PAD-deficient neutrophils. A p value < 0.05 was considered significant.

3. Results

Ionomycin (a calcium ionophore and pore-forming molecule), MSU crystals (which activate leukocytes via Toll-like receptors and the inflammasome driving gout), PMA (which activates protein kinase C and thus NF-κB), and *C. albicans* are diverse and common stimulants of NETosis with innumerable connections to autoimmune disease. To determine if these stimulants induce citrullinated and/or uncitrullinated NETs, human neutrophils were isolated from peripheral blood and incubated with no treatment or each stimulant for 4 hours followed by fixation, staining to detect DNA and citrullinated proteins, imaging, and quantification. As expected and as confirmation of a lack of stimulation upon purification, untreated neutrophils generated almost no NETs (Figures 1(a) and 1(b)). Ionomycin, MSU, PMA, and *C. albicans* all induced more NETs than untreated neutrophils (Figures 1(a) and 1(b)). As shown in Figure 1(e), MSU and *C. albicans* induced primarily citrullinated NETs, whereas PMA induced mostly uncitrullinated NETs. However, PMA also induced some citrullinated NETs, more than unstimulated neutrophils (Figure 1(c)). *C. albicans* induced more citrullinated NETs as well as more uncitrullinated NETs than untreated neutrophils (Figures 1(c) and 1(d)). The ionomycin-induced NETs were a mix of citrullinated and uncitrullinated (Figure 1(e)). Given the low levels of citrullination at 4 hours after PMA or ionomycin treatment, we also quantified NETs 8 and 20 hours after PMA or ionomycin treatment. Similar numbers of citrullinated NETs were seen at those time points as compared to the 4-hour time point (data not shown). Finally, because F95 may cross-react with homocitrulline, we repeated our experiments quantifying citrullinated NETs using an antibody against citrullinated histone H4. The numbers of citrullinated NETs and uncitrullinated NETs were similar using this antibody (Figures 1(c) and 1(d) versus Supplementary Figure 2B, C), although in general slightly fewer citrullinated and slightly more uncitrullinated NETs were detected for each condition leading to some differences in the ratio of citrullinated versus uncitrullinated NETs (Figure 1(e) versus Supplementary Figure 2D).

Since mice are commonly used as an experimental model for autoimmune diseases involving NETs, we wanted to determine if findings would be similar in mice. We purified neutrophils from murine bone marrow and induced and quantified NETs as above. Unlike in human neutrophils, ionomycin was a very strong inducer of murine NETs and these structures were primarily citrullinated (Figures 2(b) and 2(e)). Also unlike in humans, MSU induced NETs variably in mice and murine MSU-induced NETs were a mix of citrullinated and uncitrullinated structures (Figures 2(b) and 2(e)). With murine neutrophils, PMA did not induce more NETs than untreated neutrophils (Figure 2(b)), which is also different than human neutrophils. Even when we stimulated with tenfold higher concentrations of PMA, similar results were seen (data not shown). However, like human neutrophils, the few NETs that were induced by PMA were uncitrullinated (Figure 2(e)). Also, PMA, and no other stimulant, led to the formation of ring-shaped structures of variable size in about 30% of murine neutrophils. Human neutrophils did not commonly make these structures as visualized by immunofluorescence, but similar structures could be seen by electron microscopy (Supplementary Figure 1) and perhaps could be called "doNETs" given their donut-like shape. As in human neutrophils, *C. albicans* was a strong inducer of NETs (Figure 2(b)) and *C. albicans* induced a mixture of citrullinated and uncitrullinated NETs (Figure 2(e)). Of note, both human

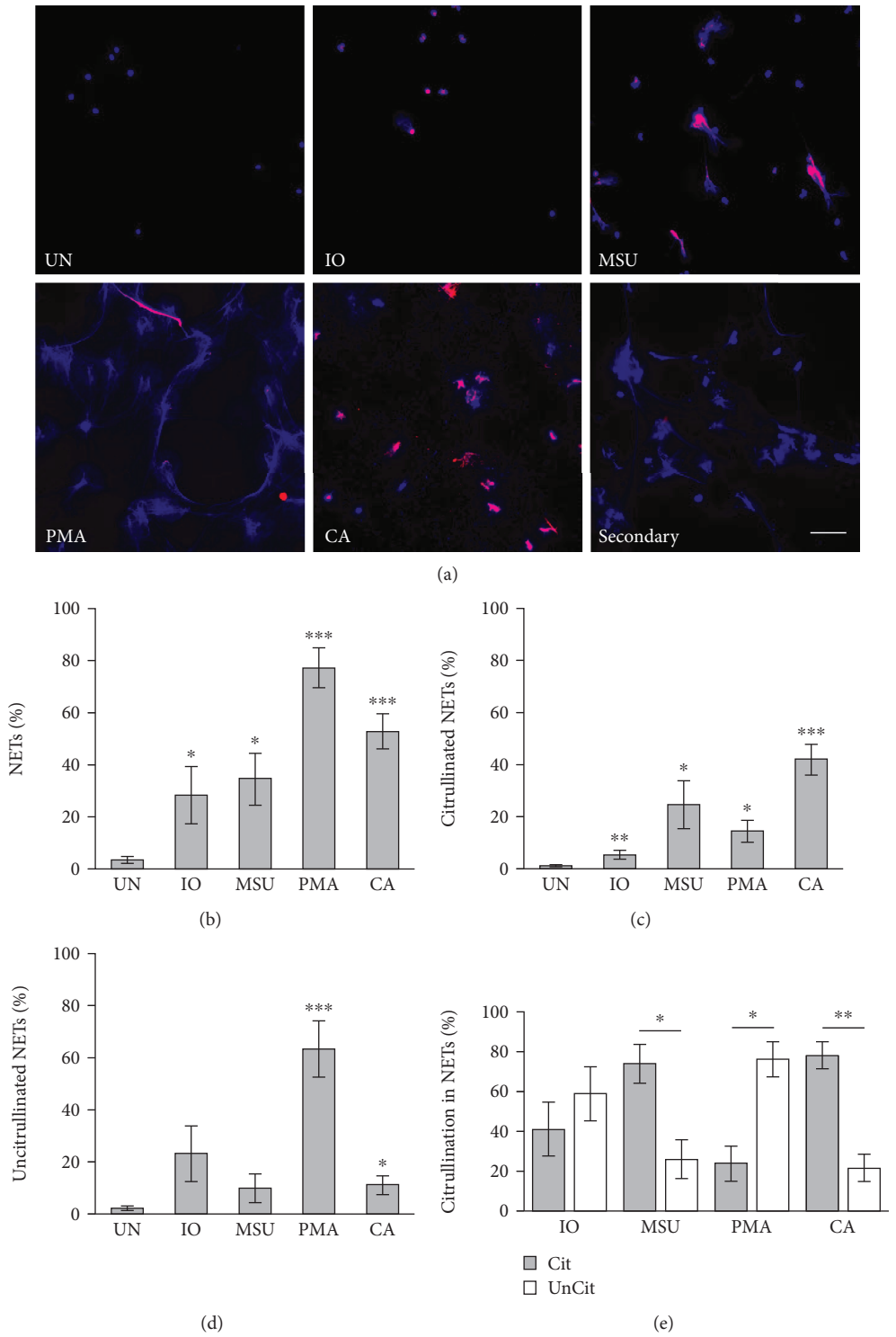

Figure 1: Induction of NETs in human neutrophils. Human neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, or *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). Image labeled "Secondary" was created by stimulating neutrophils with *C. albicans* and staining without the F95 primary antibody and only the anti-mouse IgM-TRITC secondary antibody as a negative control. (a) Representative images at 400x, scale bar = 50 μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (b), citrullinated NETs (c), and uncitrullinated NETs (d) for each condition with percent NETs for each stimulant compared to untreated. (e) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels: $n = 9$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

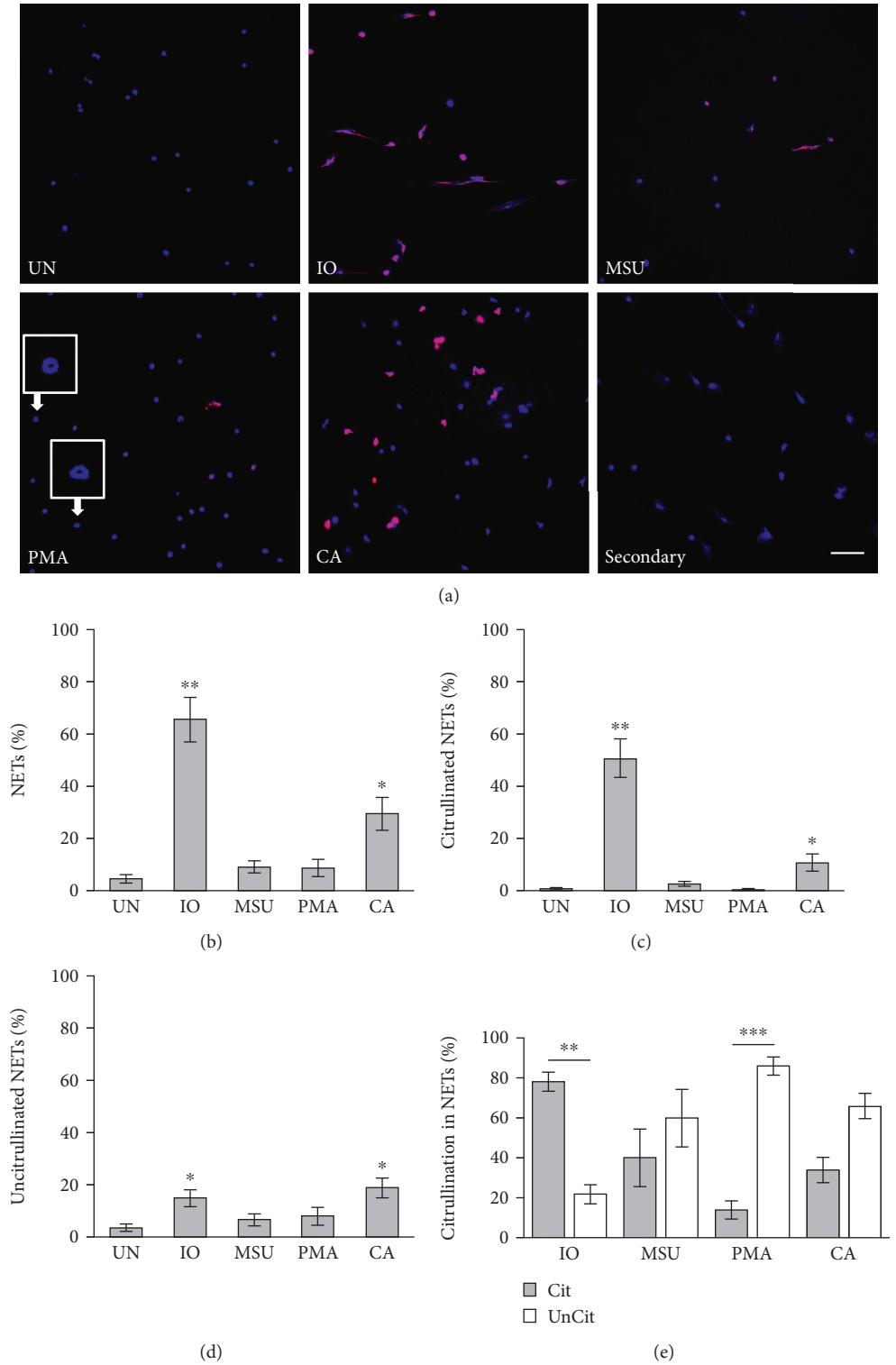

Figure 2: Induction of NETs in murine neutrophils. Murine neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). Image labeled "Secondary" was created by stimulating neutrophils with ionomycin and staining without the F95 primary antibody and only the anti-mouse IgM-TRITC secondary antibody as a negative control. (a) Representative images at 400x, scale bar = 50 μM. Enlarged insets demonstrate donut-like structures (doNETs). The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (b), citrullinated NETs (c), and uncitrullinated NETs (d) for each condition with percent NETs for each stimulant compared to untreated. (e) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels: $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

and murine *C. albicans*-induced NETs were smaller in size compared to NETs induced by other stimuli. Finally, like human neutrophils, similar proportions of citrullinated and uncitrullinated NETs were seen with activation for 8 and 20 hours as compared to 4 hours (data not shown). Results for F95 agreed with results for anti-citrullinated histone H4 with very small differences seen only for PMA likely due to the low number of NETs with this condition (Supplementary Figure 3).

We then determined if PAD4 is required for the formation of NETs in response to the selected stimuli. Identical experiments as above were performed using bone marrow-derived neutrophils from $PAD4^{+/+}$ and $PAD4^{-/-}$ mice. As shown in Figures 3(a)–3(d), $PAD4^{-/-}$ neutrophils generated almost no citrullinated NETs in response to any stimulus, with similar numbers of uncitrullinated NETs to $PAD4^{+/+}$ neutrophils for all stimuli. The absence of citrullinated NETs led to a loss of total NETs in response to ionomycin, which primarily induces citrullinated NETs in mice. We then used identical methods and $PAD2^{-/-}$ and $PAD2^{+/+}$ mice to determine if PAD2 is required for NETosis. As shown in Figures 4(a)–4(d), $PAD2^{-/-}$ mice showed no difference in the number of either citrullinated or uncitrullinated NETs induced by any stimulus. Repeating both the PAD4 and the PAD2 experiments using anti-citrullinated histone H4 showed the same findings: a loss of citrullinated NETs in the absence of PAD4 and no loss of NETs in the absence of PAD2 (Supplementary Figures 4 and 5).

4. Discussion

In this study, we quantified the formation of citrullinated and uncitrullinated NETs in response to ionomycin, PMA, MSU, and *C. albicans*. One conclusion from our studies is that human peripheral blood and murine bone marrow-derived neutrophils respond differently to stimuli. For example, ionomycin induced 66% of neutrophils to form NETs in mice and 28% in humans while PMA induced 9% of neutrophils to form NETs in mice and 77% in humans (Figures 1 and 2). Additionally, MSU was a strong inducer of NETs in human neutrophils and a variable inducer in murine neutrophils. Although some differences may be due to the location from which the neutrophils were purified (i.e., peripheral blood versus bone marrow) and thus maturation level, these findings suggest that NET production varies with the source of neutrophils, which may contribute to conflicting reports about the ability of different stimuli to induce NETs [21]. Other studies have identified a species-specific difference related to myeloperoxidase [44].

Regarding citrullination status, mice and humans often diverged again. For example, ionomycin-induced murine NETs were primarily citrullinated, whereas ionomycin-induced human NETs were a mix of citrullinated and uncitrullinated. *C. albicans* was a strong inducer of NETs in both mice and humans as previously shown [4, 23, 25, 45, 46] with primarily citrullinated NETs formed in humans and citrullinated and uncitrullinated in mice. Similarly, MSU induced mostly citrullinated NETs in humans and a mix in mice. In addition to highlighting the differences between mice and humans, our findings and the findings of others [25] do not support the theory that *C. albicans* or MSU inhibits citrullination [18]. In contrast and as expected [16, 17], for both humans and mice, PMA induced primarily uncitrullinated NETs, although some citrullinated NETs formed, particularly in humans. In neutrophils, PMA rapidly induces reactive oxygen species [17], which is required for PMA-induced NETs [38] and can inhibit PADs [47], perhaps explaining the relative lack of citrullination in addition to a reported role for PMA-induced protein kinase C alpha in PAD4 inhibition [16]. Of note, no stimulant in this study induced exclusively citrullinated or uncitrullinated NETs, a novel observation. It is possible that some citrullinated proteins were not detected by the F95 antibody and the immunofluorescence methodology, although F95 recognizes a variety of citrullinated proteins. We observed similar results using an anti-citrullinated histone H4 antibody (Supplementary Figures 2–5), although there were some differences, primarily in humans, potentially related to F95 detecting homocitrulline or the reactivity of anti-citrullinated histone H4 against only a single citrullinated protein.

Nonetheless, by quantifying both citrullinated and uncitrullinated NETs, we demonstrated that specific stimuli induce varying proportions of both citrullinated and uncitrullinated NETs in mice and humans, providing new insights into NETs. Although the citrullinated and uncitrullinated NETs could be categorized as resulting from LTH and NETosis, we did not observe that specific stimuli strictly induced either LTH with citrullination or NETosis without citrullination. Thus, the combination of stimulus and citrullination presence/absence may not be ideal for defining different NETs. Moreover, the generation of both citrullinated and uncitrullinated NETs in response to a single stimulus suggests that individual neutrophils may employ different pathways to generate NETs, sometimes involving citrullination and sometimes not. It will be important to further characterize the different mechanisms by which NETs with different characteristics form, since these differences may have important implications for autoimmune disease, especially rheumatoid arthritis with its citrulline-targeting autoantibodies.

Additionally, since we evaluated the requirement for PAD4 in both citrullinated and uncitrullinated NETs, whereas other groups evaluated either total or citrullinated NETs, we were able to demonstrate for apparently the first time that PAD4 is required for the production of citrullinated, but not uncitrullinated, NETs. These findings help to explain some of the discrepancies in the literature. Multiple studies have shown a requirement for PAD4 in NETosis [2, 28, 34–38]. However, many of these studies quantified citrullinated NETs. More recently, PAD4 was shown to be dispensable for *Klebsiella*-induced NET [39] and *C. albicans*-induced NET [25], in both cases with NETs detected primarily by DNA staining. Thus, some of the discrepancies among PAD4 studies may relate to whether only citrullinated NETs or total NETs were quantified. Indeed, PAD4 is required for histone citrullination induced by *Klebsiella* and *C. albicans* [25, 39]. Other discrepancies related to the

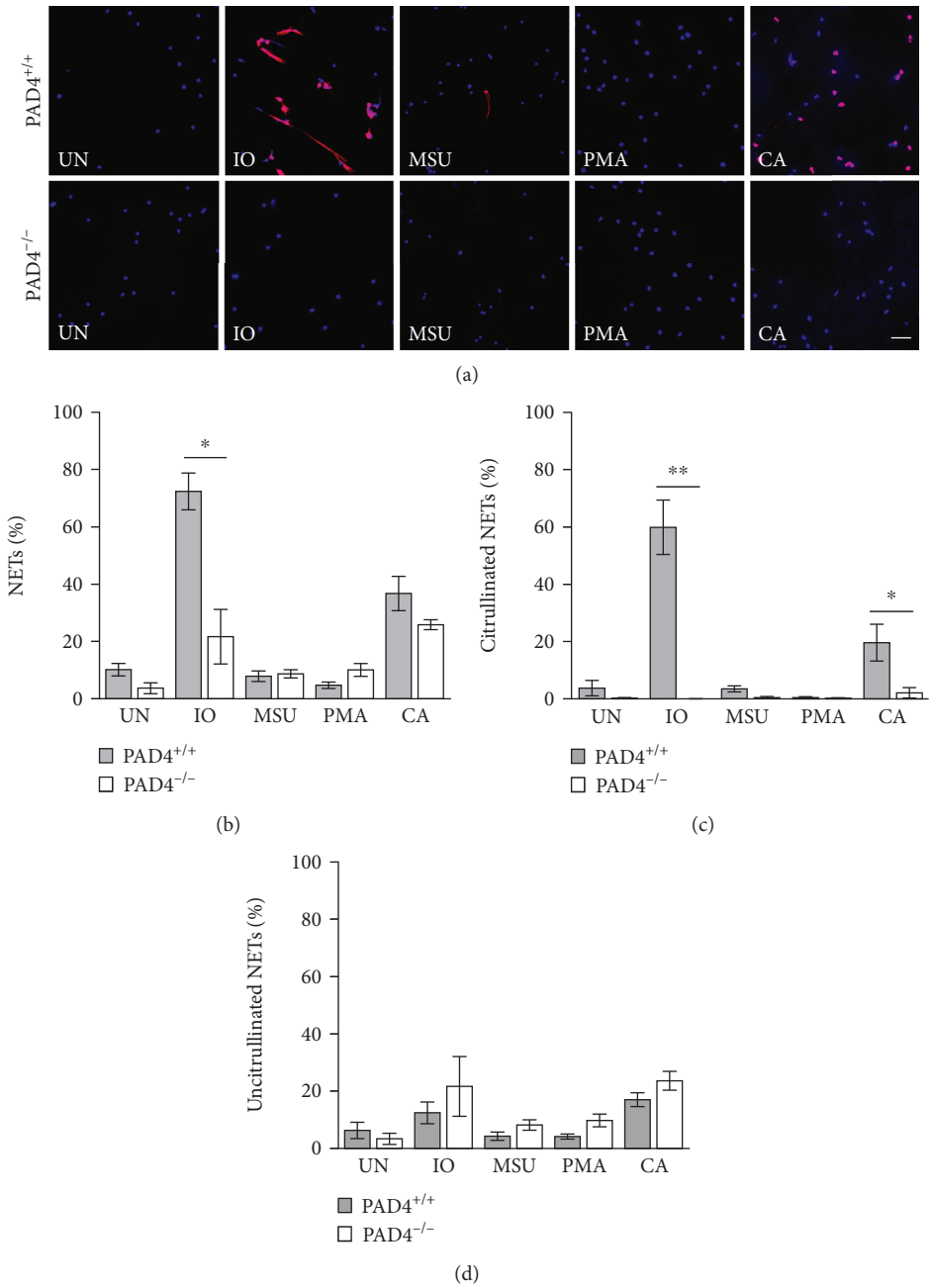

Figure 3: PAD4 is required for the formation of citrullinated NETs in murine neutrophils. Bone marrow neutrophils from PAD4$^{+/+}$ and PAD4$^{-/-}$ mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). (a) Representative images at 400x, scale bar = 50 μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (b), citrullinated NETs (c), and uncitrullinated NETs (d) for each condition with percent NETs for each stimulant compared between PAD4$^{+/+}$ and PAD4$^{-/-}$ mice. For all panels: $n = 4$; $^{*}p < 0.05$ and $^{**}p < 0.01$.

role for PAD4 in NETosis may be due to methodology. For example, in a study that concludes that PAD4 is not required for ionomycin-induced NETs [25], the NETs were quantified by increased SYTOX fluorescence, not visualized NETs. Since ionomycin can form pores, perhaps those pores allowed SYTOX entry and DNA staining without NET formation. Our observation that PAD4 is required for the production of only citrullinated NETs also suggests that the formation of different NETs can have different requirements. Thus, it is important to assess both citrullinated and uncitrullinated NETs.

Finally, we evaluated PAD2 in NETosis. Previously, we demonstrated that PAD2 is not required for NETs induced by LPS and TNFα [28]. Here, we found that PAD2 is not required for the production or citrullination of murine NETs induced by ionomycin, MSU, PMA, or *C. albicans*, suggesting that PAD2 is not required for NETosis in general. Thus, although PAD2 is present in NETs [40], it is

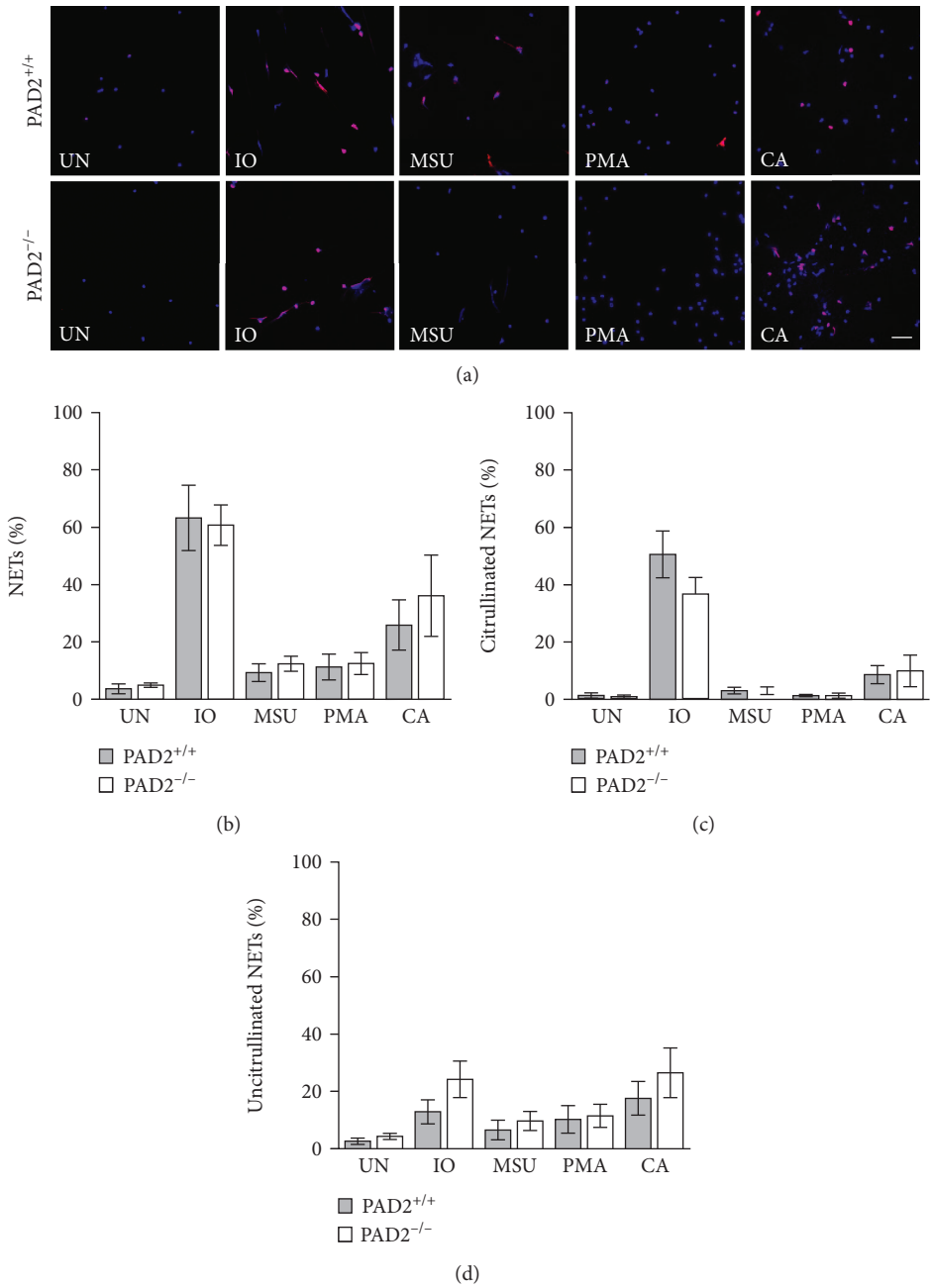

Figure 4: PAD2 is not required for the formation of NETs in murine neutrophils. Bone marrow neutrophils from PAD2$^{+/+}$ and PAD2$^{-/-}$ mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). (a) Representative images at 400x, scale bar = 50 μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (b), citrullinated NETs (c), and uncitrullinated NETs (d) for each condition with percent NETs for each stimulant compared between PAD2$^{+/+}$ and PAD2$^{-/-}$ mice. For all panels: $n = 4$; no comparisons were significant.

not required for their formation. This finding has interesting implications for rheumatoid arthritis. NETs have been hypothesized to be a significant source of citrullinated protein in rheumatoid arthritis [6, 7]. We, and others, have shown that PAD4 is required for the formation of citrullinated NETs [2, 28, 35]. However, in PAD4-deficient mice with inflammatory arthritis, total citrullination is not reduced in the serum, lung, or joint [28, 29, 48]. In contrast, PAD2 is required for a significant amount of citrullination in the joints of mice with inflammatory arthritis [28], but is not required to form citrullinated NETs. Although there are challenges related to the quantification of citrullination, taken together, these studies suggest that although NETs display citrullinated proteins targeted by anti-citrullinated protein antibodies [6], NETs may not be the main source of citrullinated proteins in rheumatoid arthritis. This theory is supported by the observations that PAD2 levels in synovial fluid correlate

with total PAD activity and disease activity in rheumatoid arthritis [49], PAD2$^{-/-}$ mice have less central nervous system citrullination in experimental autoimmune encephalomyelitis [41], and PAD2 has less restrictive substrate specificity than PAD4 [50].

5. Conclusion

This study demonstrates that various stimuli induce a mix of citrullinated and uncitrullinated NETs in mice and humans. Further, PAD4 is required for citrullinated NETs and PAD2 is not required for citrullinated or uncitrullinated NETs. Future studies are needed to further define different NETs, their mechanisms of formation, and their roles in the pathophysiology of autoimmune disease.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: Scanning electron micrographs of doNETs formed by PMA-stimulated human neutrophils. Supplementary Figure 2: Citrullinated NETs in human neutrophils detected by anti-citrullinated histone H4. Supplementary Figure 3: Citrullinated NETs in murine neutrophils detected by anti-citrullinated histone H4. Supplementary Figure 4: PAD4 is required for the formation of citrullinated NETs in murine neutrophils as detected by anti-citrullinated histone H4. Supplementary Figure 5: PAD2 is not required for the formation of citrullinated NETs in murine neutrophils as detected by anti-citrullinated histone H4. *(Supplementary Materials)*

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Review Article

Different Faces for Different Places: Heterogeneity of Neutrophil Phenotype and Function

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As the most abundant leukocytes in the circulation, neutrophils are committed to innate and adaptive immune effector function to protect the human body. They are capable of killing intruding microbes through various ways including phagocytosis, release of granules, and formation of extracellular traps. Recent research has revealed that neutrophils are heterogeneous in phenotype and function and can display outstanding plasticity in both homeostatic and disease states. The great flexibility and elasticity arm neutrophils with important regulatory and controlling functions in various disease states such as autoimmunity and inflammation as well as cancer. Hence, this review will focus on recent literature describing neutrophils' variable and diverse phenotypes and functions in different contexts.

1. Introduction

Neutrophils, considered as infantrymen in the innate immune system, are indispensable in safeguarding the human body against encroaching microbes. Generated from the bone marrow and circulating in the blood, neutrophils are a critical "second defense" standing behind the skin and mucus. As precursor leukocytes to be enlisted in inflammatory sites [1], neutrophils possess the capacity of both intra- and extracellular mechanisms [2, 3] to eliminate pathogens. Their very singular features, such as short lifespan and lower transcriptional activity, have led to the overly simplistic perception that neutrophils are homogenous with limited phenotypic heterogeneity. However, this classical view has been greatly challenged since different phenotypes have been reported in both healthy and pathologic conditions. Are all neutrophils generated equally? Do they share the same phenotypes in different environments? For those who know the Face Changing Dance of the traditional Chinese Sichuan Opera, you may appreciate the dance as a metaphor for the heterogeneity of neutrophil phenotype and function. Just like the performance of face changing in the Chinese Sichuan Opera, neutrophils resemble the actors expressing "different faces" in different conditions and places. We describe the elasticity of neutrophils and discuss their multiple phenotypes and functions.

2. Growth Footprint of Neutrophils

As the major activity of the bone marrow, almost two-thirds of the hematopoiesis is dedicated to myelopoiesis [2], and around 1 to 2×10^{11} neutrophils are generated every day. Granulopoiesis is under the control of multiple physiological and environmental cues. The feedback loop of IL-23, IL-17A, and granulocyte colony-stimulating growth factor (G-CSF) is vital to the regulation of granulopoiesis. Phagocytosis of apoptotic neutrophils by macrophage and dendritic cells depresses their production of IL-23, thus reducing IL-17A production by T cells and neutrophils, which leads to the downregulation and reduced production of G-CSF by fibroblasts and epithelial cells and reduction in neutrophil generation [4–6]. By contrast, the upregulation of G-CSF increases granulopoiesis and triggers chemokine receptor type 2 (CXCR2) signaling and neutrophil release [7, 8].

There are six stages in neutrophil maturation: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear [2], during which the transcription factors C/EBPα (CCAAT/enhancer-binding protein α) and ETS (E26 transformation specific) family transcription factor

PU.1 (Spi-1) play fundamental roles. The balance between the two factors maintains a precise state of myeloid lineage commitment to granulocyte or monocyte. PU.1 is essential for monocyte differentiation while C/EBPα promotes granulocyte differentiation [9–14]. Other transcription factors including Lef-1, Gfi-1, and C/EBPε are also conductive to terminal granulopoiesis [15–20].

3. Circulating Neutrophils: Fresh and Aged

Though neutrophils have a half-life of only a few hours in the circulation, they nonetheless achieve phenotypic heterogeneity before migrating into tissues (Figure 1). It has been demonstrated *in vivo* that during a four-hour circulation in peripheral blood from the beginning of release from the bone marrow to clearance by macrophages, neutrophils change phenotype and morphology. This progression from fresh, new bone marrow emigrants to aged neutrophils and total number of neutrophils is regulated in a circadian way [21, 22].

CXCR4 and CXCR2 play vital roles in neutrophil retention in the bone marrow. WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis) is clinically characterized by the overaccumulation of neutrophils in the bone marrow, which can be linked to a mutation of CXCR4 [23]. Deletion of CXCR4 or CXCR2 has a similar negative effect on neutrophil migration from the bone marrow to circulation [8, 24]. Neutrophils isolated from fresh blood have upregulated CXCR4 expression after four hours' culture *in vivo* [25]. Higher expression of CXCR4 combined with lower expression of CD62L promotes longer residency of neutrophils in circulation *in vivo* [21].

As for the aged neutrophils, some membrane molecules are increased including CD11b (αM) and CD49d (α4), the alpha subunits for integrins Mac-1 (macrophage-1 antigen, also known as $\alpha_M\beta_2$), VLA4 (very late antigen 4, also known as integrin α4), TLR4 (Toll-like receptor 4), ICAM-1 (intercellular adhesion molecule-1), CD11c, CD24, and CD45 [26] while the expression of CD47, which promotes resistance to phagocytosis by macrophages, is downregulated [27]. The classical TLR4 agonist lipopolysaccharide (LPS) induced age-dependent changes in human neonatal neutrophil migration, gene expression, and cytokine production [28]. In addition to the aforementioned molecular changes, morphological alterations appear as smaller size, fewer granules, and a more multilobular nucleus in aged cells [21]. Signaling pathways related to cell activation, pathogen recognition, cell adhesion, migration, and apoptosis are modified in aged cells [27]. All of these changes assist mature circulating neutrophil migration to inflamed tissues and are consistent with similar changes in sites of inflammation [22].

4. Neutrophil Extravasation: Go to the Battlefront

4.1. Rolling. The very distinguished feature of neutrophil extravasation starts from the activation of endothelial cells. There are two ways to initiate this process: endothelial cells can be directly stimulated by surrounding pathogens or indirectly, irritated by inflammatory mediators released from resident leukocytes like tumor necrosis factor α (TNF-α), IL-1 and IL-17 [1, 29, 30]. Subsequent changes take place on the luminal surface where the expression of E-selectin, P-selectin, and α and β integrins is upregulated. Binding between cell surface glycoproteins such as P-selectin ligand 1 (PS GL-1) and P-selectin helps capture free neutrophils to the endothelial surface. E-selectin's binding with E-selectin ligand 1 (ESL-1) helps slow neutrophil rolling speed, and binding with CD44 leads to a distribution change of PSGL-1 and L-selectin, which also contributes to further reduction of rolling speed [31].

4.2. Adhesion. When it comes to the process of firm adhesion, β2 integrins LFA-1 (lymphocyte function-associated antigen 1, also known as $\alpha_L\beta_2$) and MAC-1, together with their ligands ICAM-1 and ICAM-2 expressed on endothelial cells, are key molecules. LFA-1 binding to ICAM-1 initiates a change in neutrophil motion from rolling to adhesion [32], while the activation of LFA-1 depends on signals from PS GL-1 and CD44 [33]. The activation of G protein-coupled chemokine receptors on neutrophils leads to the conformational change of cell surface integrins that subsequently show higher affinity for their ligands. Overall, the link between integrins and ligands strengthens outside-in signaling pathways in neutrophils, reinforcing adhesion and initiating cell motility [1].

4.3. Crawling. When neutrophils set about migrating across endothelial cell-cell junctions, it is necessary for them to crawl effectively. Considering the existence of shear force in the blood flow, crawling vertically would be the best way to move to the endothelial junction. Neutrophils are endowed with the capacity to crawl forward while retaining the adhesion to the endothelial surface. Actin-binding proteins such as mammalian actin-binding protein 1 (MABP1, also known as drebrin-like protein) fortify the high-affinity conformation of β2 integrins to strengthen the interactions with actin, helping neutrophils crawl stably in the shear condition of blood flow. Additional signaling by VAV1 (a guanine exchange factor for the RHO-family GTPase RAC) and CDC42 (cell division control protein 42, a major regulator of organization of the actin cytoskeleton during leukocyte polarization and migration) contributes to neutrophil crawling [34] although the detailed mechanism is still poorly understood.

4.4. Migration. Particular types of migration include transmigration, abluminal crawling, and interstitial migration to inflamed foci. Transmigration requires integrins and CAMs (ICAM-1, ICAM-2, and VCAM-1 (vascular cell adhesion protein 1)) as well as various junctional proteins, such as CD31, CD99, CD155, and CD157 (reviewed in reference [1]). There are two ways for neutrophils to pass through the endothelium: paracellular and transcellular. Most neutrophils use paracellular migration, which is more efficient and takes shorter time (about 2-5 minutes) [1]. In transcellular migration, endothelial cells form microvilli-like transmigrating cups, which are projections enriched in ICAM-1 and

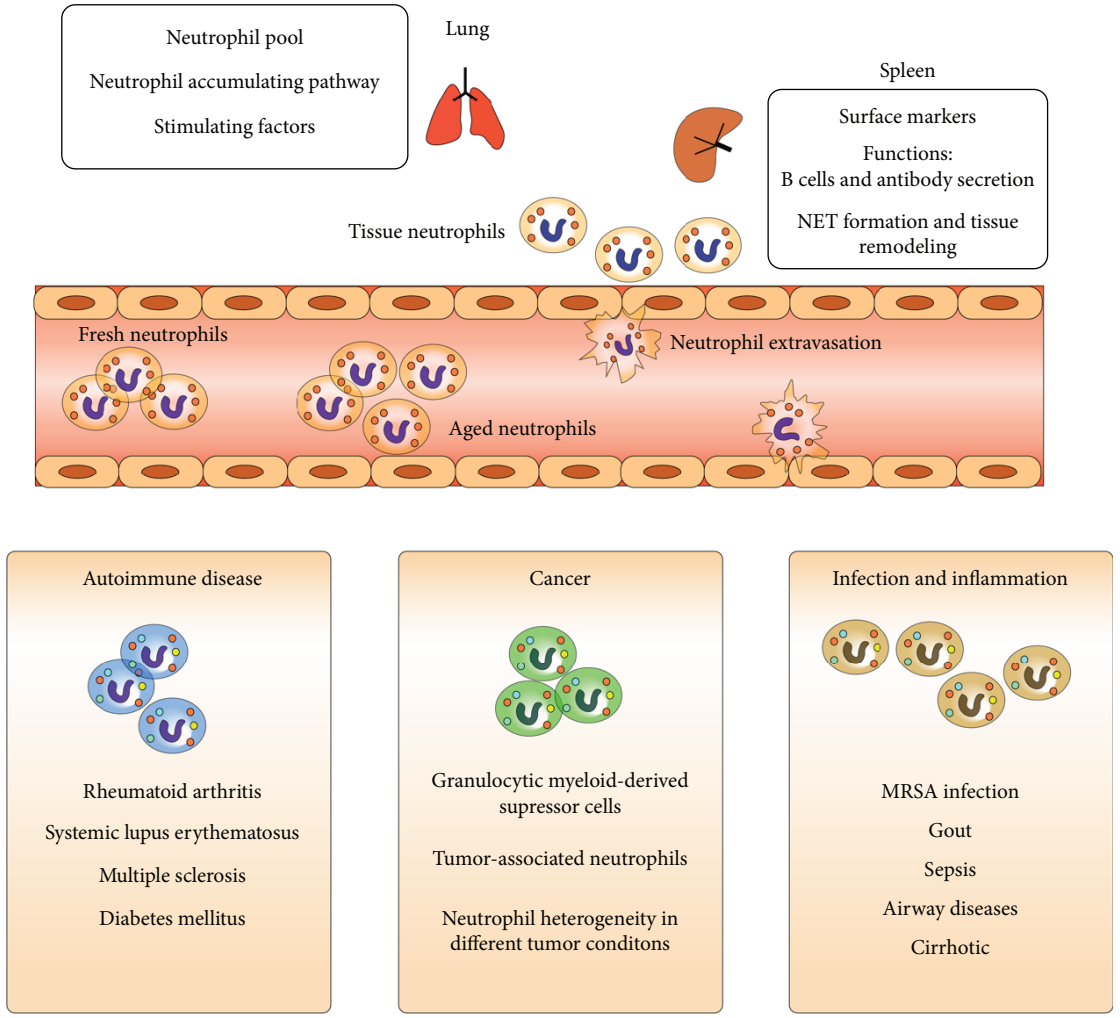

Figure 1: Heterogeneity of neutrophils in both health and disease. Neutrophils protect human body from intruding microbes and they display notable heterogeneity in blood circulation and specific tissues. After being activated by pathogens, neutrophils extravasate from the blood vessel and function as "immune soldiers" in various states. Intriguingly, neutrophils are verified to share multiple phenotypes and functions in autoimmune disease and cancer as well as inflammation and infection.

VCAM-1, and form cuplike structures around adherent neutrophils in an LFA1- and VLA4-dependent manner [35, 36]. What is noticeable is that transcellular migration differs from phagocytosis, as the neutrophils *ipso facto* never enter the intracellular compartment of the endothelial cell.

The endothelial basement membrane consists of extracellular matrix containing collagens and laminins. Proteases in granules (described in detail below) vest neutrophils with the ability to break down the matrix, such as elastase (azurophil granules), matrix metalloproteinase 8 (MMP8) (specific granules), MMP9, and the membrane-attached matrix metalloproteinase MT6-MMP (gelatinase granules and secretory vesicles) [37].

Rab small GTPases (guanosine triphosphatases) belong to the Ras superfamily and are constituted of around 60 family members in mammals. Rab family GTPases play crucial roles in intracellular membrane trafficking, including cargo sorting, vesicle budding, vesicle formation, vesicle transport, docking, tethering, and fusion of vesicles with target membranes in eukaryotic cells [38]. Rab27, a Rab subfamily member which is known to control granule exocytosis, has two main isoforms: Rab27a and Rab27b. Rab27 stimulates elastase release from azurophilic granules, thus allowing local proteolysis of the extracellular domain of CD11b leading to uropod detachment and forward movement of the cell [39, 40]. Neutrophil proteases and its intracellular transport system provide the means for neutrophils to move through the extracellular matrix, but the complete mechanism for neutrophil extravasation requires more detailed understanding.

5. Tissue-Residing Neutrophils: One or More?

Recruitment and migration to tissues and organs are necessary processes for neutrophil functions [41]. Pattern recognition receptors (PRRs) expressed in local epithelial, endothelial, and dendritic cells, for example, such as TLRs and NOD-like receptors (NLRs), can be triggered in both infected and noninfected conditions. That activation may lead to increased vascular permeability and the release of

chemokines to enhance neutrophil recruitment to the infected sites [1, 42]. So, neutrophils that have migrated into tissues are more active as phagocytic cells than blood neutrophils. Do all of extravasated neutrophils share a similar phenotype, or are they *per se* heterogeneous? Recent evidence is supportive for multiple phenotypes among tissue-resident neutrophils in different organs and sites.

In contrast to other organs, the lung is outstanding because of the high numbers of neutrophils that accumulate in pulmonary vessels. β2-integrins and very late antigen 4 (VLA4) are necessary for neutrophil adhesion, transmigration, and diapedesis into the lung tissue [1]. The smaller diameter of capillary segments (around 5 μm) enhances extravasation of neutrophils by strengthening vessel wall contacts since the diameter of a neutrophil is 7-8 μm. On the other hand, that change shows a high demand for cell deformation [43, 44], which is regulated by multiple factors, including anaphylatoxin C5a, chemotactic tripeptide fMLF (N-formyl-Met-Leu-Phe) [45–48], and bacterial compounds such as LPS [45, 49]. Neutrophils that collect in vascular lumen and interstitial space are maintained via a CXCR4-dependent pathway [50]. Since CXCR4 is expressed on aged neutrophils, it is possible that they may be the first class of neutrophils to migrate into the lung tissue [22], so the lungs may be a pool for primed neutrophils.

In the splenic marginal zone, neutrophils are capable of producing various cytokines, particularly IL-21, BAFF, APRIL, and TNF, to promote B cell differentiation and antibody production [51]. They are $CD62L^{low}$ $CD11b^{hi}ICAM\text{-}1^{hi}$ and also assist in the formation of neutrophil extracellular traps (NETs, described in detail below) [52]. The neutrophils in draining lymph nodes play a direct role in phagocytic killing and also protect against pathogens via noncell autonomous mechanisms including the release of NETs, producing matrix metalloproteases and participating in tissue remodeling [53], but the further impact of macrophages removed by neutrophils remains unknown. It has been well studied that CXCR4 is necessary for the migration of neutrophils to lymph nodes, but the function of CCR7 is controversial since neutrophils could migrate to draining lymph nodes through a CCR7-independent way following *S. aureus* infection [54]. Besides, the β2 integrin CD11b also plays an important role in neutrophil migration from lymph vessels to lymph nodes [55], but the precise ligand has yet to be unambiguously determined [56].

6. Neutrophil Clearance and Reversed Migration

Tissue-resident neutrophils undergoing apoptosis are finally removed by neighboring macrophages and dendritic cells via phagocytosis. This process forms a feedback loop to control the production of neutrophils in the bone marrow [6]. Neutrophils in inflamed tissue are traditionally considered to be phagocytosed by macrophages; however, other research has indicated that extravasated neutrophils may reenter the circulation [57–59]. These reverse migrating neutrophils are considered to migrate to remote infected tissues to kill microbes, which makes effective utilization of neutrophils' capacity to fight infection [1].

7. Granule Biogenesis and Heterogeneity

Granules are the notable features of neutrophils containing various proteins to kill phagocytosed pathogens and digest damaged tissues. There exists a continuum in granules manifesting as four unique subsets: primary (azurophilic) granules, containing myeloperoxidase (MPO) and azurocidin; secondary (specific) granules, containing lactoferrin; tertiary (gelatinase) granules, containing MMP9 (gelatinase B); and secretory vesicles. The granule subsets are formed in a consecutive manner during granulopoiesis [60–62]. Azurophil granules are formed at the promyelocyte stage; the other granule subsets are formed during the myelocyte to segmented cell stage differentiation. The chronologic heterogeneity of granulopoiesis can be clarified by a mechanism called "targeting by timing of biosynthesis" [63], and the formation of granule proteins is confined to relevant stages of myelopoiesis. Intriguingly, the timing of granule subtype synthesis is not entirely concomitant with the expression of granule protein, which results in heterogeneity within granules of the same granule subtype [62].

In general terms, granule heterogeneity is revealed by neutrophil function and activity. Primary granules accumulate multiple antibacterial proteins such as MPO, azurophil, cathepsin G, elastase and proteinase 3, lysozyme [64, 65], enzymatic inactive CAP37 (protease cationic antimicrobial protein of 37 kDa) (azurocidin) [66], NSP4 (neutrophil serine protease 4) [67], and defensins. The heterogeneity of azurophil granules exists in both protein content and subcellular targeting [62]. Some granules are transported to the cell surface while others fuse with phagosomes. A vital member of the Rab GTPases family, Rab27, along with its effector, mammalian uncoordinated 13-4 (Munc13-4), regulates the targeting of the granules. Interaction between Rab27a and Munc13-4 mediates degranulation of neutrophils [68–70]. Secretory granules express Rab27 and fuse with the cell membrane via a Munc13-4-dependent mechanism, while nonsecretory granules fuse with phagosomes by a Munc13-4-independent mechanism. These mechanisms might be associated with changes in concentration and types of proteins contained in the fusing granules. It has been reported that nonsecretory granules display a high concentration of antibacterial proteins, 500 mg/ml in phagosomes [71]. Some azurophil granules are defensin-poor [72, 73], and the amount of defensin also varies. Some classical membrane proteins of lysosomes, such as LAMP (lysosome-associated membrane proteins) I and II, are not expressed on azurophil granules [74], which is inconsistent with the view that azurophil granules are specialized lysosomes though LAMP III is expressed on azurophil granules [65, 75, 76].

Secondary and tertiary granules have important roles in neutrophil extravasation and migration, and they display a morphological and functional continuum rather than discrete subsets [65]. There are three combinations of surface markers that define granule subsets. Specific granules are lactoferrin +/gelatinase−; gelatinase granules are lactoferrin-/gelatinase

+; and "hybrid" granules containing both features are lactoferrin+/gelatinase+. Although these granules contain similar proteins, they play distinct roles in the anti-infection process. Gelatinase granules, containing less antibacterial proteins like lactoferrin, are exocytosed earlier than the other two kinds of granules followed by the hybrid granules, and specific granules are exocytosed last to kill microbes [77]. The chronology of granule exocytosis is consistent with granules' functions: when tissues are invaded by pathogens, antimicrobial granules are released after those that aid migration. Rab GTPases seem to play an important role in this process. The trafficking of specific and gelatinase granules to the cellular membrane is via the Rab27- and Munc13-4-dependent pathway [70]. Rab32 cycles with Rab38 and regulates the endosomal trafficking system (reviewed in reference [78]). Rab32 has not only been shown to be associated with the biogenesis of lysosome-related organelles, such as melanosomes, but also in controlling intracellular pathogens. For example, Rab32 has also been shown to be involved in the degradation of intracellular *Listeria monocytogenes* by enclosing the listeria that escapes from listeria-containing vesicles into the cytoplasm [79]. Far more understanding of the Rab32-mediated host defense mechanism should be a focus for both cell biology and medical research. Granule defects often lead to diseases, for example, granules that are abnormally clustered and polarized participate in the development of specific granule deficiency (SGD). Besides, the abnormally clustered granules also express proteins with limited glycol-epitopes [80], displaying abnormality in function.

Secretory vesicles (SVs) are formed in the late stage of neutrophil phenotypic progression and function to convey membrane proteins to the cell surface. The proteins packaged in SVs include the formyl peptide receptor 1 (fPR1), the integrin Mac-1 ($\alpha M\beta 2$), the phagocytic receptors CD16, together with CXCR2, which are vital for extravasation and migration [65]. These proteins enhance the expression of integrins and chemotactic receptors on neutrophil membrane and promotes neutrophil extravasation and function in inflammatory tissues.

8. Neutrophil Heterogeneity in Disease

There is no doubt that neutrophils contribute vital function in the development of disease with an indispensable role in innate immunity. It is becoming obvious that neutrophils are much more than microbe-killing cells in diseases, and they display various phenotypes and perform a wide range of functions (Table 1).

8.1. Autoimmune Disease. Considerable research has provided evidence for the critical and profound role of neutrophils in promoting the development of autoimmune disease [81]. Those neutrophil functions include, but are not limited to, the following mechanisms: secretion of various cytokines and chemokines; formation of NETs that promote the production of antibodies to citrullinated protein antigens (ACPA) in rheumatoid arthritis or ds DNA in lupus; increased expression of inflammation-related membrane molecules; interactions between cells, including activation of natural killer cells; and release of ROS (reactive oxygen species) and proteases to regulate the release of cytokines [82]. Representative autoimmune diseases are presented below and a discussion of all diseases where neutrophils are known to play a role is impractical and beyond the intended scope of the review.

8.1.1. Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by synovial inflammation and cartilage erosion. The view has been expounded that activated neutrophils assemble in inflamed joint tissues, synovial fluid, and involved skin tissues to aggravate the development of RA [83–86]. Circulating neutrophils isolated from RA patients' peripheral blood are functionally different from those in healthy people, being primed for immediate ROS release. First, there are some autologous alteration in RA neutrophils themselves. For example, transcriptional changes take place with high-level expression of TNF [87] and myeloblastin [88], and the expression of membrane-bound receptor activator of NF-κB (nuclear factor κB) ligand (RANKL) is also upregulated in synovial fluid neutrophils [89, 90]. Microenvironmental factors also contribute to strengthen the specific function of RA neutrophils. In the synovial cavity, cytokines like TNF-α, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [91, 92] help delay the apoptosis of neutrophils and promote neutrophil activation and release of granules [93]. As a result, these stimulated neutrophils secrete various kinds of cytokines and chemokines such as RANKL and BAFF (B cell-activating factor) to activate osteoclasts and B lymphocytes [89, 94] and upregulate the transcription of major histocompatibility complex (MHC) II molecules [95], which may contribute to $CD4^+$ T cell activation. All these changes contribute to initiating the advancement of inflammation. In addition, neutrophils at the pannus cartilage junction aggravate matrix degradation through the secretion of MMP-8, MMP-9, neutrophil elastase, cathepsin G, and proteinase 3 [96], all of which have a significant link to cartilage damage in RA. The granule proteins, such as collagenase, gelatinase, and elastase, in RA neutrophils were found in high concentrations and are largely responsible for cartilage and tissue damage [82]. The high concentration of synovial fluid calgranulins induces protease release from specific and gelatinase granules, as well as secretory vesicles [97].

To date, the synovial neutrophils display activating surface markers as CD11b/CD18, CD43, CD63, CD35, CD55, CD66, and CD45 [98–101]. Even though the RA neutrophils are unique for delayed apoptosis, increased ROS release, and intensified intracellular transport, there have been no straightforward evidence or special surface markers for RA-related neutrophils to identify them as a distinct or unique subtype. Despite that, it is conclusive that RA neutrophils have a distinct phenotype compared with neutrophils in other functional states.

8.1.2. Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE) is considered to be a disease with defects in both innate and adaptive immune regulation [102], which is characterized by the production of autoantibodies to

Table 1: Neutrophils' phenotypes and functions in different states.

States	Representative phenotype	Function	Reference
Health			
Neutrophils in circulation	$CXCR4^{high}CXCR2^{high}CD62L^{low}$	Migration	[21, 23]
Aged neutrophils	$CD11b^{+}CD49d^{+}Mac\text{-}1^{+}VLA\text{-}4^{+}ICAM\text{-}1^{+}TLR4^{+}CD47^{low}$	Migration Anti-inflammation Resistance to phagocytosis	[26–28]
Tissue-resident neutrophils			
Lung	$\beta 2$ integrins, $VLA4^{+}CXCR4^{+}$	Adhesion Transmigration Deformation	[1, 22, 50]
Spleen	$CD62L^{low}$ $CD11b^{hi}$ $ICAM\text{-}1^{hi}$, $CD11b^{+}$, $CXCR4^{+}$, $CCR7^{+}$	Migration Activated apoptosis	[51, 52, 54, 55]
Disease			
Autoimmune disease			
RA	$CD11b/CD18^{+}CD43^{+}CD63^{+}CD35^{+}CD55^{+}CD45^{+}CD66^{+}$	Complement regulation Adhesion Inflammatory activation	[88–96, 98–101]
SLE	LDGs:$CD15^{+}CD14^{low}CD10^{+}CD16^{+}CD31^{+}CD11c^{+}G\text{-}CSFR^{+}GM\text{-}CSFR^{+}$	Inflammatory activation Activated apoptosis Correlated with disease activity	[85, 109–113]
MS	$TLR2^{+}fMLPR^{+}IL\text{-}8R^{+}CD43^{+}$	Activated apoptosis	[117]
EAE	$ICAM\text{-}1^{+}$	Autoimmune demyelination	[118]
DM	$PSGL\text{-}1^{+}$	Dysfunction in neutrophil-endothelial interaction	[119]
Cancer			
Typical clusters			
G-MDSC	$CD14^{-}CD11b^{+}CD15^{+}CD66b^{+}HLA\text{-}DR^{-}CD33^{+}$	—	[118, 119]
TAN	$CD66b^{+}CD15^{+}CD16^{+}CD11b^{+}HLA\text{-}DR^{-}$ $Arg\text{-}1^{+}$	N1: antitumoral function N2: support tumor progression	[130, 131]
Melanoma	High expression of $\beta 2$ integrins	Transmigration N2 activation	[137]
Metastases	$CD11b^{+}Ly6G^{+}$	N2 activation	[138]
GC	$CD66b^{+}$	Associated with GC prognosis	[140]
HCC	$CD66b^{+}$	Proinflammatory activation	[141]
Lung tumor	$CD62L^{low}$ $CD54^{high}$	N1 activation	[142]
Colorectal tumor	$CD45^{+}Lin^{-}HLA\text{-}DR^{-}CD11b^{+}CD33^{+}$ $CD66b^{+}$	N2 activation	[143]
ALL	Altered expression of CD10, CD33, CD13, CD15/CD65, and CD123	No correlation with clinical features	[147]
HNSCC	$CD16^{high}CD62L^{dim}$	N1 activation Correlated with increased survival rate	[148]

Table 1: Continued.

States	Representative phenotype	Function	Reference
Infection/inflammation			
MRSA			
PMN-1	$CD49d^{high}CD11b^{low}$ $TLR2^{high}TLR4^{high}TLR5^{high}TLR8^{high}$	Protective activation IL-2 producing	[149]
PMN-2	$CD49d^{low}CD11b^{high}$ $TLR2^{high}TLR4^{high}TLR7^{high}TLR9^{high}$	Aggravating infection IL-10 producing	[149]
Gout	—	Activated apoptosis	[151–154]
Bronchiectasis	$CD11b^{high}CD62L^{high}$	Inflammatory activation Impaired phagocytosis	[155, 156]
Sepsis	$ICAM\text{-}1^{+}$	Accumulation and migration Inflammatory activation	[157]
Acute viral respiratory tract infection	$CD49d^{+}CysLTR1^{+}$	Further researches needed	[158]
Asthma	$CD66c^{high}$	A biological feature of treatment-resistant asthma	[155]
Cirrhotic	$EMR2^{+}CD11b^{high}CD181^{high}CD182^{high}CD49d^{high}$	Inflammatory activation Impaired phagocytosis; correlated with infectious complications	[158]

RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; MS: multiple sclerosis; EAE: autoimmune encephalomyelitis; DM: diabetes mellitus; G-MDSC: granulocytic myeloid-derived suppressor cells; TAN: tumor-associated neutrophils; GC: gastric cancer; HCC: hepatocellular carcinoma; ALL: acute lymphoblastic leukemia; HNSCC: head and neck squamous cell carcinoma; MRSA: methicillin-resistant *Staphylococcus aureus*.

nuclear antigens and immune complex-induced chronic inflammation [103]. Evidence over the last few years have implicated an influential role for neutrophils in the pathogenesis of SLE. Lupus neutrophils display impaired phagocytic capabilities and reduced ability to be removed by the C1q/calreticulin/CD91 pathway [104] as well as abnormal oxidative activity [105–107]. In contrast with RA neutrophils, there are increased numbers of apoptotic neutrophils in SLE, which are related to disease activity and serum levels of anti-double-stranded DNA (anti-dsDNA) antibody. Anti-dsDNA and anti-SS/B antibodies can modulate neutrophil cell death and function [108]. The phenotypic characteristics of SLE neutrophils include enhanced apoptosis, secondary necrosis, and impaired phagocytic capabilities. Neutrophils in SLE also have enhanced NET formation and impaired NET degradation (discussed later).

A distinct subpopulation of neutrophils in SLE is the low-density granulocytes (LDGs) that have attracted much attention in recent years. Although LDGs have been well researched in various autoimmune diseases including idiopathic arthritis [109] and antineutrophil cytoplasmic antibody-associated (ANCA) vasculitis [110] and infections including tuberculosis [111], this review will focus on LDGs in SLE. Lupus LDGs are mononuclear, not polymorphonuclear, cells and included in almost all stages of granulocyte development. They have an enhanced capacity to synthesize proinflammatory cytokines such as interferon gamma (IFN-γ) [112, 113]. FACS (fluorescence-activated cell sorting) distinguished LDGs from monocytes by their high expression of CD15 and low expression of CD14, compared to monocytes. In addition, LDGs express CD10, CD16, CD31, CD11c, G-CSFR, and GM-CSFR. Activated lupus LDGs also have surface molecules of CD11b and CD66b [85, 113]. The most prominent characteristic of LDG is similarity in surface markers with a mature neutrophil phenotype (e.g., CD10) but differ the common neutrophil phenotype in the nuclear morphology, which tends to show an immature neutrophil nuclear phenotype. Gene array studies have shown higher mRNA levels of various immune-stimulatory bactericidal proteins and alarmins present in azurophilic granules in the LDGs [112]. Considering that the levels of mRNAs encoding neutrophil serine proteases are highest at the promyelocytic stage in the bone marrow and reduced as granulocytes mature, some investigators hold the view that lupus LDGs are actually immature neutrophils [114]. Since LDGs have both "old" surface markers and "young" nucleus, it is worthy to consider whether lupus LDGs constitute a new subset of neutrophils, or they are just activated cells with distinct phenotype and function. As for their function, LDGs secrete high levels of proinflammatory cytokines including TNF-α, IL-6, IL-8, and type I and II IFNs to set up inflammation in lupus. Midgley and Beresford also reported that increased numbers of LDGs in SLE were positively correlated with disease activity and anti-dsDNA antibody level in juvenile SLE (JSLE) [115]. Intriguingly, LDGs are demonstrated to have an effect on cardiovascular incidents in SLE patients. Noncalcified plaque burden (NCB) in SLE patients is positively associated with LDGs, and activated LDGs might contribute to vascular damage and unstable coronary plaque [116].

8.1.3. Multiple Sclerosis. Multiple sclerosis (MS) is characterized by autoimmune inflammation and demyelinating disease. Naegele et al. verified that neutrophils in MS patients had a distinct phenotype with high expression of TLR-2, fMLP (N-formyl-methionyl-leucyl-phenylalanine) receptor, IL-8 receptor, and CD43, as well as displaying a primed state based on reduced apoptosis, stronger degranulation, oxidative burst, and higher levels of NETs in serum [117]. The phenotypic changes might be associated with the specific function of MS neutrophils, and the chronic inflammatory environment in MS may contribute to the active phenotype, thus verifying the elasticity of neutrophils in different states and inflammatory environments.

The experimental autoimmune encephalomyelitis (EAE) model is usually used to study MS. A cluster of extravascular ICAM1$^+$ neutrophils in the central nervous system (CNS) in EAE has been demonstrated to gain macrophage-like properties after extravasation. These neutrophils play a role in autoimmune demyelination [118]. They may support inflammation via the enzyme aspartic peptidase retroviral-like 1 (ASPRV1, also known as SASPase), which is, as demonstrated, only expressed by neutrophils in the immune and nervous systems and is necessary for the transition from acute to chronic inflammation in EAE [118]. The expression of ICAM1 distinguishes extravascular macrophage-like neutrophils in EAE, and ICAM1$^+$ and ICAM1$^-$ neutrophils are differentially distributed in the spinal cord, which again illustrates neutrophil heterogeneity and plasticity.

8.1.4. Diabetes Mellitus. Diabetes mellitus (DM) is a prototypic dysmetabolic syndrome characterized by chronic hyperglycemia and remains a serious health burden globally. Hou et al. used a rapid microfluidic sorting analysis to isolate a subset of rolling neutrophils from peripheral blood of DM patients. This high-rolling-speed phenotype of neutrophil was clarified to have significant correlation with neutrophil activation, rolling ligand P-selectin glycoprotein ligand 1 (PSGL-1) expression, and cardiovascular risk factors associated with DM [119]. Morphologic changes were also detected with a higher number of elongated cells in this high-rolling-speed neutrophil group. A conclusion can be deduced that phenotypic changes in DM patients lead to impaired initial neutrophil capture and rolling, which result in dysfunction in neutrophil-endothelial interaction.

8.2. Cancer. Neutrophils have a vital and controversial role in the development of cancer. Circulating PMNs in patients and experimental animals with cancer can be divided into at least three groups according to their density: high-density neutrophils (HDNs), low-density neutrophils (LDNs), which display a segmented nuclear, mature morphology, and granulocytic myeloid-derived suppressor cells (G-MDSCs) with immature morphology [120], and CD14$^-$/CD11b$^+$/CD15$^+$/CD66b$^+$/HLA-DR$^-$/CD33$^+$ cell surface phenotype [121]. LDNs and HDNs commonly show distinct levels of CD11b$^+$ expression. A group of sediment granulocytes from renal cell carcinoma patients, which share the same density as PBMC, has higher levels of CD11b$^+$ and CD66b$^+$ compared to HDNs. LDNs were less segmented than the normal-density PMNs although both of them express membrane markers of CD11b$^+$ and CD66b$^+$ [122]. MDSCs, on the other hand, are much more heterogeneous among different individuals and can be separated into three groups as CD16$^+$/CD11b$^+$, CD16$^-$/CD11b$^+$, and CD16$^-$/Cd11b$^-$ [123]. The number of circulating neutrophils is increased in both tumor-bearing mouse models and patients with tumor progression [124]. A general mechanism for this phenomenon could be that cytokines produced within the tumor induce the release of G-CSF [125], IL-1, and IL-6 [126]. Tumor-infiltrating neutrophils are considered an independent prognostic factor in tumor recurrence [127–129].

Tissue tumor-associated neutrophils (TAN), traditionally divided into N1 and N2 neutrophils, share a similar surface phenotype with circulating neutrophils including CD66b$^+$, CD15$^+$, CD16$^+$, CD11b$^+$, HLA-DR$^-$, and arginase-1$^+$ (Arg-1). Recent research indicates that the function of TANs varies in different disease states [130, 131]. To be more specific, N1 neutrophils have antitumor function, whereas N2 neutrophils support tumor progression [131]. Since these findings are mostly reported in murine models [124], the nature and biological function of N1 and N2 phenotypes in tumor immunity and progression need better understanding in humans.

The transition of the TAN phenotype and neutrophil dysfunction are strongly influenced by endogenous cytokines released in the tumor microenvironment. Zou et al. [132] found increased numbers of neutrophils in peripheral blood, enhanced tumor infiltration by TANs, and a N2 phenotype transition of infiltrating neutrophils in vivo. These changes in neutrophil number and phenotype were induced by IL-35, which has a high expression in tumor issue. This process is initiated by IL-35-induced production of IL-1β and IL-17. IL-17, serving as a protumorigenic factor, is capable of upregulating the expression of IL-6 and G-CSF as well as energizing the recruitment of neutrophils into the tumor immune microenvironment [120]. IL-35 also downregulates TNF-related apoptosis-inducing ligand (TRAIL) expression to enhance the proangiogenic function of neutrophils [132], strengthening tumor growth and disease progression. So, a conclusion can be drawn that the N2 phenotype of TANs in cancer is, at least in part if not wholly, a consequence of IL-35 production by not only tumor cells but also stroma cells and immune cells [133, 134]. Tumor-associated inflammation of neutrophils is also modulated by NK cells. NK cells might act as inhibitors of vascular endothelial growth factor-A (VEGF-A) expression by neutrophils via an IFN-γ-stimulated pathway that promotes angiogenesis and, consequently, tumor growth. A higher production of TGF-β was also observed in NK cell–depleted tumors, given that TGF-β would promote the tumorigenic N2 phenotype [135]. Distant regulation was also found. A unique subset of tumor-infiltrating SiglecF^{high} (Sialic acid-binding immunoglobulin-type lectin) neutrophils was verified to display cancer-promoting properties. The SiglecF^{high} tumor-infiltrating neutrophils in lung cancer are sustained remotely by bone-resident osteocalcin-expressing (Ocn$^+$) osteoblastic cells. This group of neutrophils is considered to be effector cells in osteoblast-driven protumoral responses [136].

The interesting function of TANs to facilitate metastasis formation has been well studied. A research of melanoma revealed that tumor cells synthesized IL-8 to upregulate the expression of β2 integrin on neutrophils, thus enhancing the neutrophil–melanoma cells' interaction with ICAM-1. This function accelerated the transmigration of melanoma cells through the endothelium. IL-8 also contributes to neutrophil retention in the lung tissue [137]. Additionally, TANs may support the survival and dissemination of tumor cells. $CD11b^+/Ly6G^+$ TANs improve intraluminal survival of tumor cells by inhibiting NK cell function. In addition, the secretion of IL1β and matrix metalloproteinases from neutrophils enhances the extravasation of tumor cells [138]. It seems that $CD11b^+/Ly6G^+$ TANs in metastases tend to be N2 phenotype. But intriguingly, this effect can be reversed by TGF-β blockade in the tumor microenvironment, which induces $CD11b^+/Ly6G^+$ neutrophils to display an antitumor phenotype [131]. These findings indicate that the phenotype of TAN may be altered artificially from N2 to N1, depending on the tumor microenvironment, thus manifesting the remarkable flexibility and heterogeneity of neutrophils.

CD66b is a very important molecule among TAN surface markers. Huang et al. [139] clarified that $CD66b^+$ TANs are significantly increased in number in gastric cancer (GC) and are independently associated with GC prognosis. $CD163^+$ TAMs (tumor-associated macrophages) combined with $CD66b^+$ TANs could serve as a precise marker to predict the prognosis of GC patients. $CD66b^+$ TANs are also found in hepatocellular carcinoma (HCC). They exhibit high expression of programmed cell-death ligand 1 (PDL1), IL8, TNFα, and CCL2 but a low expression of CD62L. The upregulation of PDL1 could be a key molecule to maintain the survival and function of activated TANs through an IL6-STAT3-PDL1 signaling cascade driven by cancer-associated fibroblasts (CAFs) [140]. Increased survival rate in colorectal cancer is also associated with $CD66b^+$ TAN infiltration, and these neutrophils enhance the activation of $CD8^+$ T cells in cocultural system [141], which might function as antitumor cells and have a positive effect on the prognosis of patients.

Much detail of TAN functions has been derived from research in animal models, but research on the function of human TANs is controversial. There have been two controversial reports on human TAN's immunosuppressive capacity. Eruslanov et al. [142] isolated TANs from digested human lung tumors. These TANs displayed a phenotype of $CD62L^{low}CD54^{hi}$ and produced a number of proinflammatory cytokines that enhanced T cell proliferation and IFN-γ production. Wu et al. [143], on the other hand, collected TANs from colorectal tumor tissue that presented a phenotype of $CD45^+Lin^-HLADR^-CD11b^+CD33^+CD66b^+$, which was similar to the classical neutrophil morphology. Those TANs produced arginase 1 and ROS and downregulated T cell proliferation and IFN-γ production. One possible explanation for the different results could be that different tumor microenvironments may determine the TAN's heterogeneity in both phenotype and function.

Although neutrophils have historically not been considered as MHC class II APC, they are now well established as being able to express MHC class II under certain conditions and function as "atypical" APC [144]. Yuan et al. [145] clarified that CXCL1-induced, tumor-infiltrated neutrophils have increased expression of MPO (myeloperoxidase) and Fas/FasL (also known as CD95/CD95L), which may be involved in TAN-mediated inhibition of $CD4^+$ and $CD8^+$ T cells. TANs displaying characteristics of both neutrophils and antigen-presenting cells (APCs) were identified in early-stage human lung cancer [146]. These hybrid neutrophils defined as $CD11b^+Arg\text{-}1^+CD66b^+CD15^+HLA\text{-}DR^{+-}CD14^+$ have the capacity to trigger antitumor T cell responses as well as cross-present antigens, which builds an intriguing connection between innate and adaptive immunity. Cohort studies on TAN phenotypes and functions have generated considerable phenotypic data on TAN in different human cancers. Changes to neutrophil membrane markers in childhood acute lymphoblastic leukemia (ALL) were reported in a 118 BCP-ALL cohort study [147]. Around 77% of the cases showed altered markers, including CD10 (53%), CD33 (34%), CD13 (15%), CD15/CD65 (10%), and CD123 (7%), although no correlation was found between altered markers and clinical features. So, the biological relevance of the abnormal phenotypes has yet to be resolved. Another cohort study [148] in head and neck squamous cell carcinoma (HNSCC) patients verified the antitumor function of $CD16^{high}CD62L^{dim}$ neutrophils. The $CD16^{high}CD62L^{dim}$ neutrophils inhibited migration and proliferation and induced apoptosis of cancer cells via NET formation. An increased proportion of $CD16^{high}CD62L^{dim}$ neutrophils was correlated with increased survival rate in that cohort. The role of neutrophils, particularly tumor-infiltrating neutrophils, in inhibiting or promoting tumor growth and the variation in phenotypes associated with the disparate functions, is emphasis of the remarkable heterogeneity and plasticity of neutrophils.

8.3. Inflammation. As the vanguard of immune cells in sites of inflammation, it is not surprising for neutrophils to switch into different phenotypes with multiple functions during infection and inflammation. Specific functions are usually associated with altered phenotypes, thus defining prominent functional subpopulations adapted to the microenvironment and characteristics of relevant innate or adaptive immune stimuli.

Infection and inflammation related neutrophils show quite different characteristics with those in other diseases and may have great heterogeneity even in the same microenvironment. In the condition of methicillin-resistant *Staphylococcus aureus* (MRSA) infection, two distinct subsets of neutrophils named PMN-1 and PMN-2 were first reported in 2004 [149]. PMN-1 neutrophils were characterized as $CD49d^{high}CD11b^{low}$ with upregulated expression of TLR2, TLR4, TLR5, and TLR8, while PMN-2 cells were $CD49d^{low}CD11b^{high}$ with upregulated TLR2, TLR4, TLR7, and TLR9. There was also functional heterogeneity in cytokine secretion between PMN-1 and PMN-2. PMN-1 were prone to IL-2 production while PMN-2 were prone to IL-10. Suppression of PMN-2 or enhancement of PMN-1 led to the protection of immunocompromised hosts against MRSA infection.

Gout is an acute inflammatory disease mostly presenting with symptoms of joint swelling, redness, and pain attributed to the precipitation of uric acid in the form of monosodium urate (MSU) crystals [150] that stimulate inflammation mainly in joints. MSU crystals stimulate a NALP3 inflammasome-dependent acute inflammation [134]. Numerous chemokines are released into affected, inflamed sites, such as CXCR2, CXCL-8, CXCL-1, CXCL-2, and CXCL-3 [151], inducing accumulation of neutrophils and release and aggregation of NETs. The role that NETs play in gout seems to be protective. MSU crystals are embedded within NET chromatin, and proteins from granules including serine proteases associated with NETs help degrade inflammatory cytokines [152]. NETs also have an anti-inflammatory function by blocking further accumulation of neutrophils through lactoferrin synthesis [153]. MSU-induced NETs are enriched for actin and are less sensitive to DNase degradation [154]. Distinctly, NETs contribute to the resolution of inflammation in gout.

Only a few neutrophils are maintained in the lung in normal homeostatic conditions. Bronchiectasis is a predominantly neutrophilic condition. Circulating neutrophils in bronchiectasis have a significantly prolonged lifespan, delayed apoptosis, increased CD62L shedding, upregulated CD11b expression, increased myeloperoxidase release, and impaired phagocytosis and killing of *Pseudomonas aeruginosa* (PAO1) that is associated with a worse outcome [155]. These aberrant functional activities of neutrophils in bronchiectasis indicate an attenuated capacity for bacterial elimination. Neutrophils overexpressing ICAM-1 had enhanced effector functions including phagocytosis and reactive oxygen species (ROS) generation [156]. An increased number of ICAM-1^+ neutrophils were shown to accumulate in the lung tissue during sepsis, leading to acute respiratory distress syndrome (ARDS). The abnormal accumulation of ICAM-1^+ neutrophils was related to cold-inducible RNA-binding protein (CIRP), a damage-associated molecular pattern (DAMP) [157]. $CD49d^+$ $CysLTR1^+$ (cysteinyl leukotriene receptor 1) neutrophils isolated during acute viral respiratory tract infection produced TNF, CCL2, and CCL5 and were necessary for the complete development of postviral atopic disease [158]. However, further research is needed to validate the function of this group of neutrophils. $CEACAM6^{high}$ (CEACM6, nonspecific cross-reacting antigen, also known as CD66c) neutrophils were isolated from bronchi in severe asthma patients and were considered to be a vital biological feature of treatment-resistant severe asthma [159]. The expression of CEACAM6 protein was upregulated in both bronchi epithelial cells and lamina propria neutrophils in patients with severe asthma. Homophilic binding of CEACAM6 to N-domain CEACAM6 peptides could potentially enhance neutrophil activation with the generation of superoxide [158] potentially contributing to neutrophil activation and epithelial damage as well as respiratory dysfunction in asthma [155].

Circulating neutrophils from visceral leishmaniasis patients have been shown to have reduced CXCL8 expression but increased IL-10 and arginase-1 expression with enhanced capacity to phagocytose *Leishmania* spp. promastigotes [160]. These functions may support an immunosuppressive role of neutrophils in active visceral leishmaniasis, but prominent phenotypic markers for this subset of neutrophils are unknown.

In a cohort liver cirrhotic study in 2016 [161], $EMR2^{high}$ (EGF-like molecule containing mucin-like hormone receptor 2) neutrophils were verified to be associated with disease severity and to predict the rate of mortality in cirrhotic patients. These EMR2-expressing neutrophils displayed an activated phenotype with a higher-level expression of activation molecules such as CD11b, CD181, CD182, and CD49d, but these neutrophils also showed deranged function including increased resting oxidative burst and impaired phagocytosis ability. Besides, $EMR2^{high}$ neutrophils were also correlated with higher mortalities in cirrhotic patients. Thus, these neutrophils can be considered as a significant parameter to predict the outcome of liver cirrhosis in patients.

9. Neutrophils and NETs

An alternative pathway for the death of neutrophils besides necrosis or apoptosis is the formation of NETs. NETs are extracellular strands of unwound chromatin in complex granule proteins including those with inflammatory and bactericidal activity. The NET granule proteins include neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, lactoferrin, pentraxin 3, gelatinase, proteinase 3, peptidoglycan-binding proteins, and DNA-free histones [162]. There are at least 24 neutrophil proteins associated with NET formation according to mass spectrometry analysis [163]. Classical NET formation pathways include activation of integrins and Toll-like receptors (TLR) in response to bacterial-associated pathogen-associated molecular patterns (PAMPs) [164]. The prominent mechanism of NET formation is dependent on ROS and the Raf/MERK/ERK (rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase ERK kinase (MERK)/extracellular signal-regulated kinase (ERK)) pathway. After neutrophil activation, nicotinamide adenine dinucleotide phosphate oxidase (NOX) increases via protein kinase C (PKC), resulting in cytosolic calcium intake, peptidyl arginase deaminase 4 (PAD4) activation, and chromatin decondensation. When cytosolic calcium increases, PAD4 activation and chromatin decondensation occur [165]. Secondly, ROS promotes the loss of the nuclear membrane. Then chromatin spreads throughout the cytoplasm together with cytoplasmic and granule proteins and finally NETs are released out of the cell. Another mechanism independent of ROS and the Raf/MERK/ERK pathway evolves through three stages: nuclear envelope growth and vesicle release, nuclear decondensation, and nuclear envelope disruption [166]. The NETosis, although not unique to neutrophils, is *bona fide* a well-recognized and important phenomenon in neutrophil function, so this section will discuss NETs and NETosis in detail in diseases where they are recognized as particularly important.

9.1. NETs in Inflammation and Infection. It is generally considered that NET formation plays a positive role in attenuating inflammation during infection since neutrophils

primed by microbiota are more prone to form NETs [26]. A UK cohort study [167] identified neutrophil dysfunction in sepsis that included significantly decreased NET formation accompanied by defects in neutrophil migration and delayed apoptosis. These abnormal changes forebode poor outcomes with increased 30-day and 90-day mortality. In addition, NETs appeared to display a positive role in *Streptococcus suis* serotype 2 (SS2) infection. Although SS2 biofilms are capable of inhibiting NET formation, NETs derived from neutrophils stimulated by planktonic bacteria and host inflammatory factors still display the ability to eliminate bacterial biofilms [168]. This research elucidates a novel view on the battles between NETs and bacterial biofilms. In the examples of bacterial infection cited above, NETs have a protective function. In other types of infection, NETs may have a deleterious effect. NET formation was associated with *Sendai virus* (Sev) infection and amplified early inflammation in the lung in the Sev-induced asthma model, consequently priming an inflammatory cascade, immune cell activation, and airway remodeling [169]. Neutrophil-derived cysteine protease dipeptidyl peptidase I (DPPI) is an important mediator in NET formation, and inflammatory conditions such as sepsis were less severe in the absence of DPPI [170]. To summarize, the role of NETs in infection and inflammation is variable and dependent upon the activating stimulus.

Findings described above identify the value of NETs as potential new biomarkers of disease activity, prognosis, and NETosis as potential therapeutic targets. Future studies are needed to illustrate and understand the detailed mechanisms and relationships between NETs and diseases.

9.2. NETs in Autoimmune Disease. The function of NETs in autoimmunity has been reviewed recently [133]. Externalized DNA is a DAMP with potent innate immune-activating potential, and it is more resistant to degradation [171]. The persistence of nuclear components encompassed in NETs, particularly DNA, creates a potent source of autoantigens. Apoptotic neutrophils in the circulation could also be a source of autoantigens in SLE patients. These autoantigens such as dsDNA and cathelicidin (LL-37) show an increased level in peripheral blood and correlate with disease activity in SLE patients [172]. NETs released from LDGs displayed high levels of autoantigens and immune-stimulating molecules, including MMP-9, α- and β-defensins, and LL-37 [112], and the ability for SLE patients to clear NETs is impaired [173]. A high level of type I IFN, which enhances autoimmune B cell activation, is a critical feature in SLE [174, 175] and is correlated with demethylated CpG DNA, LL-37, and other NET contents, all of which upregulate type I IFN expression by plasmacytoid dendritic cells (pDC) [176]. These processes initiate a progressive cycle to induce more NETs that stimulate more type I IFN production and exacerbated chronic inflammation and B cell activation and autoantibody production. The inhibition of FcgRII, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, or TLR7 are inhibitors of NETs [177]. All these findings have provided new insights into the role of neutrophils and NETosis in the generation of the type I IFN signature in SLE. As such, neutrophils and NETosis are potential targets for future therapeutics.

9.3. NETs in Cancer. NETs are *per se* a double-edged sword. NETs could induce the proliferation and malignant transformation of B cells toward malignant lymphoma via NF-κB signaling [178], and in addition, some NET-induced cytokines reveal a relationship between NETs and tumor development. IL-8 has been demonstrated to play a role in both NET generation and angiogenesis as well as tumor progression [179, 180], and granulocyte colony-stimulating growth factor (G-CSF) was also demonstrated to be associated with tumor generation [181]. Notably, NETs may play a crucial role in hematogenous metastasis. It has been verified that metastatic breast cancer cells induced neutrophils to form NETs, which enhanced tumor cell growth in target organs [182]. Kanamaru et al. found that $CD66^+$ mature LDNs were observed to cluster in the peritoneal cavity within patients who underwent laparotomy due to gastric cancer. These LDNs released NETs with the typical features of threadlike structures positive for nucleic acid staining, histones, and myeloperoxidase [183]. *In vitro* experiments indicated that tumor cells attached to NETs did not die but continued to proliferate. Additionally, it was verified that NETs helped upregulate the level of MMP-9 to degrade extracellular matrix, which facilitated tumor invasion [1]. These findings support the conclusion that NETs may enhance the clustering and growth of free tumor cells.

Recently, intriguing discoveries of an association between cancer thrombosis and NETs have drawn attention. It has been reported that NETosis was associated with microcirculatory thrombosis [184] leading to thromboembolic complications in cancer. Spontaneous intestinal tumorigenesis was verified to correlate with the accumulation of N2 phenotype LDGs, as well as NET formation and hypercoagulation. The potential mechanism was inferred that stimuli such as circulating LPS could upregulate complement C3a receptor on neutrophils. C3aR plays an important role in NETosis [185] since C3a-activated neutrophils become more susceptible to NETosis. This process aggravated thrombus formation, which induced a N2 phenotype in the neutrophils. These neutrophils underwent spontaneous NETosis, further exacerbating hypercoagulation and initiating a progressive cycle [186]. In another way, NETs could cooperate with tumor-derived exosomes to induce the establishment of venous and arterial thrombus formation in breast cancer [187] through the stimulation of platelet aggregation, activation of contact pathways, and degradation of natural coagulation inhibitors [188, 189]. More detailed research on these associated mechanisms is still on the exploratory stage.

10. Conclusion

The brand-new discoveries of neutrophil plasticity in various conditions broaden the horizon that neutrophils are not just simple reproductions. They display strong heterogeneity in morphology and function in both healthy and disease circumstances including infection, tumorigenesis, tumor immunity, and autoimmunity. Even though some researchers hold the view that several characteristic classes of neutrophils, such as LDGs and N1/N2 TANs, are *bona fide*, independent subpopulations based upon a host of

supporting evidence, others interpret the heterogeneity of form and function as just manifestations of differential activation. Researches have elucidated distinct functions of neutrophils with up- or downregulated membrane molecules in various contexts, but further relation and mechanism that correlate those phenotypic differences are still being revealed. The interaction of phenotype and function in neutrophils will continue to be worthy of attention in the future. There is a reason to consider neutrophils as highly malleable cells and most type features can be acquired at specific sites after stimuli, but the variations occurring in the early immature stage or the late mature stage are still poorly understood. We expect more valuable studies to expand existing recognition of neutrophils' multiple roles.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Nanomaterial Exposure Induced Neutrophil Extracellular Traps: A New Target in Inflammation and Innate Immunity

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Nanotechnology has become a novel subject with impact in many research and technology areas. Nanoparticles (NPs), as a key component in nanotechnology, are widely used in many areas such as optical, magnetic, electrical, and mechanical engineering. The biomedical and pharmaceutical industries have embraced NPs as a viable drug delivery modality. As such, the potential for NP-induced cytotoxicity has emerged as a major concern for NP drug delivery systems. Thus, it is important to understand how NPs affect the innate immune system. As the most abundant myeloid cell type in innate immune responses, neutrophils are critical for concerns about potentially toxic side effects of NPs. When activated by innate immune stimuli, neutrophils may initiate NETosis to release neutrophil extracellular traps (NETs). Herein, we have reviewed the relationship between NPs and the induction of NETosis and release of NETs.

1. Introduction

Nanotechnology has emerged as one of the most exciting industrial innovations worldwide [1, 2] in diverse areas of structural and material design and device and systems engineering. Nanoparticles (NPs) are defined as structures whose sizes are within the range from 1 to 100 nm in one, two, or three dimensions while nanomaterials are a group of small-scale substances which are applied to carry out their distinct properties in many kinds of fields, including but not limited to optical, magnetic, mechanical, and electrical engineering [3–5]. NPs also have the unique biological characteristic of high surface-to-volume ratio and small size. Due to their unique structural and size properties, they can easily penetrate molecular, cellular, and extracellular matrix barriers to reach most body systems. While NPs can be easily taken up by cells, they may also bind to cell surface proteins, initiate signaling, activate or inactivate the relevant cells, and in some cases cause unexpected cellular interactions [6, 7]. At present, environmental exposure and deliberate administration are two approaches by which NPs may be introduced. As the potential for NP exposure from inhalation, ingestion, and direct skin contact has increased [8, 9], nanotoxicology has emerged as a new type of toxicology to evaluate the safety of nanostructures and nanodevices [10].

The innate immune system is the first line of immune defense for mammalian and other eukaryotic hosts including mice and humans. Innate immunity includes both soluble proteins such as secreted cytokines and acute-phase and complement system proteins [11–14] and cells from the myeloid, lymphoid, and mast cell lineages [13–21]. The myeloid cells include granulocytes (neutrophils, basophils, and eosinophils), monocytes, macrophages, and dendritic cells [16–18]. Innate lymphoid cells (ILC) [19], natural killer cells (NK) [20], and to some extent $\gamma\delta$ T cells [21] are the lymphoid representatives to innate immunity. Mast cells, although similar in many respects to granulocytes, are a distinct lineage of innate immune cells [15]. Cells from all

of these cell lineages are the main effector cells in innate immune responses [22] to both pathogenic and non-pathogenic challenges through pattern recognition receptor (PRR) recognition of pathogen-associated molecular patterns (PAMPs) to initiate an inflammatory response [23]. Polymorphonuclear leukocytes and neutrophils (PMNs) are not only the most abundant leukocytes in the blood, up to 65% of white blood cells in humans, but also short-lived. PMNs are derived from a granulocyte-monocyte precursor in adult bone marrow [24] and account for more than fifty percent of hematopoietic activity. Each day, there are about 5×10^{10} PMNs released from bone marrow into the peripheral circulation [25, 26]. Due to the PMN's short lifespan, close to 24 hours, homeostatic control is essential to maintain relatively stable cell numbers in the circulation. Acute bacterial or fungal infection, for example, stimulates an immediate inflammatory response by the vascular endothelium and the migration of PMNs to the site of infection in response to local chemokines and local changes in endothelial integrins [27]. The recruited PMNs phagocytose and kill the potential pathogens. Upon phagocytosis of potential pathogens, PMNs initiate a respiratory burst to generate reactive oxygen species (ROS) that are bactericidal [28].

2. Critical Role of Nanoparticles in Immune Response and Inflammation

The effects of NPs on the immune system, especially the innate immune system, are critical to a thorough understanding of the physiological and pathophysiological consequences of NP exposure. Intentional or unintentional NP exposure will initiate engagement of cellular and soluble protein components of the innate immune system to activate intracellular and extracellular signaling cascades [9, 29, 30] in response to the NPs. Both extracellular and intracellular innate immune receptors, pattern recognition receptors (PRR), may be engaged and stimulated by NPs [31–33]. Likewise, proteins in serum, particularly those in the complement [34, 35] and kallikrein [36] systems, may be engaged by NPs. Whether the NP interaction gradually leads to stimulation or inhibition of innate immunity and or inflammation is determined by the physicochemical properties of the relevant NPs [37–41]. NPs such as sand, dust particles, or pollen are generally ignored by the immune system. On the other hand, when NPs engage PRR, the NPs may mimic pathogen-associated molecular patterns (PAMPs) and initiate an innate immune reaction [42–44].

Upon exposure to NPs, neutrophils may initiate an inflammatory response, secreting signaling chemokines and evoking downstream reactions [14]. The physical and chemical properties of NPs are major factors that may affect the innate immune response. Differences in size, size distribution, charge, surface area, reactivity, crystallinity, aggregation in relevant medium, composition, surface coating, method of synthesis, and impurities not only affect biodistribution and cellular uptake of NPs but also affect innate immune responses [9, 45–48]. It still remains controversial whether the toxicity of NPs originates from the NPs themselves, metal ions released by dissolution of the NPs, or a combination of both. Several studies demonstrate that the released metal ions are the major or even the only cause of their toxicity. Soluble NPs, such as ZnO and FeO that have higher surface ion dissolution, were reported to be more toxic than NPs with less surface ion dissolution, such as CeO_2 and TiO_2 [49–52]. Some studies have also indicated that size is an important determinant for toxicity and the inflammatory potential of NPs. Larger-size NPs with a smaller surface-to-volume ratio have higher dissolution of toxic ions and induce more inflammatory ROS production [53]. Shape and composition are also critical determinants for NPs' toxicity and inflammatory potential [54]. The NPs' surface composition influences NPs' interactions with cell membranes and surface receptors. More positively charged NPs have higher potential to induce inflammatory reactions [55]. Thus, if aggregated NPs are dissociated through sonication, the cytotoxicity and ROS production may increase on account of the increased solubility and ion dissolution [56]. However, several studies report that the major source of toxicity of NPs is derived from their particulate characteristics [57, 58]. Wang et al. [59] reported that ZnO NP toxicity was due solely to the released Zn ions, and CuO NP toxicity originated from both the released Cu ions and the CuO particles. Toxicities of Fe_2O_3, Co_3O_4, Cr_2O_3, and NiO were caused by the particulate characteristics of the NPs. In consideration of the above, medical use of NPs must consider how NPs' physical properties, especially solubility, affect toxicity.

Usually, neutrophils take up NPs through pinocytosis, macropinocytosis, clathrin/caveolar-mediated endocytosis, or phagocytosis. Both micropinocytosis and pinocytosis are nonspecific and related to immune response. When neutrophil PRR engage NP PAMPs, that engagement may initiate inflammasome-dependent neutrophil activation [31, 60]. Recently, NETosis, a new cell death specific to neutrophils, has become another significant way by which NPs may stimulate immune and inflammation response [61, 62]. Herein, we focus on the correlation and interaction between NPs and NETs in innate immune responses.

2.1. NP-Induced NET Formation in Inflammatory Response and Inflammation Resolution. Neutrophil extracellular traps (NETs), a network structure released during NETosis, consist of 15-17 nm chromatin strands decorated with as many as 20 different antimicrobial proteins and peptides including myeloperoxidase (MPO), neutrophil elastase (NE), proteinase 3 (PR3), cathepsin G, LL37, and histones 1, 2A, 2B, 3, and 4 [62]. Conventional suicidal NETosis is usually initiated by several stimuli (bacteria, viruses, and fungi) binding to neutrophil toll-like receptors (TLRs) [62, 63], which activate the endoplasmic reticulum to release stored calcium ions. Elevated calcium levels increase protein kinase C (PKC) activity, inducing NADPH oxidase to assemble into the functional phagocytic oxidase (PHOX) complex [64–66]. PHOX generates ROS that initiates nuclear and granular membrane rupture with subsequent chromatin decondensation and diffusion into the cytoplasm [64, 65]. The aforementioned neutrophil granular proteins and peptides attach to the cytoplasmic chromatin, and the complexes break through the plasma membrane and diffuse into the extracellular space

as NETs [61, 67]. Vital NETosis is another pathway to release NETs induced by *Staphylococcus aureus* [68] and *Candida albicans* [69] via blebbing of the nuclear envelope and vesicular exportation. Consequently, this pathway preserves the integrity of the neutrophil plasma membrane [66, 68, 70]. Meanwhile, it still remains controversial whether and how suicidal NETosis and vital NETosis coexist. Recent data suggest that other immune cells such as mast cells [71], eosinophils [72], and macrophages [73] can also release extracellular traps. When NPs stimulate NETs, the NPs may be captured within the NETs in a phagocytosis-independent process [70]. Recent results indicate that NETs may function in the setting of noninfectious disease and its regulation [70]. Several kinds of NPs such as gold, silver, cationic lipid, polystyrene, nanodiamonds, and graphene oxide (GO) were found to trigger NETosis [37, 55, 74–78] (Table 1).

2.2. Gold Nanoparticles (AuNPs). Gold nanoparticles (AuNPs) have great potential in diagnostics and therapeutic nanomedicine [79]. AuNPs are recognized as nonbiodegradable and mostly insoluble in biological media, and they cause activation of neutrophils by altering the surface charge density on neutrophil membranes [80, 81]. AuNPs function as excellent nanocarriers not only because of their small size, which is similar to cellular components, but also because of their biocompatibility. AuNPs larger than 10 nm in size have less cytotoxicity and are more biocompatible [82, 83]. Bartneck et al. [77] explored the interaction between gold NPs with diameters of 15–50 nm and neutrophils. They built a successful model library of AuNPs with different surface chemistries or different shapes and studied their effect on human primary peripheral PMNs. Accordingly, they use transmission electron microscopy (TEM) or electroless deposition to observe that neutrophils trapped AuNPs mostly within extracellular networks. NETosis was detected 15 minutes after AuNPs come in contact with neutrophils and progressively trapped more NPs with time. AuNPs in different shapes and modified surface properties such as cetyltrimethyl ammonium bromide (CTAB) and polyethylene oxide (PEO) were compared to determine how size and surface properties affect NET formation. From that research, they concluded that NP's surface chemical characteristic had only a slight effect on NET formation but had significantly more impact on the range and ratio of gold PMN aggregates. The positively charged CTAB- and PEO-NH_2-coated AuNPs were more frequently located internally in the NETs than PEO-OH- or PEO-COOH-modified NPs. In this study, we also found that unless we use DNase-pretreated neutrophils before staining, gold PMN aggregates could be detected. Meanwhile, gold NPs remain in the structure. It proves that there are some proteins in the gold PMN aggregate structure that may not be influenced by DNase and still play a role in trapping NPs. Since DNA structure is the main component part and carries a net negative charge, positively charged particles of AuNPs trapped by NETs can be explained by electrostatic forces [77]. Ali et al. [84] did researches on gold nanorods (AuNRs). In this study, AuNRs showed ability to treat cancer [84]. Another study has investigated how AuNPs with a size of 60 nm induce the generation of free radicals that may be involved in NET formation [81].

Table 1: Nanoparticles (NPs) that induce NETs.

NPs	Reference
Gold	[77]
Silver	[55, 74]
Cationic lipid	[75]
Polystyrene	[76]
Nanodiamonds	[76]
Graphene oxide	[78]

2.3. Silver Nanoparticles (AgNPs). Silver nanoparticles (AgNPs) are widely used in many fields such as electronics, biosensing, and food adjuvants. AgNPs may also be used in medical applications such as drug delivery because of their size and antimicrobial properties [85]; however, AgNPs do have significant dose-dependent cytotoxicity. Meanwhile, it still remains controversial whether AgNPs or silver ions (Ag^+) have been attributed to the cytotoxicity, because the majority of cell culture studies are done in suspension that makes it difficult to differentiate between particle and soluble Ag^+ effects [86, 87]. A study in which AgNP particle dissolution (and aggregation) in cell culture media was prevented by using an air-liquid exposure cell system did not cause cytotoxicity or induce the release of proinflammatory markers [88]. However, more experiments are needed to clarify the fate of intracellular AgNPs and Ag^+. Several studies have evaluated the effects of AgNPs on neutrophils including NET formation in vivo and in vitro. Liz et al. [74] reported that 15 nm AgNPs ($AgNP_{15}$) induce atypical cell death in neutrophils in a caspase-1- and caspase-4-dependent process. AgNPs also induced ROS and IL1β [89]. The atypical cell death was also inhibited by the antioxidant n-acetylcysteine indicating ROS dependency on the AgNP-induced atypical cell death. AgNPs also induced NETosis in adherent neutrophils that could not be inhibited by caspase-1 and caspase-4 inhibitors [74]. During AgNP-induced activation, the volume of neutrophils increased when the expression of the neutrophil surface marker CD16 remained the same unlike apoptotic neutrophils where the CD16 expression decreased [90, 91]. These changes were related to oxidative stress.

2.4. Cationic Lipid Nanoparticles. Solid lipid nanoparticles (SLNs), which are made up of solid crystalline lipids at room and body temperature, are among the colloidal nanosystems [92]. Nowadays, SLNs are commonly used in nanomedicine as drug carriers for a variety of medical treatments including cancer therapy, medical diagnosis, and tissue impairing [93]. Cationic SLNs (cSLNs) have been useful as carriers for DNA and RNA in promoting gene transfection and expression, respectively [94, 95]. Investigation into the possible roles for cSLNs in inflammation is still lacking. Hwang et al. designed a study examining the effect of cSLNs on human primary neutrophils and whether cSLNs can induce NETosis. As noted above, NETosis is initiated when the nuclear

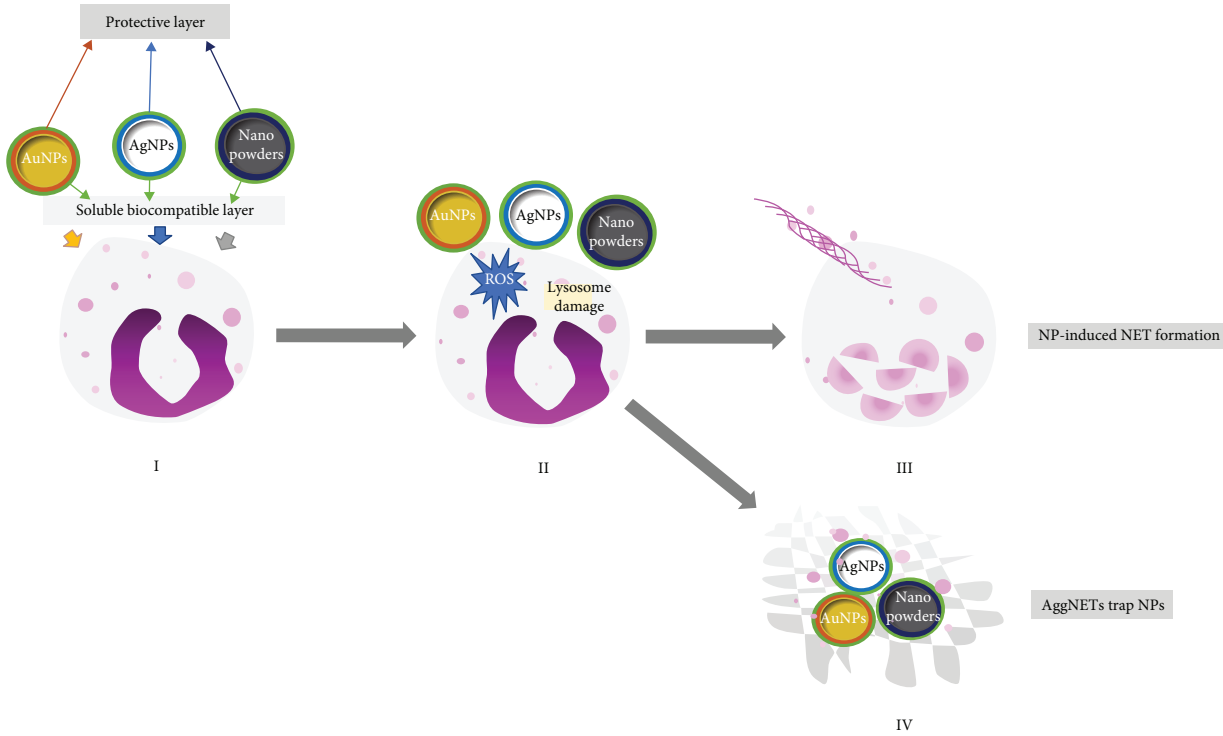

Figure 1: Several kinds of nanoparticles (gold nanoparticles, silver nanoparticles, nanopowders, etc.) can induce NET formation (I). Once the NPs contact the cellular membrane, cellular activation and/or cellular membrane damage can lead to lysosomal damage and ROS production (II). NP stimulation may induce histone deimination and chromatin decondensation resulting in the formation of NETs (III). Neutrophils may form aggNETs and trap NPs in order to eliminate and immobilize inflammatory particles (IV).

and granular membranes rupture with subsequent chromatin decondensation and diffusion into the cytoplasm [61, 67]. Their results indicated that oxidative stress, Ca^{2+} influx, and MAPK pathway signaling were essential to cSLN-induced NET formation. All these findings indicate the significance of cSLNs in the activation of neutrophils [75].

2.5. Carbon and Polystyrene Nanopowders. Muñoz et al. [76]. recently studied the interaction between neutrophils and carbon and polystyrene NPs. Environmental exposure to carbon NPs, including nanodiamonds, is unavoidable. Carbon NPs are a ubiquitous, necessary by-product of common procedures used in manufacturing and business including abrasive grinding and laser printing to carbon combustion that generates smoke. While NETs are induced by inflammation, aggregated NETs (aggNETs), which are generated under high neutrophil densities, may restrict and promote the absorption of inflammation [96]. Neutrophil NETs and aggNETs can capture and "neutralize" NPs in a size-dependent mechanism. When small NPs such as nanodiamonds (d) with a size of 10 nm (d10) and polystyrene beads (b) with a size of 40 nm (b40) were used to stimulate neutrophils, classical NET-like structures appear similar to those induced with PMA, whereas larger NPs (d_{1000} or b_{1000}) did not induce NETs. Thus, there is a conclusion that both unipolar diamonds and polystyrene beads may induce NETs in a size-dependent way in vivo. This process activates a short-term inflammatory response and limits inflammation by immobilizing and entrapping NPs. They also got a conclusion that small-sized NPs may damage the cell molecular barrier and the function of cell membrane ion selectively. Oxidative stress and lysosomal damage are vital in NP-induced NETosis. The membranes were damaged by NPs and used for recycling in body systems firstly, then fused with primary lysosomes to form into phagolysosome. When lysosomes ruptured, the oxidative stress is being activated and the production of ROS is increasing beyond intracellular pathways. In order to prevent further tissue damage, neutrophils formed aggNETs to restrict and immobilize NPs that lead to an endpoint of inflammation [76] (Figure 1).

2.6. Oxidative Stress Is the Major Mechanism of NP-Induced NET Formation. A number of studies indicate that oxidative stress is a major pathway in NP-induced NET formation by nanoparticles such as AgNPs, cSLNs, and nanopowders [76, 77, 85, 95]. In classical PMA-induced NETosis, reactive oxygen species is a vital factor. Thus, there was a hypothesis that ROS is the major pathway in NP-induced NET formation. Research with AgNPs revealed that AgNP-induced NETosis could not be reversed by the inhibitors of caspase-1 and caspase-4 [74, 85]. IL-1β, an inflammatory cytokine, is also measured, and it was found that its expression is decreased due to the function of caspase-1 and caspase-4 inhibitors. ROS was assayed by flow cytometry and found to be increased by AgNPs. Therefore, it was concluded that AgNPs rapidly induced an atypical cell death in neutrophils by a mechanism involving caspase-1, caspase-4, and ROS [74]. In the research of cSLNs, Hwang et al. [97]

found that cSLNs can activate neutrophils through respiratory and degranulation pathways. cSLNs induce a dose-dependent increase in superoxide anion production. Uptake of cSLNs activated Ca^{2+} channels and increased Ca^{2+} influx. Pretreatment with the Ca^{2+} influx inhibitor BAPTA-AM inhibited increases in Ca^{2+} influx and ROS induced by cSLNs [75]. Muñoz et al. concluded that both carbon and polystyrene nanopowders induced NETs by an oxidative stress-dependent mechanism. The NPs damaged neutrophil cell membranes and caused lysosome to rupture to activate the production of ROS and induce NETosis [76].

3. Conclusion and Perspective

Nowadays, nanoparticles have become widely used in engineering, vaccine carrying, and drug delivery due to their biochemistry and biocompatibility. The interaction between NPs and the innate immune system, especially neutrophils, is a vital area of research to be further pursued. Currently, neutrophils release NETs and trap sterile NPs and nonsterile pathogens as soon as they can, and NPs can be trapped due to their different biochemical properties. Further studies are needed to understand the interaction between NPs and NETs. Meanwhile, it is important to know the most vital properties of NPs in NETosis. Thus, NP-induced NET formation needs to be further investigated to figure out their physiological roles to utilize NPs well in nanomedicine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

HY and YZ wrote the first draft of this article. HY, YL, LZ, XC, and YZ designed the figures. YZ, MH, and TM critically revised the manuscript for important intellectual content. All authors approved the final version.

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Research Article

Effect of CXCR2 Inhibition on Behavioral Outcomes and Pathology in Rat Model of Neuromyelitis Optica

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Objective. To reduce immune-mediated damage in a rat model of neuromyelitis optica (NMO) by blocking neutrophil migration using SCH527123, a drug that inhibits CXCR2. *Background*. Neuromyelitis optica is a relapsing autoimmune disease that preferentially targets the optic nerves and spinal cord leading to blindness and paralysis. Part of the immunopathogenesis of this disease is thought to involve neutrophils, which are present within NMO lesions. We tested the effect of blocking neutrophil migration in an NMO rat model. *Methods*. The Lewis rat model of NMO uses a myelin-reactive experimental autoimmune encephalomyelitis (EAE) background with passive transfer of pooled human antibody from patients with aquaporin-4 (AQP4) seropositive NMO at onset of EAE symptoms. We treated rats early in the course of EAE with CXCR2 inhibitor and assessed the extent of neutrophil infiltration into the spinal cord and the extent of AQP4 depletion. *Results*. CXCR2 inhibitor decreased neutrophil migration into the spinal cord of AQP4 IgG-treated EAE rats. However, there was no difference in the acute behavioral signs of EAE or the extent and distribution of AQP4 lesions. This suggests that neutrophils are not centrally involved in the immunopathogenesis of the Lewis rat NMO disease model. *Conclusions*. CXCR2 inhibitor blocks neutrophil migration into the spinal cord during EAE but does not significantly reduce inflammation or AQP4 lesions in the Lewis rat model of NMO.

1. Background

Neuromyelitis optica spectrum disorder (NMOSD) is a devastating autoimmune disease that targets the optic nerves and spinal cord leading to blindness and paralysis [1]. The pathology of NMOSD is distinguished from other demyelinating diseases, such as multiple sclerosis, by the presence of the aquaporin-4 (AQP4) serological antibody and by humorally mediated inflammatory markers associated with AQP4-depleted lesions, including perivascular immunoglobulin and complement [2, 3]. In addition, neutrophils and other granulocytes are present in lesions in the spinal cord and optic nerve where they have been speculated to contribute to the permanent destruction of the myelin, glial cells, and vulnerable neurons [4].

Neutrophils have been identified as a key player in the effector pathway of NMO disease and are being targeted for intervention in clinical trials [5, 6]. Animal models of NMO demonstrated that neutrophils are important in lesion development; inhibition of neutrophils reduces the severity and size of lesions [7]. CXCR2, also known as IL8RB, is a receptor for interleukin-8, CXCL1, and GRO-beta, which are secreted by neutrophils as a chemoattractant to amplify neutrophil recruitment to sites of injury and inflammation [8]. We tested the potential benefit of inhibiting neutrophil recruitment with a small molecular CXCR2 inhibitor, SCH527123, in a rat model of NMO. The purpose was to evaluate the impact of CXCR2 inhibition on the severity of immune-mediated damage in the central nervous system.

2. Methods

2.1. Rats and Induction of EAE. All animal studies were approved by the Johns Hopkins Animal Care and Use Committee. 8-week-old female Lewis rats (~150 g; Envigo, Indianapolis, USA) were housed in the pathogen-free animal

facility at Johns Hopkins on a 12-hour day light cycle. Complete Freund's adjuvant containing 10 mg/ml heat-killed Mycobacterium tuberculosis H37RA (Difco) in Incomplete Freund's adjuvant (Imject; Thermo Scientific) was mixed 1 : 1 with a 1 mg/ml solution of guinea pig myelin basic protein (gpMBP, Sigma USA, or extracted from guinea pig spinal cord) in PBS and mechanically emulsified for 10 minutes (Norm-Ject luer lock; cat. no. 4100-X00V0; Thermo, MX5341L). EAE was induced by subcutaneous injection of 100 μl of this mixture with a 20-gauge needle at the base of the tail under isoflurane anesthesia (2.5% Forane in oxygen). All rats were weighed and scored daily starting at day 5 post-immunization. Scoring was assigned using a standard 5 point scale: 0, no deficit; 0.5, partial loss of tail tone or slightly abnormal gait; 1.0, complete tail paralysis or both partial loss of tail tone and mild hind limb weakness; 1.5, complete tail paralysis and mild hind limb weakness; 2.0, tail paralysis with moderate hind limb weakness (evidenced by frequent foot dragging or turning over while walking); 2.5, no weight-bearing on hind limbs (dragging) but with some leg movement; 3.0, complete hind limb paralysis with no residual movement; 3.5, hind limb paralysis with mild weakness in forelimbs; 4.0, complete quadriplegia but with some movement of the head; 4.5, moribund; and 5.0, dead. Animals reaching a score of 3.5 for >1 day or a score of 4 at any time were euthanized. Water source is low to the floor of the cage such that paralyzed animals can easily reach it; food was placed on the floor of the cage when signs of any paralysis were noted.

2.2. NMO-IgG Preparation. AQP4 antibody (NMO-IgG) was among the total IgG from plasmapheresate donated by aquaporin-4 antibody-seropositive donors undergoing treatment for a relapse; fluids were stored at −80°C until use. Plasmapheresate was diluted 3-fold in PBS, clarified at 10,000 ×g for 15 minutes at 4°C, and filtered with 70 μm strainer (Millipore) to remove particulate materials. Clarified solution was loaded on to 8 ml columns of Protein G-Sepharose (Biovision Inc., cat. no. 6511-25) equilibrated with phosphate buffered saline (PBS). Columns were then washed with 10 column volumes (CV) of PBS. Purified IgG was eluted with 0.1 M glycine-HCl, pH 2.6 into 1.5 ml tubes containing 90 μl of neutralization solution (1 M Tris-HCl pH 9.0). Fractions with 280 nm absorbance greater than 5-fold over background were pooled and buffer-exchanged to PBS using Amicon spin filters (50 KDa MWCO, 15 ml; Millipore). The final volume was adjusted to 7–10 ml of PBS and sterile filtered. The final yield typically was 140–160 mg of IgG from 50 ml of plasmapheresate.

2.3. AQP4 IgG/Drug Treatment of Lewis Rats. Vehicle (0.5% hydroxypropyl methylcellulose) or CXCR2 antagonist, SCH527123 drug (generously provided by Genzyme), was injected as indicated in Figures 1(a) and 1(b), intraperitoneally, at 10 or 30 mg/kg on 3-4 days preceding tissue collection. Pooled purified IgG from three AQP4 seropositive patients was injected intraperitoneally on the two days preceding tissue collection. Depending on the cohort, each animal received 12 mg (cohort 1) or 15 mg (cohort 2) of IgG on each of the two days. Rats were euthanized the day following the last injection. One cohort included a control treatment arm with methylprednisolone acetate on days 8 and 10 postimmunization (Depo-Medrol, 10 mg/kg, subcutaneous, Pfizer).

2.4. Tissue Collection, Embedding, and Sectioning. Rats were terminally anesthetized by inhalation of isoflurane via necrosis chamber (VWR) and perfused with 75 ml of cold 100 mM HEPES, pH 7.3, 5 mM sodium pyruvate, 2 mM KCl, 2 mM MgCl2, and 124 mM NaCl through the left cardiac ventricle. Following decapitation, the unfixed spinal cord was immediately removed, placed lengthwise on a flat wooden stick, and frozen by immersing in a 500 ml bath of dry ice-cooled 2-methylbutane and stored at −80°C until OCT embedding (Sakura Tissue-Tek). Ten micron sections of tissue from 6–8 spinal levels were placed on Fisherbrand Plus slides and immediately placed in a dry ice-cooled slide box. Slides were stored at −80°C until used.

2.5. Immunohistochemistry. Slides were removed from −80°C and air-dried for 5 minutes. A hydrophobic barrier (Liquid blocker Super PAP pen; Electron Microscopy Sciences, USA) was applied around sections either before (paraformaldehyde) or after (acetone) fixation. Fixation was performed by immersion in either freshly prepared ice cold 4% (w/v) paraformaldehyde (PFA) in PBS, pH 7.4, for 1 hour (T cells, C4d, microglia, human IgG, GFAP) or 20 minutes (His48; neutrophils), or in acetone (C5b-9 membrane attack complex, C7, C9) for 10 minutes at −10°C. PFA-fixed slides were then washed in PBS while acetone-fixed slides were air-dried for 5 minutes then washed in Tris-buffered saline (TBS).

Staining conditions are summarized in Table 1. Alkaline phosphatase procedures used TBS for washes and diluents and signal was developed with 5-bromo-4-chloro-3-indolyl phosphate (4-toluidine salt) with 4-nitro blue tetrazolium chloride (BCIP+NBT; Sigma-Roche) using 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM MgCl2 supplemented with 4 mM tetramisole hydrochloride as a development buffer and levamisole to block endogenous phosphatases (Sigma). Horseradish peroxidase procedures (ABC-HRP) used PBS + 0.1% Triton®-X100 (SigmaUltra) for washes and diluents; endogenous peroxidases were quenched by immersing slides in 3% H_2O_2 in PBS for 20 minutes and washed before blocking with 5% goat serum. When ABC-HRP was used, endogenous biotin reactivity was blocked by supplementing the 5% goat serum (Sigma) during blocking with 1 : 20 avidin and the primary antibody staining solution with 1 : 20 biotin (Avidin-biotin blocking kit; Vector Labs), with 3 × 2 min washes with PBS in between. All primary antibody incubations were performed overnight at 4°C except MAC, C9 (90 minutes, room temperature), and anti-human-IgG (30 minutes, room temperature). Anti-rabbit Immpress Alkaline Phosphatase (Vector Labs) was used without dilution (C4d), while alkaline phosphatase-conjugated anti-mouse IgM (Sigma) used for His48 staining was diluted in 5% goat serum in TBS with 0.1% Triton-X100. Staining with anti-C5-C9 omitted all detergents and used 2% (w/v) bovine serum

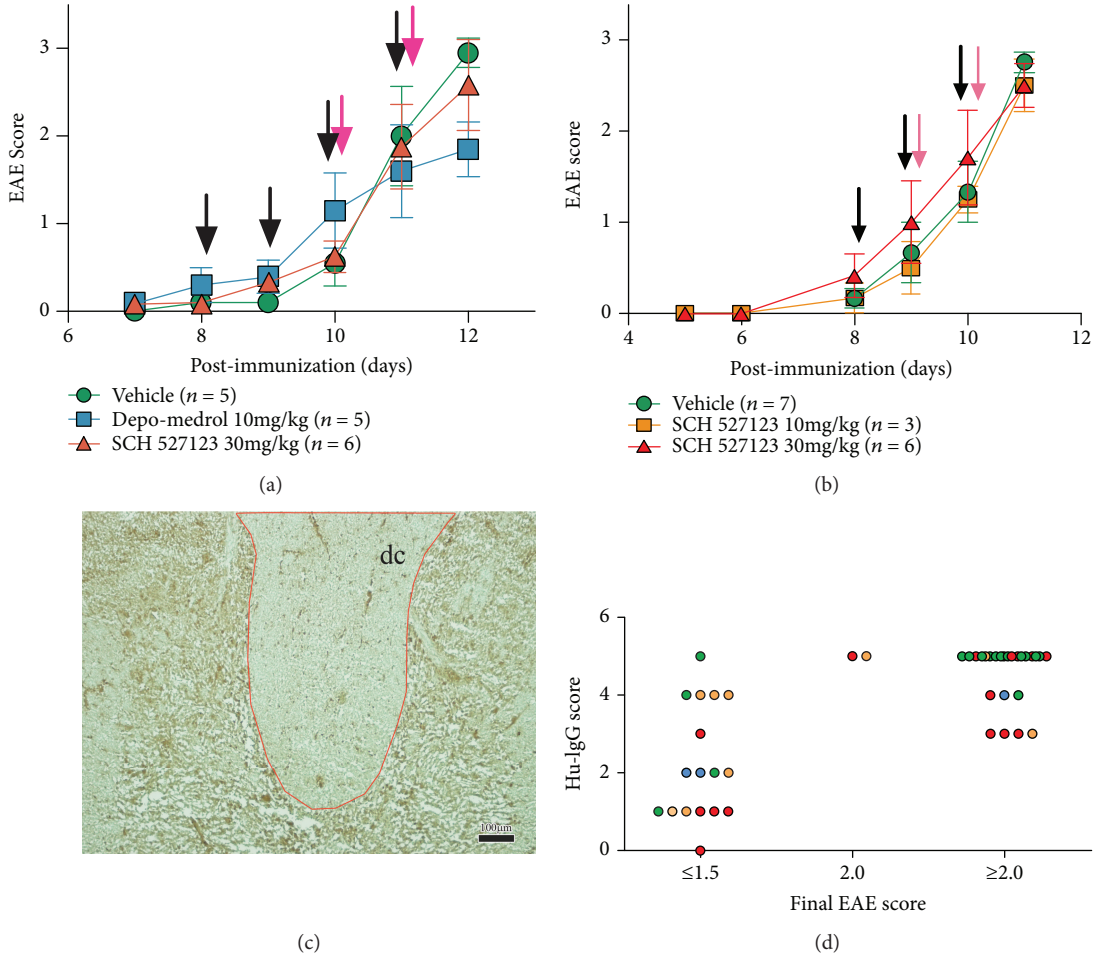

Figure 1: (a, b) After immunizing Lewis rats with gpMBP to induce EAE, CXCR2 antagonist (red triangles) or vehicle control (green circles) was injected at the time points indicated by the black arrows and pooled IgG from AQP4-seropositive NMO patients was injected at the time points indicated by the pink arrows. Treatment with corticosteroid, Depo-Medrol, on days 8 and 10 was included in cohort 1 ((a) blue squares), while cohort 2 tested SCH527123 at both 10 mg/kg (orange squares) and 30 mg/kg (red triangles). EAE scores were not exacerbated by IgG from AQP4-seropositive NMO patients. (c) Diffusion of human IgG (Hu-IgG) into the spinal cord was assessed by immunohistochemistry (brown stain) and scored semiquantitatively. (d) Human IgG (Hu-IgG) was associated with higher EAE scores, while lower EAE scores were associated with lower levels of human IgG signal in the spinal cord.

Table 1: Immunohistochemistry markers.

Marker	Working dilution (clone)	Source company	Fixation/ time	Detection	Chromogen
Human IgG	1 : 1000	Vector Labs	PFA, 1 h	Elite ABC-HRP (Vector Labs)	DAB
Neutrophils	1 : 250 (His48)	BD Pharmingen	PFA, 15 min	Alk. phos. anti-ms IgM (Sigma)	BCIP+NBT
Aquaporin-4	1 : 250 (H-80)	Santa Cruz Biotechnology	PFA, 1 h	Elite ABC-HRP	DAB
GFAP	1 : 250	Dako USA	PFA, 1 h	Elite ABC-HRP	DAB
Macrophage/microglia (rabbit anti-iba1)	1 : 1500 (IHC)	Wako Lab. Chem. USA	PFA, 1 h	Elite ABC-HRP	DAB
T cells (CD3)	1 : 60 (sp7)	GeneTex, Inc.	PFA, 1 h	Elite ABC-HRP	DAB
C4d	1 : 1000	American Research Products Inc.	PFA, 1 h	Immpress anti-rabbit alk. phos. (Vector Labs)	BCIP+NBT
Anti-C5-C9	1 : 1000 (#204903)	Calbiochem	Acetone, 10 min	Immpress anti-rabbit alk. phos.	BCIP+NBT

albumin (Fraction V, Sigma A9056; use of serum in this procedure was extremely detrimental to staining).

Biotinylated antibodies were all diluted 1 : 1000 (Vector Labs) and incubated on slides for 30 minutes followed by washing and incubation with Avidin-Biotin HRP complex (ABC, Vector Labs) for a further 30 minutes. HRP signal was developed by immersing slides in freshly prepared and filtered 3,3′-diaminobenzidine tetrahydrochloride hydrate (Sigma) for 5 minutes (0.05% in PBS with 0.03% H_2O_2). Peroxidase-reacted slides were dehydrated, Fast Green-counterstained, and mounted as previously described [9]. Some BCIP+NBT-reacted slides were counterstained with 0.01% Ponceau S in 0.5% acetic acid (Sigma), washed with 50 mM Na acetate pH 5.3, and mounted without coverslip with In Situ Mounting Medium (Electron Microscopy Sciences, cat. no. 17988-30).

2.6. Quantitative Immunohistochemical Analysis. Human IgG signal was scored on a semiquantitative scale of 0 (no antibody signal) to 5 (complete and intense coverage of all spinal cord segments examined for a given animal) in a blinded manner. Other immunoreactive markers were quantified as described [9]. Briefly, high-resolution pictures of each spinal cord segment were acquired at 4× and background corrected. ImageProPlus5.1 (Media Cybernetics, Rockville, MD, USA) was employed to determine the total area of each tissue section (8–10 axial spinal sections per rat) and the total immunoreactive area, with the same color choices for quantification applied to all slides stained in the same batch. Data points are expressed as total % Immunoreactive Area (total stained area/total area × 100%), and the mean ± standard error of the mean are represented by a horizontal line with y-error bars.

2.7. Statistics. Student's t-test was performed on immunohistochemistry results. A result of $p < 0.05$ was considered significant. Results are presented via tools and analysis using GraphPad Prism software.

3. Results

3.1. Study Design. Lewis rats immunized with gpMBP to induce EAE and then injected with pooled IgG from AQP4-seropositive patients developed AQP4-depleted lesions in the spinal cord. The injection of human IgG is most pathological in the first 24–48 hours from behavioral onset of disease. To examine neutrophil involvement in lesion development, a CXCR2 small molecule antagonist, SCH527123, or vehicle control (0.5% methylcellulose) was injected into EAE rats starting on day 8 postimmunization and on the days of IgG injections (black arrows, Figures 1(a) and 1(b)). We tested the hypothesis that treatment with SCH527123 would prevent the influx of neutrophils into the spinal cord and would subsequently attenuate the formation of AQP4-depleted lesions.

3.2. Behavioral Outcomes. Injections of IgG from NMO patients did not significantly exacerbate EAE scores within the short time period examined in this experiment (Figure 1(a)). In addition, treatment with SCH527123 at either 10 mg/kg or 30 mg/kg did not affect the development of EAE behavioral disease (Figure 1(b)). Conversely, corticosteroid (Depo-Medrol) treatment before injection with IgG significantly attenuated EAE development relative to the vehicle control ($p < 0.02$ on day 12 postimmunization). Despite the limited use of this NMO rat model for evaluation of neurological deficits due to AQP4 IgG, the model is useful to study the pathological mechanisms of astrocyte damage due to AQP4 IgG and complement.

3.3. Pathological Outcomes at the Blood-Brain Barrier. Behavioral signs of EAE progressed quickly once initial signs were observed, often progressing from a score of 1.0 (limp tail) to 3.0 (complete hind limb paralysis) in a span of 24 hours beginning at day 10 or 11 postimmunization. At the onset of neurological disability, blood-brain barrier (BBB) integrity is briefly compromised allowing injected human IgG into the CNS (Figures 1(c) and 1(d)), along with other serum proteins and any drugs that had been injected that might not enter the CNS under basal conditions. In contrast, animals injected with human IgG before behavioral disease onset had moderate to no detectible human IgG in the CNS (Figure 1(d)). Immunohistochemical signal for human antibody in the spinal cord was most robust in animals that have a score of 1.0 to 2.0 on the day of injection of pooled IgG from AQP4 seropositive patients and have a score of ≥2.5 on the following day.

3.4. Pathological Outcomes in the Spinal Cord. In the first cohort, neutrophil accumulation in the CNS was significantly decreased in animals treated with SCH527123 compared to the vehicle-treated group (Figure 2(a); $p < 0.0005$). Corticosteroid (Depo-Medrol) treatment also decreased neutrophil influx ($p < 0.05$). In a second cohort which compared 10 and 30 mg/kg of SCH527123, the higher dose of drug in animals with high IgG levels showed a trend towards blocking neutrophil influx compared to the vehicle-treated group. The degree of variability in the vehicle-treated group was significantly increased compared to the SCH527123-treated group (Figure 2(c), F ratio: $p < 0.02$). When responses from animals with high IgG deposits in the CNS from both cohorts are combined, the overall result demonstrates that treatment with SCH527123 at 30 mg/kg decreased the influx of neutrophils induced by NMO-IgG by 60% (Figure 2(d), $p < 0.02$).

Pooled IgG from AQP4-seropositive NMO patients historically causes focal areas of Aqp4 loss in this rat EAE model. Figure 3(a) shows an example of a typical AQP4-depleted lesions in this rat EAE model, and Figure 3(b) shows an adjacent section stained for GFAP (glial fibrillary acidic protein). We quantified AQP4-depleted lesions as a percentage of total spinal tissue area in the sections examined and found that SCH527123 does not reduce the lesion area compared to vehicle control (Figure 3(c)). As expected, corticosteroids do have a protective effect. In rats that showed only low deposits of human IgG, AQP4-depleted lesions did not develop, regardless of control or treatment group (Figure 3(d)).

Treatment with SCH527123 did not significantly alter the levels of activated macrophage/microglial lineage cells in the

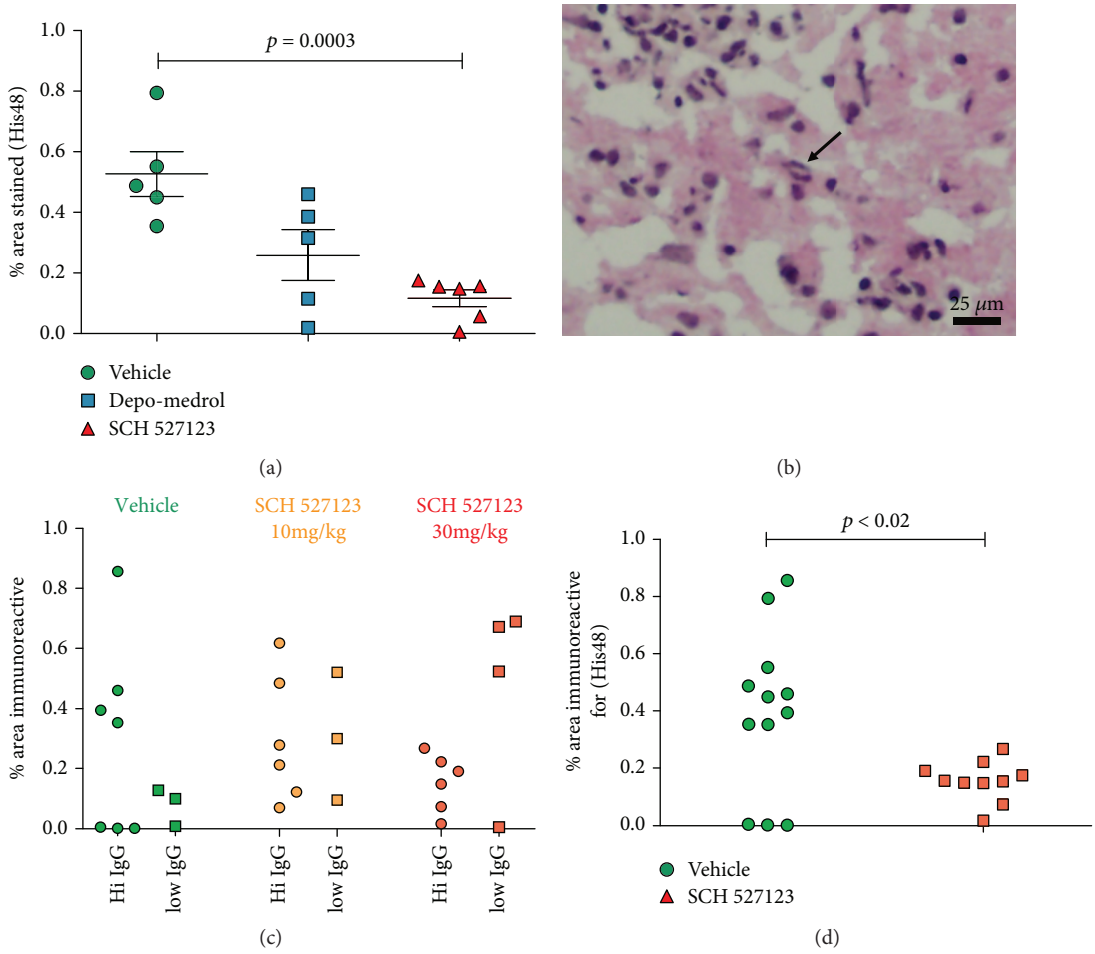

Figure 2: SCH527123 decreases neutrophil migration into the spinal cord of EAE rats with high human IgG levels. (a) In this cohort, Hu-IgG levels were high in both the vehicle- and SCH527123-treated animals. At 30 mg/kg, SCH527123 treatment (red triangles) significantly decreased neutrophil accumulation in the spinal cord of EAE rats when compared to the vehicle-treated group (green circles, $p < 0.0003$). (b) Examples of His48+neutrophils in the spinal cord of EAE rats (arrows, bar = 25 μm). (c) In this cohort, where Human IgG levels in the CNS were more variable, there is a trend towards decreased neutrophil recruitment in SCH527123 treatment at 30 mg/kg compared to vehicle-treated animals only in animals with high Hu-IgG scores, but high variability in the vehicle-treated group limits the differences between these groups (Student's t-test $p = 0.362$; variance is significantly increased $p < 0.02$). (d) Pooling results for rats with high IgG in their spinal cords from the two cohorts for vehicle and SCH527123 treatment at 30 mg/kg indicate that this higher dose significantly decreases His48+neutrophil recruitment following high levels of human IgG deposition in the spinal cord of EAE rats ($p < 0.02$).

spinal cords of EAE rats, as measured by the marker IBA-1 (Figures 4(a) and 4(c)). In addition, despite reports that a subset of T cells expresses CXCR2, numbers of infiltrating T cells (CD3+) were also not significantly affected by SCH527123 treatment (Figures 4(b) and 4(c)). Complement markers were also not significantly reduced by SCH527123 treatment including classical complement marker C4d (Figures 5(a) and 5(b)) or terminal complement markers C5b-9 (Figures 5(c) and 5(d)).

4. Discussion

The Lewis rat model of NMO is a useful animal model in which to test the role of neutrophils in the pathology of immune-mediated damage in NMO [10]. This particular rat model is carried out in a Lewis rat on a background of experimental autoimmune encephalomyelitis (EAE) using T cells reactive to myelin basic protein, in this case, generated by direct immunization. Pooled immunoglobulin from NMO patients who are seropositive for the aquaporin-4 antibody is then passively transferred into rats early in the course of EAE. In this model, the aquaporin-4 (AQP4) antibody specifically binds astrocytes in the rat central nervous system leading to antibody-mediated astrocytopathy, consistent with the human disease pathology [11, 12].

The complement activation pathway, initiated by human antibody fixation of rat complement, generates C5a, which attracts neutrophils [13]. Both CXCR2 ligands and C5a alone are neutrophil chemoattractants. Although the mechanistic link between C5a and CXCR2 ligands is poorly understood, in our complement-driven model, it appears that blocking CXCR2 with the small molecule CXCR2 antagonist, SCH527123, decreases neutrophil migration into the CNS. However, the prevention of neutrophil migration into the

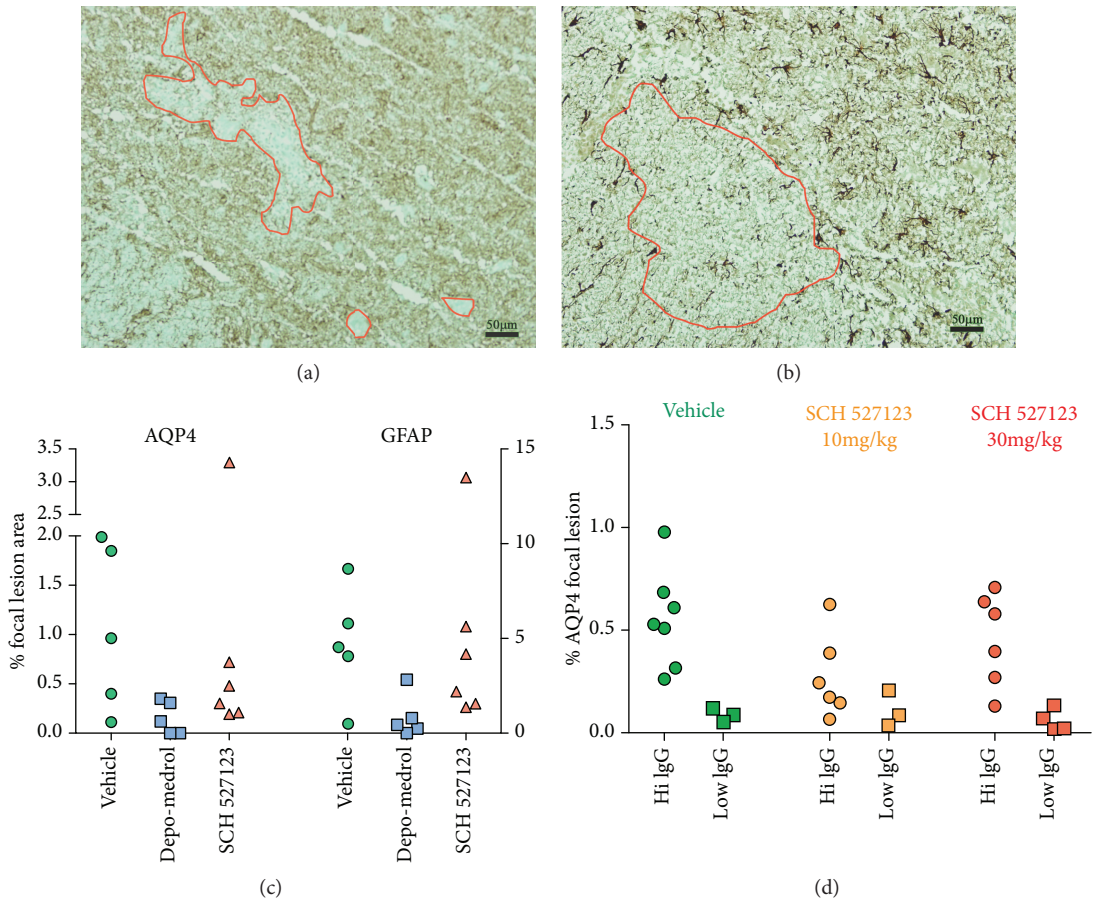

Figure 3: Astrocyte damage is not attenuated by treatment with CXCR2 antagonist, SCH527123. Examples of focal astrocyte death demonstrated by loss of AQP4 (brown stain (a)) or GFAP (brown stain (b)) immunohistochemistry in adjacent tissue sections (bar = 50 μm). In the first cohort (c), AQP4- and GFAP-depleted lesions (left and right y-axis, respectively) were not significantly decreased by SCH527123 treatment at 30 mg/kg (red triangles) compared to vehicle-treated rats (green circles). Depo-Medrol treatment at 10 mg/kg (blue squares) significantly decreased lesion load ($p = 0.042$). In the second cohort (d), there was no significant astrocytic protective effect of SCH527123, even when the groups were subdivided by IgG deposition, where the "hi IgG" animals showed relatively higher levels of IgG deposition and AQP4/GFAP loss compared to the "low IgG" animals.

CNS did not result in the reduction of AQP4 lesions, suggesting that neutrophils are not central to this part of the pathogenesis of NMO. Close physical association of CXCR2 and C5a receptors (C5aR) on the surface of neutrophils has been reported [14]. Both receptors use similar intracellular signaling pathways involving Gi protein-mediated induction of protein kinase C, biphasic intracellular calcium increases, and of activation of ERK1 and 2 [15, 16]. CXCR2 is known to form heterodimers with other receptors, which can positively or negatively regulate each partner's response to its ligands and antagonists [17, 18]. It has been reported that C5a treatment of human neutrophils interferes with calcium responses to subsequent CXCR2 ligand treatment but that CXCR2 ligand does not block C5a responses [14]. SCH527123 is thought to lock CXCR2 into a form that is not conducive to signaling but allows ligand to bind (a so-called "allosteric inverse agonist" [19]); if CXCR2 and C5aR form heterodimers on neutrophils, our results suggest that SCH527123 may block responses to both neutrophil chemoattractants (CXCR2 ligands and C5a) while only interfering with the signaling to the receptor for one (CXCR2).

Behaviorally, there was no apparent benefit for rats that received SCH527123. However, this model is not ideal for assessing this outcome measure. Our rats did not show significant neurological worsening with passive transfer of the AQP4 IgG. Other investigators have shown that AQP4 IgG exacerbates behavioral scores of EAE rats [20, 21]. We speculate that the difference in outcomes may be due to varying toxicity of AQP4 IgG clones used by other investigators [22–24]. Alternatively, we may have terminated the study too early to see this effect [21].

Neutrophils are primarily thought of as a first-line defense against invading pathogens, using a combination of Fc- and complement receptor-driven pathways to pour toxic granule contents and oxygen-derived free radicals on to target cells [25, 26]. Neutrophils also invade tissues in a wide variety of injury models and they can have both positive and negative roles in repair, depending on the tissue and the injury. For example, neutrophils have been implicated with impeding functional recovery from spinal cord injury by promoting glial scarring in the CNS [27] but also have been shown to promote repair in the peripheral nervous

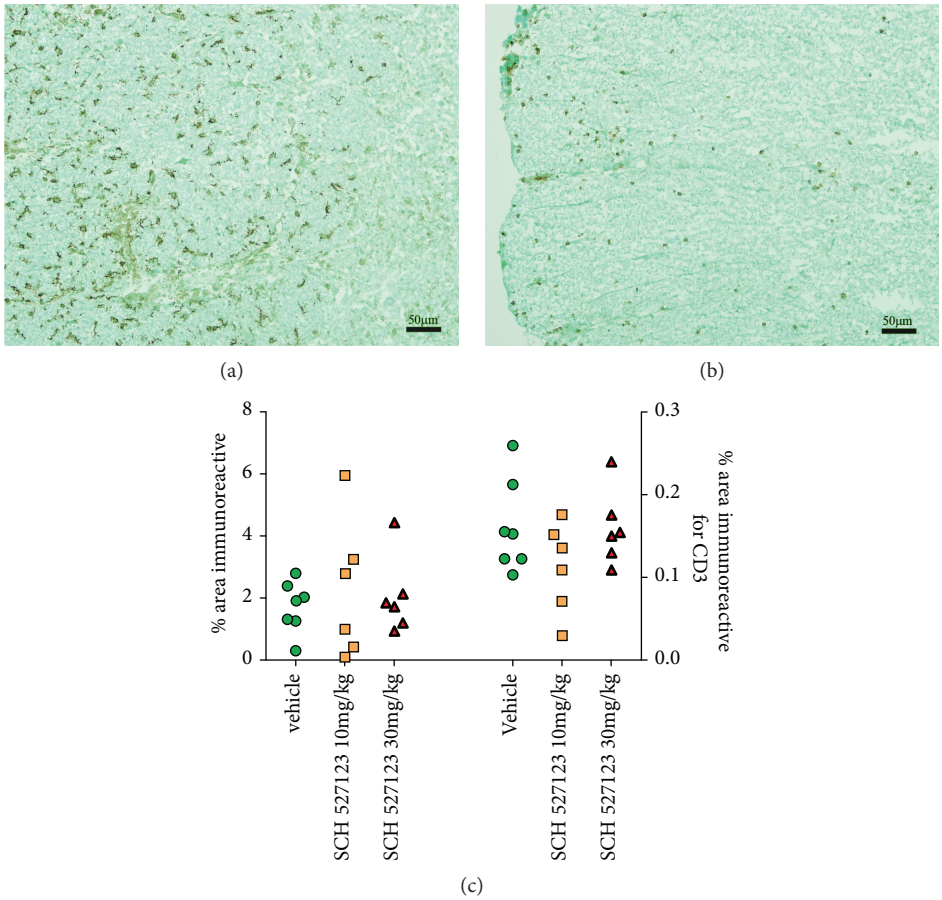

Figure 4: CXCR2 antagonist, SCH527123, does not alter macrophage/microglial activation or CD3 T cell influx into the spinal cord. Representative sections of immunohistochemistry using IBA1 to mark macrophages and microglia are shown (a) and CD3 to mark T cells (b) (bar = 50 μm). Focusing only on animals in cohort 2 for which Hu-IgG signal and astrocyte damage were high, neither IBA1 (left axis) nor CD3 (right axis) signal was altered by SCH527123 treatment (blue squares, 10 mg/kg; red triangles, 30 mg/kg) as compared to vehicle-treated animals (green circles).

system through phagocytic functions [28]. Neutrophils and neutrophil expression of CXCR2 are essential to the development of EAE [29, 30] and in the progression of cuprizone-induced demyelination [31]. While reducing neutrophil accumulation up to 60% with SCH527123 treatment did not significantly protect astrocytes from damage in our study, it is possible that low levels of neutrophil involvement were sufficient to cooperate with the classical complement pathway to initiate damage.

This study used a CXCR2 antagonist to inhibit neutrophil recruitment to the CNS. Many cell types have been reported to express CXCR2 constitutively or after induction (reviewed by [32]), including subsets of T cells, macrophages, and endothelial cells [33]. In CXCR2 null mice, the predominant phenotype is on neutrophil migration. Additional studies confirmed that while CXCR2 function on monocytes responds to activation by migration inhibitory factor (MIF), monocyte migration can function without CXCR2, likely due to the compensation by other chemokine receptors including CCR2, CX3CR1, and CCRs1-5. Other than neutrophils, we did not observe reduction of migration of any other cell type in this Lewis rat NMO model. CXCR2 is also expressed in nonhematopoietic cells that may have an impact on CNS demyelinating models. For example, CXCR2 is expressed on oligodendrocyte precursor cells where it limits remyelination by interfering with their expansion and migration [34]. Oligodendrocyte precursor cells within demyelinated lesions of CXCR2 null mice proliferated earlier and more vigorously [34]. CXCR2 also represents a viable target for promoting remyelination following traumatic injury, dysmyelinating conditions, or infection [35–38].

There are two significant limitations to the Lewis rat model of NMO. First, the disease course is initiated by myelin-reactive T cells, which is not AQP4 specific. It should be noted, however, that in this Lewis rat EAE model, MBP-reactive T cells (whether generated by direct immunization or by adoptive transfer of MBP-reactive T cell lines) do not mediate any cell damage without the subsequent passive transfer of pathogenic autoantibodies. Second, the AQP4 specificity depends on human IgG fractions from patients with NMO. While mouse monoclonal antibodies directed against rat AQP4 transferred into EAE rats induced large confluent AQP4-depleted lesions, the passive transfer of human IgG into EAE rats causes relatively much smaller lesions not reproducible between human IgG specimens. This may be due to a lower affinity of the human antibody

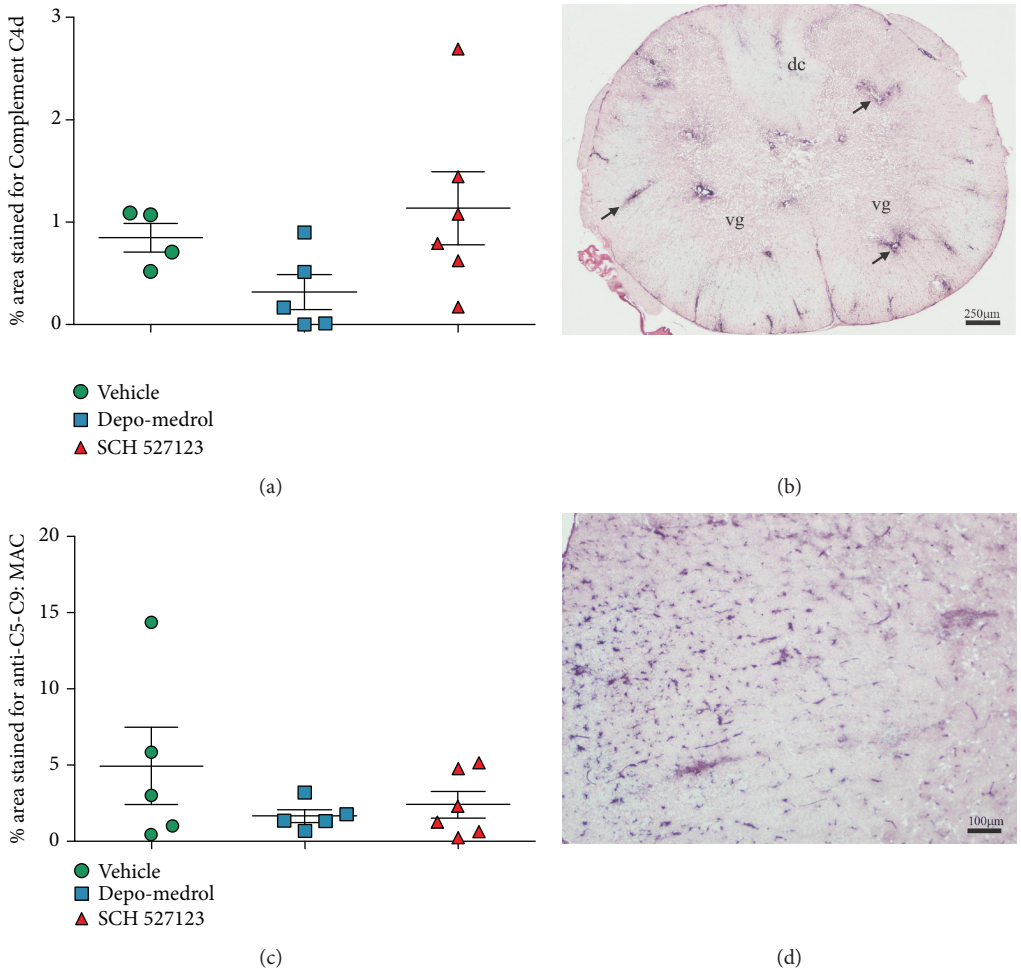

Figure 5: Complement markers are unchanged in NMO rats treated with CXCR2 antagonist, SCH527123. C4d, a marker for activation of the classical complement pathway, showed similar staining patterns in and around spinal cord lesions from rats treated with SCH527123 compared to vehicle control, quantified (a) with a representative sample (b). Depo-Medrol at 10 mg/kg showed lower levels of C4d staining. Staining with anti-C5-C9 was used to assess overall terminal activation of complement pathways. C5-C9 was not significantly different among the three groups, quantified (c) with a representative sample (d).

for the rat AQP4 [12]. Alternatively, this may be due to specimen-specific variation in the mode of toxicity of the antibodies [22], which might influence our ability to block astrocyte damage by targeting only neutrophils.

In conclusion, impeding neutrophil migration into the CNS by CXCR2 antagonism was successful but failed to protect AQP4 from damage by passively transferred human IgG from NMO patients. CXCR2 antagonism may have benefits beyond neutrophil involvement for the treatment of autoimmune-mediated CNS disease and should be explored further.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

Melina Jones has no conflicts of interest to report. Michael Levy currently receives research support from the National Institutes of Health, Maryland Technology Development Corporation, Sanofi, Genzyme, Alexion, Alnylam, Shire, Acorda, and Apopharma. He also received personal compensation for consultation with Alexion, Acorda, and Genzyme and he serves on the scientific advisory boards for Alexion, Acorda, and Quest Diagnostics.

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Review Article

Neutrophil Function in an Inflammatory Milieu of Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by the presence of autoantibodies against citrullinated protein antigens and proinflammatory cytokines which cause chronic synovitis, bone erosion, and eventual deformity; however, the precise etiology of RA is unclear. In the early stage of RA, neutrophils migrate into the articular cavity, become activated, and exert their function in an inflammatory process, suggesting an essential role of neutrophils in the initial events contributing to the pathogenesis of RA. Solid evidence exists that supports the contribution of neutrophil extracellular traps (NETs) to the production of autoantibodies against citrullinated proteins which can trigger the immune reaction in RA. Concurrently, proinflammatory cytokines regulate the neutrophil migration, apoptosis, and NET formation. As a result, the inflammatory neutrophils produce more cytokines and influence other immune cells thereby perpetuating the inflammatory condition in RA. In this review, we summarize the advances made in improving our understanding of neutrophil migration, apoptosis, and NET formation in the presence of an RA inflammatory milieu. We will also discuss the most recent strategies in modulating the inflammatory microenvironment that have an impact on neutrophil function which may provide alternative novel therapies for RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the presence of autoantibodies against citrullinated protein antigens (ACPAs) and proinflammatory cytokines which cause chronic synovitis, bone erosion, and eventual deformity. In the early stage of RA, there is a large infiltrate of neutrophils to the articular cavity, accumulating in both synovial tissue and fluid [1]. The presence of neutrophils in the synovial area correlates with the early clinical manifestations of joint inflammation, suggesting that neutrophils play a significant role in the initiation of RA [2]. Neutrophils are the first cells attracted by chemotactic cytokines and thus are quickly recruited into sites of infection or inflammation. As a member of the phagocytic innate immune system, neutrophils play an indispensable role during infection, injury, autoimmunity, and chronic disease. Neutrophils within circulating blood are captured by adhesion molecules on the endothelial surface of blood vessels and migrate from the bloodstream into pathological sites where invading pathogens are recognized by the host [3]. Neutrophils fulfill their protective functions through phagocytosis and release of granular enzymes and reactive oxygen species (ROS) as well as producing web-like structures called neutrophil extracellular traps (NETs) [4]. The release of granular enzymes and ROS into the extracellular space can cause damage to host tissues during inflammation [5]. Effective constitutive apoptosis of these cells is required for the resolution of inflammation. NETosis is a special type of neutrophil death which can produce NETs composed of a network of extracellular DNA fibers, histone proteins, elastase, and myeloperoxidase. NETs help neutrophils to immobilize and ensnare bacteria, fungi, or viruses, which results in a more effective elimination of these pathogens. It is reported that RA synovial fluid neutrophils show increased NETosis. Neutrophils from patients with RA are preactivated by immune complexes such as rheumatoid factor (RF), resulting in excessive ROS release, degranulation, and NETosis ex vivo [6]. Thus, the activation of neutrophils is associated with the initial inflammation in RA.

In addition, NETs can serve as a potential major source of citrullinated autoantigens which can trigger the development of autoimmune disease such as RA [7]. In recent years, studies have found that the formation of NETs is associated with autoantigens being detected in patients with RA. NET formation may drive ACPA production in the lung and promote the development of the early stage of RA [8]. Furthermore, it is reported that increased sputum NET levels are related to several citrullinated antibody reactivities in patients with RA [9]. Citrullinated autoantigens contained in NETs can be taken up by fibroblast-like synoviocytes (FLS) and presented to T cells in a major histocompatibility complex (MHC) II-dependent manner, leading to amplified antigen-specific T and B cell responses in RA [10]. Therefore, neutrophils may act as a bridge connecting FLS to T cell-mediated responses. In RA, the hallmark profile is the markedly increased proinflammatory cytokines which may regulate the neutrophil functions such as migration, apoptosis, and the formation of NETs [11–13]. In addition, activated neutrophils in the inflamed tissue can express a wide variety of proinflammatory cytokines and chemokines, which are also important to RA pathogenesis and neutrophils themselves [14]. For example, neutrophil-derived production of IL-8 (also known as CXCL8) leads to further rounds of neutrophil migration from the circulation, which enhances the inflammatory process [15]. In this review, we summarize the advances made in improving our understanding of neutrophil migration, apoptosis, and NET formation in the presence of an RA inflammatory milieu. We will also discuss the most recent strategies in targeting cytokines to modulate the function of neutrophils which may provide alternative novel therapies for RA.

2. Neutrophil Migration in RA

Neutrophils are major components in the synovial fluid of RA patients making up to 90% of cells. These cells are also abundant at the junction of the pannus and cartilage, where invasion occurs [16]. During the past decades, it has been reported that neutrophil depletion can greatly inhibit the development of arthritis in two different murine models, collagen antibody-induced arthritis (AIA) and the K/BxN models [2, 17]. Therefore, it is suggested that neutrophils play a crucial role in initiating the inflammatory response and the progression of arthritis in RA. It is important to know which cytokines can affect the neutrophil migration. By blocking or weakening the migration of neutrophil into the inflamed tissue, one would predict that this would lead to a decrease in disease activity of RA, thereby developing a new strategy for the treatment of RA.

It is well accepted that factors including cytokines, chemokines, and immune complexes (RF and ACPA) can contribute to the process of neutrophil migration. In this review, we focus on the cytokines that can orchestrate neutrophil migration in human RA and animal arthritis models. In RA, cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-17a, IL-22, IL-23, IL-1β, IL-8, interferon-γ (IFN-γ), granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-15, IL-18, IL-33, and IL-37 [11, 13, 18–36] are all detected either in the serum or in the synovial fluids of these patients. Thus, we discuss the impact of these cytokines on neutrophil migration in RA separately (Table 1).

2.1. TNF-α Enhances the Neutrophil Migration in RA. It is well known that TNF-α and IL-6 are two of the most predominant proinflammatory cytokines involved in the pathogenesis of RA. Anti-TNF-α and anti-IL-6R agents have been proven to be clinically useful in the disease control of RA. It is reported that TNF-α and IL-6 can both enhance the RA neutrophil migration *in vitro* [11, 21]. An additional study also finds that treatment with an anti-TNF-α agent can decrease the migratory capacity of neutrophils in patients with RA [18]. However, anti-IL-6R agents do not appear to affect neutrophil migration *in vitro* [21]. It indicates that TNF-α is a strong cytokine which can enhance RA neutrophil migration.

2.2. Th17-Related Cytokines Increase Neutrophil Migration in RA. Th17 cells (IL-17 and IL-22) and neutrophils are frequently observed in the synovial fluid of RA patients. A crosstalk exists between Th17 cells and neutrophils. Activated Th17 cells can directly attract neutrophils via the release of IL-8 [37]. Moreover, IL-17 and IL-23 can both induce neutrophil migration after intra-articular injection in a dose-dependent manner. In antigen-induced arthritis, early release of IL-23 can stimulate IL-17 production, in turn, causing a release of TNF-α, a variety of chemokines, and leukotriene B4 (LTB4), which together contribute to neutrophil recruitment. Therefore, IL-23/IL-17-induced neutrophil migration plays an important role in the pathogenesis of a murine model of RA [22]. Furthermore, injection of adenoviral vectors with IL-17 (AdIL-17) into the knee joints before induction of immune complex- (IC-) mediated arthritis has been shown to induce greater neutrophils migrating to the cartilage surface, exacerbating inflammation and cartilage destruction [38]. Interestingly, another Th17-related cytokine IL-22 is highly expressed in the synovial tissue of an antigen-induced arthritis mouse and human RA [25]. Furthermore, IL-22 inhibition and IL-22 genetic deficiency can reduce neutrophil migration in arthritic mice and improve articular pain [25]. Therefore, IL-17 and IL-22 both can promote the neutrophil migrating to the cartilage surface, exacerbating joint inflammation of RA.

2.3. IL-8 Promotes the Neutrophil Migration in RA. IL-8, a potent chemoattractant for neutrophils, plays a vital role in the recruitment and activation of neutrophils. IL-8 is considered to be one of the most crucial inflammatory chemokines involved in the development of arthritis [12]. Injection of lipopolysaccharide (LPS) or IL-1α into joints of rabbits induces arthritis accompanied by upregulation of IL-8. Blockade of IL-8 by neutralizing antibody can ameliorate arthritis and reduce the infiltration of neutrophils into the joints in the early phase of inflammation [39]. In RA patients, IL-8 is significantly elevated in the ACPA^{high} synovial fluid. The elevated level of IL-8 is associated with worse clinical

Table 1: Known cytokines' capacity to induce biological changes in neutrophils in RA.

Cytokines/blockade	Human RA or mouse model	Expression	Migration or recruitment	Apoptosis or survival	NET formation	Outcome
TNF-α	Human RA	↑ in serum and synovial fluid [13, 18, 19]	↑ *in vitro* [11]	Promote or delayed apoptosis *In vitro* [27]	↑ *in vitro* [13]	
Blockade of TNF-α	Human RA	—	↓*in vivo* [18]	Antiapoptotic Mcl-1 ↑ and proapoptotic caspase-9 ↓ [57]	↓ *in vitro* [13, 73]	Improved [73, 98]
TNFR$^{-/-}$	Collagen Ab and LPS-induced arthritis model	—	↓ *in vivo* [52]	—	—	Improved [52]
IL-6	Human RA	↑ in serum and synovial fluid [19–21]	No effect *in vitro* [21]	No effect *in vitro* [21], delayed apoptosis *in vitro* [27]	↑ *in vitro* [73] ↑ *in vitro** [75]	—
Blockade of IL-6	Human RA	—	—	No effect *in vivo* and ex vivo [21]	↓ *in vivo* and *in vitro* [73, 76]	Improved [99]
IL-17a	AIA mouse model and human RA	↑ in the joint [22, 23]	↑ *in vivo* [22]	—	↑ *in vitro* [13]	
Adenoviral vectors with IL-17	IC-mediated arthritis mouse model	—	↑ *in vivo* [38]	—	—	Deteriorated [38]
Anti-IL-17 antibody administration	CIA model	—	↓ *in vivo* [44]	—	↓ *in vitro* [13]	Improved [44]
IL-23	AIA mouse model and human RA	↑ in joint [22, 24]	↑ *in vivo* [22]	—	—	—
IL-22	AIA mouse model and human RA	↑ in synovial tissue [25, 26]	↑ *in vivo* [25]	—	—	—
Block IL-22 or IL-22$^{-/-}$	AIA mouse model	—	↓ *in vivo* [25]	—	—	Improved [25]
rmIL-22 administration	Normal mice	—	↑ *in vivo* [25]	—	—	Deteriorated [25]
IL-1β	Human RA and AIA mouse model	↑ in synovial fluid [19]	↑ *in vitro* [25]	Delayed apoptosis *in vitro** [62]	↑ *in vitro** [81–83]	—
IL-1R$^{-/-}$ or IL-1R antagonist	Collagen Ab and LPS-induced arthritis model	—	↓ *in vivo* [52]	—	↓ *in vitro** [82, 83]	Improved [52]
IL-8	Arthritis rabbit model and human RA	↑ in synovial fluid [19, 27]	↑ *in vivo* [39], ↑ *in vitro* [58]	No effect *in vitro* [27]	↑ *in vitro* [13]	—
Blockade of IL-8	Arthritis rabbit model	—	↓ *in vivo* [39]	—	—	Improved [39]
IL-10	Human RA	↑ in synovial fluid [19]	—	—	↑ *in vitro** [100]	—
IFN-γ	Human RA	↑ in synovial fluid [19]	—	Delayed apoptosis *in vitro* [58]	↑ *in vitro** [79]	—

Table 1: Continued.

Cytokines/blockade	Human RA or mouse model	Expression	Migration or recruitment	Apoptosis or survival	NET formation	Outcome
IFN-$\gamma^{-/-}$	AIA, CIA model	—	↑ *in vivo* [43, 44]	—	—	Deteriorated [43, 44]
GM-CSF	Human RA	↑ released by RAFLS *in vitro* [28], ↑ in serum and synovial fluid [18, 29, 30]	—	Survival ↑ [28], delayed apoptosis in vitro [27, 57, 58]	No effect *in vitro* [13]	—
Blockade of GM-CSF	CIA	—	↓ neutrophils in the joint [48]	—	—	Improved [48]
GM-CSF$^{-/-}$	K/BxN serum transfer arthritis	—	↓ neutrophils in the joint [48]	—	—	Improved [48]
G-CSF	Human RA	↑ in serum and synovial fluid [30, 31]	—	—	↑ *in vitro** [101]	—
rhG-CSF	CIA	↑ serum G-CSF [51]	↑ *in vivo* [51]	—	—	Deteriorated [51]
G-CSF$^{-/-}$	CIA	—	↓ *in vivo* [51]	—	—	Improved [51]
mAb to G-CSF receptor	Collagen Ab-induced arthritis model	—	↓ *in vivo* [47]	—	—	Improved [47]
IL-9	Human RA	↑ in serum and synovial fluid [32, 33]	—	Delayed apoptosis [32]	↑ *in vitro** [85]	—
IL-15	Human RA	↑ in synovial fluid [34]	↑ *in vivo* [45]	Delayed apoptosis *in vitro* [27]	↑ *in vitro** [100]	
IL-18	Human RA	↑ in serum, synovial tissue and fluid [35]	↑ *in vivo* [53]	No effect *in vitro* [59]	↑ *in vitro** [100]	
IL-33	mBSA-immunized mouse model	↑ mRNA expression [11]	↑ *in vivo* [11]	—	↑ *in vitro** [86]	
rmIL-33	rmIL-33 local injection	—	↑ *in vivo* [11]	—	—	Deteriorated [11]
Blockade of IL-33	mBSA-immunized mouse model	—	↓ *in vivo* [11]	—	—	Improved [11]
IL-37	Human RA	↑ in serum and synovial fluid [36]	—	—	—	
IL-37 administration	CIA and streptococcal cell wall fragments induced arthritis	—	↓ *in vivo* [54]	—	—	Improved [54, 102]

RA: rheumatoid arthritis, NETs: neutrophil extracellular traps, TNF-α: tumor necrosis factor-alpha, Mcl-1: myeloid cell leukemia-1, TNFR: tumor necrosis factor receptor, Ab: antibody, LPS: lipopolysaccharide, IL: interleukin, AIA: antigen-induced arthritis, IC: immune complex, rmIL-22: recombinant murine interleukin 22, IL-1R: interleukin 1 receptor, IFN-γ: interferon gamma, CIA: collagen-induced arthritis, GM-CSF: granulocyte/macrophage colony-stimulating factor, RAFLS: rheumatoid arthritis synovial fibroblasts, G-CSF: granulocyte colony-stimulating factor, mAb: monoclonal antibody, mBSA: methylated bovine serum albumin, rmIL-33: recombinant murine interleukin 33, rhIL-37: recombinant human interleukin 37. * is found in other inflammatory diseases or healthy individuals, but not RA

manifestations of the disease. There is a positive correlation between IL-8 and the number of neutrophils in synovial fluid [40]. So IL-8 enhances the neutrophil migration and promotes the inflammatory response in RA.

2.4. IFN-γ Biphasically Affects Neutrophil Migration, While IL-15 Enhances the Neutrophil Migration in RA. IFN-γ has a dual function in the regulation of neutrophil migration. Firstly, IFN-γ is generally considered to be a proinflammatory factor produced by Th1 cells and natural killer cells. IFN-γ modulates neutrophil migration, as shown by a decrease of neutrophil recruitment in IFN-γ-deficient mice and recovery after reconstitution of the IFN-γ signaling pathway [41]. IFN-γ-induced neutrophil migration *in vivo* may be mediated by the release of the neutrophil chemotactic factor from resident macrophages [42]. In contrast, IFN-γ can also exert an anti-inflammatory effect in an RA animal model [43]. In the murine AIA model, the arthritis symptoms are exacerbated by the absence of IFN-γ. Furthermore, CXC chemokine receptor 2 $(CXCR2)^+$ neutrophil recruitment is significantly higher in the joints of IFN-γ-deficient mice. IFN-γ receptor knockout (IFN-γR KO) mice develop collagen-induced arthritis (CIA) more severely with an increased neutrophil influx, while anti-IL-17 antibody administration can ameliorate arthritis partly by reducing the neutrophil infiltration [44]. Based on these findings, it appears that there is a crosstalk between IFN-γ and IL-17 in inflammation. Meanwhile, IFN-γ modulates TNF-α-driven chemokine syntheses in RAFLS, resulting in a downregulation of IL-8 production [43]. IL-15 is a proinflammatory cytokine that has a wide variety of functions in autoimmune diseases. IL-15 has been found to be highly expressed in the synovial fluid of RA patients [34]. IL-15 can mediate blood and synovial neutrophil migration by triggering LTB4 production in antigen-induced arthritis models [45]. Furthermore, IL-15 can also induce NF-κB activation and IL-8 production directly in human neutrophils, thereby activating these cells [46]. In a summary, IFN-γ bilaterally affects neutrophil migration, while IL-15 enhances the migration of neutrophil into joint.

2.5. GM-CSF and G-CSF Both Enhance the Neutrophil Migration in RA. GM-CSF and G-CSF are two hematopoietic growth factors involved in the regulation of hematopoiesis, used to treat neutropenia and to elicit the release of the hematopoietic stem cells from the bone marrow for transplantation [47]. They are both found in human RA serum and synovial fluid [29–31]. GM-CSF can activate and sustain the viability of neutrophils. $GM\text{-}CSF^{-/-}$ mice are resistant to K/BxN serum transfer arthritis, whereas blockade of GM-CSF can ameliorate the severity of arthritis in the CIA mouse model with a simultaneous reduction in synovial neutrophils [48]. Recently, two clinical studies demonstrate that a monoclonal antibody targeting the GM-CSF receptor α called mavrilimumab is effective in the treatment of RA [49, 50]. However, the effect of this novel agent on neutrophil function has not yet been reported. Interestingly, G-CSF and G-CSF receptor-deficient mice are significantly protected in RA animal models partly due to a reduction in the number of neutrophils [47, 51]. A neutralizing antibody to the G-CSF receptor also prevents the progression of the disease by reducing neutrophil accumulation in the joints and inhibiting STAT3 phosphorylation in collagen Ab-induced arthritis [47]. Furthermore, the production of proinflammatory cytokines and chemokines is decreased in anti-G-CSF receptor-treated mice [47]. As such, targeting either GM-CSF or G-CSF may be a promising novel approach to treat RA via the downregulation of neutrophil migration.

2.6. IL-1 Family Cytokines Affect Neutrophil Migration in RA. IL-1 family is a group of 11 cytokines that play a central role in the regulation of immune responses. IL-1β is the most studied member because it is discovered first and it has a strongly proinflammatory effect. Importantly, IL-1β can promote neutrophil migration [25]. This is likely to contribute to RA disease progression since the level of IL-1β in the joint increases with the onset of arthritis and correlates highly with arthritis scores. Moreover, recombinant IL-1β (rIL-1β) administration into mice can induce arthritis, whereas arthritis development is arrested in $IL\text{-}1R^{-/-}$ mice [52]. IL-18, another member of the IL-1 family, functions by binding to its receptor to induce IFN-γ production by T cells which can cause a rapid activation of the monocytes and macrophages. Recently, IL-18 is thought to be a potent mediator of inflammation in RA. It has been reported that IL-18 is significantly elevated in sera, synovial tissues, and synovial fluid of RA patients. IL-18 contributes to the inflammatory process by recruiting monocytes, lymphocytes, and neutrophils to the inflamed joints of RA [35]. In the CIA model, IL-18 activates neutrophils through a synthesis of LTB4 in response to TNF-α, which in turn enhances neutrophil recruitment [53]. IL-33 and IL-37 are both new members of the IL-1 family of cytokines. In methylated bovine serum albumin-(mBSA-) immunized mice, enhanced local neutrophil infiltration is associated with an increase in IL-33 mRNA expression. When recombinant IL-33 is injected into the joints of mice, it induces local neutrophil infiltration which subsequently resulted in joint damage. Moreover, neutrophil migration is inhibited by systemic and local administration of soluble IL-33R [11]. IL-33-induced neutrophil migration is dependent on increased levels of CXCL1, CCL3, TNF-α, and IL-1β concentrations in the joint [11]. Unlike other cytokines, IL-37 is an anti-inflammatory cytokine which suppresses joint and systemic inflammation [54]. IL-37 has a protective effect in an arthritis mouse model by reducing cell influx and inhibiting joint inflammation [54]. In a short conclusion, IL-1 family members, IL-1β, IL-18, and IL-33, all can induce local neutrophil migration, but IL-37 has an opposite effect.

3. Neutrophil Survival in RA

As we know, neutrophils usually have a limited lifespan in the circulation and rapidly undergo constitutive apoptosis when exposed to a stimulator such as TNF-α or Fas [55]. Once recruited into sites of the inflammation, the activated neutrophils can encounter with a variety of proinflammatory cytokines in the microenvironment that may affect their

function and longevity [56]. Many cytokines are generated during inflammation, and these can induce a similar "primed" phenotype in neutrophils affecting their ability to undergo apoptosis. While proinflammatory factors such as GM-CSF, IFN-γ, IL-9, and IL-15 can reduce the induction of apoptosis and extend the lifespan of the neutrophils from RA patients *in vitro*, both IL-8 and IL-18 show no effect on this phenotype [27, 28, 32, 57–59]. In contrast, TNF-α and IL-6 exert a biphasic effect on neutrophil apoptosis [21, 27, 60–63].

3.1. GM-CSF, IFN-γ, IL-9, and IL-15 Delay Apoptosis of RA Neutrophils, but IL-8 and IL-18 Have No Effect. There is strong evidence that supports the role of GM-CSF in prolonging the neutrophil survival and activity in RA *in vitro* [27, 28, 57]. Both GM-CSF and IL-15 inhibit spontaneous apoptosis by downregulating proapoptotic proteins Bax and caspase-3, but only GM-CSF can abolish immune complex-induced apoptosis [64]. Importantly, IL-15 and IFN-γ can delay the apoptosis of RA neutrophils *in vitro* [27, 58]. Recently, Th9 cell frequency has been shown to be higher in synovial fluid of RA patients. Synovial IL-9 can decrease the apoptosis by inducing the expression of the antiapoptotic protein, Mcl-1, and prolonging the survival of the neutrophils [32]. IL-1β can reduce the spontaneous apoptosis of neutrophils from healthy controls *in vitro*, but not RA neutrophils [62, 65]. IL-8 and IL-18 do not affect the apoptosis of RA neutrophils *in vitro* [27, 59]. In summary, GM-CSF, IFN-γ, IL-9, and IL-15 can delay the apoptosis of neutrophils and extend their lifespan which may be harmful to inflammation resolution in RA [32].

3.2. TNF-α and IL-6 Exert a Biphasic Effect on Neutrophil Apoptosis. TNF-α shows an opposing effect where it enhances the rate of apoptosis in human neutrophils during the first 6 hours of exposure, followed by delayed apoptosis after 20 hours in culture [60, 61]. Furthermore, the regulation of TNF-α on human neutrophil apoptosis is dependent on the concentrations of TNF-α *in vitro*. Higher concentrations of TNF-α ($\geq$10 ng/mL) increase the rate of caspase-dependent turnover of the antiapoptotic protein Mcl-1, which controls neutrophil survival, thereby accelerating neutrophil apoptosis, whereas lower concentrations of TNF-α ($\leq$1 ng/mL) can stimulate the expression of the antiapoptotic protein Bfl-1 leading to enhanced neutrophil survival [61]. In contrast, other studies show that TNF-α can reduce the spontaneous apoptosis in neutrophils from healthy individuals (at $\leq$1 ng/mL) and RA patients (at 10 ng/mL) *in vitro* [27, 62]. Actually, the concentrations of TNF-α in RA plasma (<100 pg/mL) are far below those required to increase neutrophil apoptosis [57]. Meanwhile, IL-6 can inhibit apoptosis of neutrophils from a healthy donor [62, 64, 66] and RA patients *in vitro* [27]. However, others find that IL-6 enhances apoptosis in human neutrophils *in vitro* [63]. Recent evidence reveals that IL-6 indeed does not affect the rate of neutrophil apoptosis in patients with RA [21]. It has been reported that RA patients receiving the anti-IL-6 receptor agent tocilizumab (TCZ) may have decreased neutrophil counts [67]. However, TCZ *in vitro* does not induce apoptosis or phagocytosis of neutrophils. Also, TCZ does not affect neutrophil functions in RA patients receiving TCZ treatment ex vivo [21].

The mechanisms underlying the effects of priming cytokines on neutrophil apoptosis have been partially elucidated. GM-CSF reduces the rate of neutrophil apoptosis through activation of ERK1/2 or PI-3K/Akt pathways [68, 69]. GM-CSF has also been shown to decrease mRNA levels of the proapoptotic protein Bad while increasing its phosphorylation [60]. An RNAseq analysis of human neutrophils primed by proinflammatory cytokines (GM-CSF and TNF-α) *in vitro* revealed altered levels of 58 transcripts implicated in the control of apoptosis [70]. Synovial fluid of patients with RA contains a variety of antiapoptotic and proapoptotic cytokines. The hypoxic environment within these joints can have beneficial effects on neutrophil survival. Compared to arterial blood, inflamed tissues are usually more hypoxic and acidic. Hypoxia causes delayed apoptosis of neutrophils in humans and mice. Additional studies have found that hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity [71]. Other studies demonstrate neutrophils isolated from RA synovial fluid and blood undergo delayed apoptosis as shown by increased expression of neutrophil survival protein Mcl-1 and decreased levels of activated caspase-9 [56, 57, 72]. The delay of neutrophil apoptosis is restored to control levels after treatment with methotrexate [72]. Interestingly, Janus kinase inhibitor (JAKi) prevents GM-CSF- and IFN-γ-induced apoptosis delay in RA and healthy control neutrophils in a dose-dependent manner. Incubation with JAKi prevents chemotaxis of RA neutrophils towards IL-8 but does not increase the level of apoptosis *in vitro* [58]. Therefore, the RA neutrophils in synovial fluid and blood undergo delayed apoptosis due to the proinflammatory and hypoxic microenvironment. GM-CSF, IFN-γ, IL-9, and IL-15 delay apoptosis of RA neutrophils, but others have no effect or a biphasic effect on neutrophil apoptosis.

4. The Role of NETs in the Pathogenesis of RA

Solid evidence exists that supports the important role of NETs in the pathogenesis of RA [8, 9, 13, 20, 73]. NET formation has been identified as a link between the innate and adaptive immune responses in autoimmunity. NETs provide the immune system with access to enriched sources of citrullinated proteins and thereby representing an early event preceding epitope spreading. Enhanced NETs are observed in both the bloodstream and synovial fluid of RA patients compared to healthy controls and patients with osteoarthritis [13, 73]. However, not all NETs shape the immune response toward disease-specific autoantibodies. One hypothesis speculates that NETs triggered by smoking can be an initiating factor in the pathogenesis of RA [74]. These NETs may drive anti-ACPA production in the lung which can play an important role in the early stage of RA development [8]. Increasing NET levels in the sputum are found to be significantly correlated with several citrullinated and noncitrullinated antibody reactivities in patients with RA [9]. Sera and immunoglobulin fractions from RA patients with high levels of ACPA or

RF significantly enhanced NETosis. *In vitro* incubation of neutrophils with $ACPA^{high}$ synovial fluid results in increased ROS production and extracellular DNA release compared to neutrophils incubated with ACPA-negative synovial fluid [20].

The inflammatory cytokines such as TNF-α, IL-17a, IL-6, and IL-8 can induce NETs in RA neutrophils. Subsequently, NETs promote more cytokine production and persistent inflammation via activating FLS or macrophages [13, 73, 75–78]. The neutralizing antibodies to TNF-α or IL-17R can reduce RA serum-induced-NETs [13]. NETs are strong activators of inflammatory responses in RA synovial fibroblasts since they can significantly augment the inflammatory reactions of RA synovial fibroblasts by inducing the production of IL-6, IL-8, chemokines, and adhesion molecules [13]. IL-6 can induce NET formation in neutrophils isolated from healthy individuals [75]. In RA, IL-6 may induce B cell maturation into plasma cells that can produce ACPA, thereby indirectly promoting NET formation [73]. Furthermore, anti-TNF-α (Infliximab) and anti-IL6R drugs (TCZ) can reduce the production of NETs [73, 76]. Interestingly, IL-8 can affect neutrophils through two different mechanisms of actions in RA. IL-8 recruits neutrophils to the inflammatory site through chemotactic effects while simultaneously triggering NET formation [13, 77].

IFN-γ and complement-5a (C5a) stimulation results in the formation of NETs in mature but not in immature human neutrophil populations [79]. Additional cytokines such as IL-1β and IL-18 are both able to stimulate NETs in human neutrophils [80–83]. CD177 is exclusively expressed in neutrophils and can be upregulated during inflammation. $CD177^{+}$ neutrophils produce NETs through IL-22 signaling [84]. IL-9 and IL-33 are involved in the pathogenesis of several autoimmune diseases, such as RA [11, 32, 33]. Recently, neutrophils isolated from patients with systemic sclerosis (SSc) express IL-9R and exposure of these neutrophils to rIL-9 can significantly induce NET formation [85]. IL-33 enhances noninfectious inflammation in the liver by amplifying NET formation [86]. However, the role of IFN-γ, IL-9, IL-1β, IL-18, IL-22, and IL-33 in the formation of NETs in RA remains to be studied.

5. Parodontitis, NETs, and Anticitrulline Response in RA

Citrullines are produced not only in the lung but also in the oral cavity. Periodontitis, an oral bacterial infection, is very common in RA [87]. Patients with periodontitis appear to have increased the risk for RA and patients with RA have increased the risk for periodontitis [62]. It has been reported that periodontitis is associated with anti-CCP antibody and RA disease activity [87]. Most importantly, citrullinated proteins can be detected in the periodontium in RA patients with periodontitis [88]. As we know, the formation of citrullinated proteins is catalyzed by peptidylarginine deiminase (PAD) enzymes. Porphyromonas gingivalis (P. gingivalis), an oral bacteria that is a crucial factor for periodontitis and express PAD and citrullinated enolase, can mediate citrullination of bacterial and host protein [89]. It has been shown that deletion of the bacterial PAD gene results in complete abrogation of protein citrullination [90]. Moreover, antibody titers to P. gingivalis are correlated with ACPA titres of RA patients [91]. Therefore, PAD from P. gingivalis may cause citrullinated protein formation, which may elicit an antibody response to these proteins potentially leading to ACPA formation. Although P. gingivalis does not seem to directly regulate NET generation in RA [92], it has been shown that P. gingivalis could induce human neutrophils to generate NETs *in vitro* [93]. Interestingly, a pilot case-control study has found that periodontal treatment markedly reduced the serum levels of NETs in patients with RA and periodontitis [94]. Furthermore, Konig et al. find another periodontal germ called Aggregatibacter actinomycetemcomitans (Aa) can induce hypercitrullination in host neutrophils through its leukotoxin A (LtxA). Moreover, LtxA induces neutrophil lysis and the extracellular release of hypercitrullinated proteins mimicking extracellular trap formation. It has been confirmed in patients with RA that serum anti-LtxA antibodies are associated with both anticitrullinated protein antibodies and rheumatoid factor [95].

6. Neutrophils and Their Anti-inflammatory Effects

NETs have not only proinflammatory effects, but also contribute to the resolution of inflammation. Neutrophils can resolve inflammatory processes via the annexin A1-(AnxA1-) mediated pathway [96]. Microvesicles (MVs), those extracellular particles within a size range between 50 nm and 1000 nm, can mediate cell to cell communication by transferring proteins and lipids to target cells. MVs also have a role in antigen presentation and activation of endosomal receptors such as Toll-like receptors. Recently, MVs have been considered a functional molecule with their potential for the treatment of autoimmune disease. Neutrophil-derived MVs are present at higher levels in synovial fluids of RA [97]. Neutrophil-derived MVs can protect cartilage degradation through the reduction of interleukin-8 and prostaglandin E2. In addition, it has been demonstrated that synovial MVs overexpress the anti-inflammatory protein annexin A1 (AnxA1). Furthermore, neutrophil-derived MV-associated AnxA1 interacts with its receptor, increasing transforming growth factor-β production by chondrocytes ultimately leading to cartilage protection [97].

7. Conclusion and Perspectives

Neutrophils play a crucial role in initiating inflammatory response and progression of arthritis in RA (Figure 1). Cytokines which can regulate migration and activation of neutrophils are highly expressed in the inflamed joint tissues of RA patients. Proinflammatory factors such as GM-CSF, IFN-γ, IL-9, and IL-15 can reduce neutrophil apoptosis and extend their neutrophil lifespan in RA, whereas TNF-α and IL-6 display a biphasic effect on the apoptosis of neutrophils. NETs can be a source of released self-antigens found in RA. Formation of NETs is closely related to an autoantigen-triggered immune response in patients

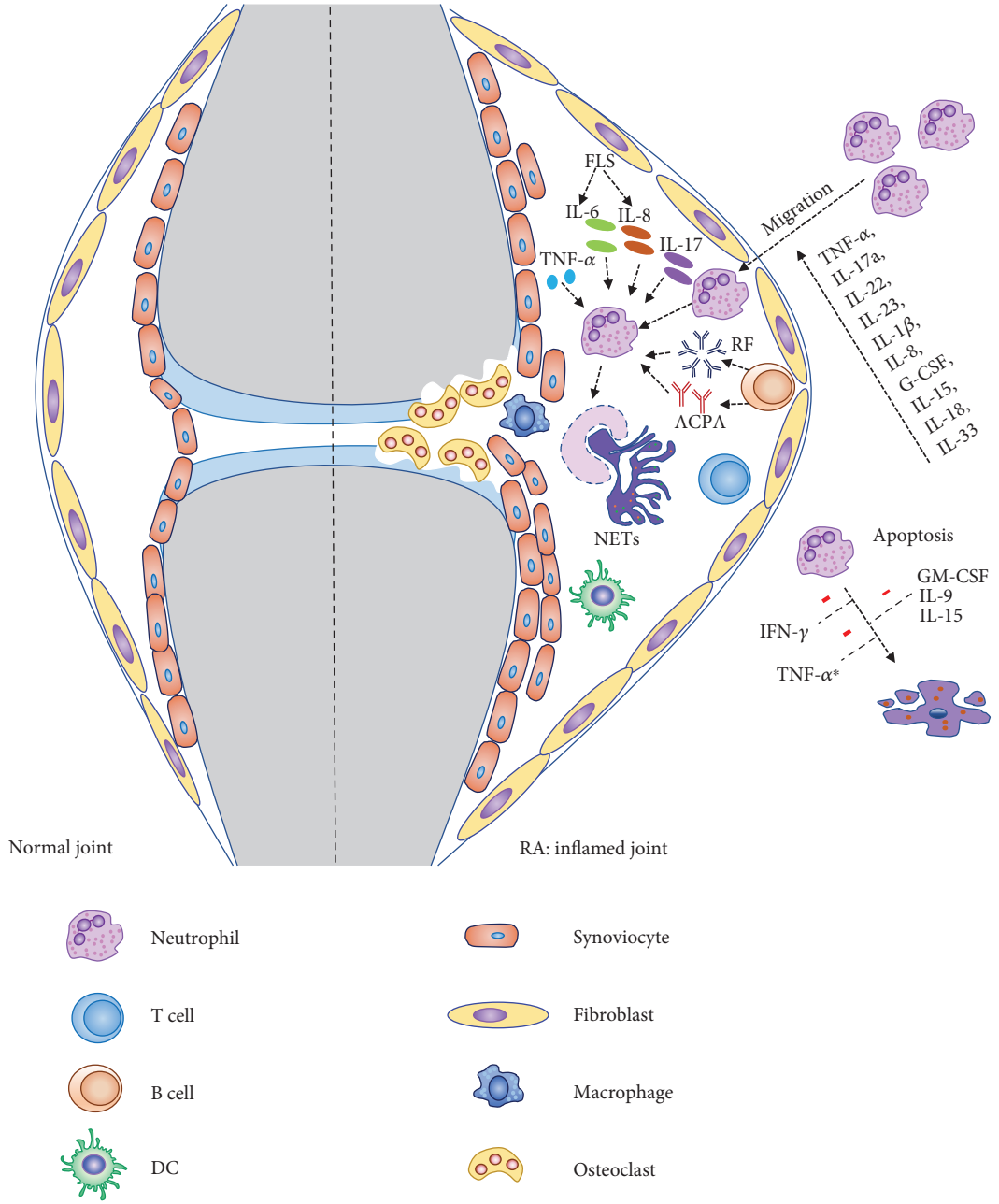

Figure 1: Role of neutrophil in the pathogenesis of RA. Proinflammatory cytokines in the joint can influence the migration of neutrophils. Neutrophils are activated by immune complexes and inflammatory cytokines (TNF-α, IL-6, IL-8, and IL-17a) within the synovial fluid, frequently causing enhanced NET formation in RA. In turn, NETs are served as a source of citrullinated autoantigens, further triggering the production of ACPA. Meanwhile, neutrophils undergo delayed apoptosis in an inflammatory milieu (GM-CSF, IL-9, IL-15, IFN-γ, and TNF-α) leading to persistent inflammation and joint damage in RA. *Controversial.

with RA. Furthermore, the inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-17a can induce NETs in RA neutrophils. Subsequently, NETs can promote an increase in cytokine production and enable the persistence of inflammation via the licensing of FLS and/or macrophages. Importantly, the anti-TNF-α and anti-IL-6R have been successful in treating RA by partially acting on neutrophils. Additional cytokines, for example, IL-22, IL-23, IL-18, G-CSF, IL-33, and IL-37, may also play an important role in the regulation of neutrophil functions. In addition, neutrophils may also play a role in the resolution of inflammation in RA. In the future, further studies are needed to understand how neutrophils act in an inflammatory milieu in patients with RA and animal models which may be critical for the development of novel treatment strategies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Weiqian Chen and Qin Wang equally contributed to this manuscript.

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Research Article

A Granulocyte-Specific Protein S100A12 as a Potential Prognostic Factor Affecting Aggressiveness of Therapy in Patients with Juvenile Idiopathic Arthritis

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Background. Defining new prognostic biomarkers has become one of the most promising perspectives for the long-term care of patients with juvenile idiopathic arthritis (JIA). The new efficient indicators of disease activity and potential response to treatment are crucial in establishing new therapeutic plans in accordance with the "treat to target" strategy. One of the most studied proteins is called S100A12, which is an alarmin specific for granulocytes, considered as a marker of their activity. *Materials and Methods*. Study involved 80 patients diagnosed with JIA. Children with systemic subtype were not included in the study. In 53 cases, blood samples were obtained in two time points. Results from the study group were compared to 29 age- and sex-matched healthy individuals. *Results*. Serum S100A12 levels were higher in JIA than in healthy controls at the study baseline (11.67 ± 6.59 vs. 6.01 ± 2.33 ng/ml). There were no significant differences in S100A12 values between assessed subtypes of JIA. The highest concentrations were observed in patients within a disease flare. S100A12 levels were not dependent from using glucocorticosteroids. The studied protein appeared to be an efficient biomarker for JIA patients: 100% specificity as a diagnostic marker (cut-off level: 10.73 ng/ml) and 100% sensitivity as an indicator of exacerbations within a 3-month observation (cut-off level: 5.48 ng/ml). *Conclusions*. S100A12 may become an important factor influencing decisions on aggressiveness of JIA therapy. Further elaboration on the clinical algorithm is necessary for that protein to be included in everyday practice.

1. Background

1.1. Juvenile Idiopathic Arthritis. Juvenile idiopathic arthritis (JIA) is a heterogenous group of arthritides affecting children under 16 years of age. It should be suspected in patients with symptoms persisting for at least 6 weeks without an apparent cause [1]. Essential part of the diagnostic process involves eliminating arthropathies caused by the known etiological factors included in the so-called "list of exclusions" [2]. A lack of unequivocal clinical features or laboratory findings frequently results in the delay of the final diagnosis. According to Aoust et al. [3], JIA was suspected only in 37% of patients from the disease onset. The remaining patients were presenting symptoms for 3 months (on average) before being diagnosed with JIA.

1.2. Disease Activity. JIA patients may considerably differ from each other in terms of the severity of their disease. In the cohort study of 609 children reported by Guzman et al. [4], who analyzed disease activity and patients' quality of life within a 5-year observation, four distinct types of JIA course were defined: mild (which is the most common, affecting 43.8% of patients), moderate (35.6%), severe controlled (9.0%), and severe persisting (11.5%). Taking into consideration the aforementioned classification, the adequate assessment of disease activity and response to treatment is crucial for effective long-term care of JIA patients.

Juvenile arthritis disease activity score (JADAS) is a widely used tool created to evaluate disease activity [5] in everyday practice of a pediatric rheumatologist. It contains four parameters: (1) physician global assessment, (2) patient or parent

global assessment, (3) normalized value of erythrocyte sedimentation rate (ESR), and (4) active joint count. In the recent study, the version involving 27 joints (JADAS27) was utilized due to its simplicity and strong correlation with the entire assessment of 71 joints [6].

1.3. Treat to Target. The "treat to target" concept of therapy, which is recently applied in rheumatoid arthritis patients, has already attracted interest of pediatric rheumatologists as well [7]. According to this innovative approach, the aggressiveness of treatment should be associated with the therapeutic goal, which may be determined as clinical remission off medication or, if it does not seem achievable, as at least minimal disease activity [8].

Although the effectiveness of early aggressive therapy has been supported by the study of Wallace et al. [9], it may increase the risk of overtreatment in patients, who would have responded to less intensive and, by extension, less toxic therapy [10]. Ravelli et al. did not report significant difference in frequency of remission in patients with oligoarticular JIA treated with intraarticular glucocorticosteroids (GCS) in monotherapy (remission in 32%) or combined with methotrexate (remission in 37%) [11]. Blazina et al. [10] underscored the necessity of utilizing prognostic biomarkers in making decisions on aggressiveness of treatment.

1.4. "Classic" and New Biomarkers. "Classic" serological markers, which include rheumatoid factor (RF), anticyclic citrullinated peptide autoantibodies (ACPA), and antinuclear antibodies (ANA) [12], are helpful in assigning patients into separate JIA subtypes or estimating risk of comorbidities (such as uveitis). However, they do not provide sufficient data on disease activity or response to treatment to predict the future course of the disease. Therefore, more and more potential markers are studied in order to facilitate a long-term care of JIA patients. In a systematic review presented by Gohar et al. [13], there were 68 biomarkers evaluated in systemic JIA. Fifty of them were assessed by only a single research group. The authors postulated consolidation of findings and further validation of markers which are already identified. Thus, the recent study involved one of the most studied and the most promising biomarkers, which is called S100A12.

1.5. S100A12. S100A12, also known as calgranulin C, is one of the calcium-binding S100 family proteins. This alarmin is specific for granulocytes; therefore, it may be considered as an indicator of their activity [14]. It presents strong chemotactic activity as a ligand binding receptor for advanced glycation end products (RAGE) [15, 16]. S100A12 participates in recruitment of inflammatory cells in murine models [17]. Previous findings reflect overexpression of S100A12 in inflamed tissues in adult patients with inflammatory bowel diseases, psoriatic arthritis, and rheumatoid arthritis [18, 19]. Moreover, serum concentration of S100A12 is considered as a marker of disease activity in children with Kawasaki disease [20].

Being an indicator of granulocytes' activity, S100A12 may appear useful as a marker of disease activity in JIA patients. The S100A12 level in synovial fluid was 10 times higher than in serum in a study published by Foell et al. [21], which demonstrated its potential as a reliable marker of local inflammation. Furthermore, serum concentration of that protein was higher in patients with active arthritis than in children with stable remission. Baseline S100A12 levels were elevated in patients with good response to intraarticular GCS. Serum S100A12 concentrations were also increased in children who exacerbated within 6 months after measurement. Similar findings were reported by Yamasaki et al., who observed higher S100A12 levels in patients who were unable to maintain remission for 2 years [22]. Additionally, S100A12 was the best isolated biomarker for prognosing disease flares in the further validation performed by Gerss et al. [23].

The most recent study by Gohar et al. [24] confirmed the relation between S100A12 concentration and the effectiveness of therapy. Patients with a good response to methotrexate and tumor necrosis factor (TNF) inhibitors had higher baseline S100A12 levels than treatment-refractory children. Combining methotrexate with systemic GCS did not affect the marker values. The authors suggested further elaboration on the ideal algorithm involving S100A12 in therapeutic decisions.

The present study was conducted in order to evaluate whether it is reliable to measure S100A12 serum levels in JIA patients in everyday practice of the pediatric rheumatologist. The objective of the study was to assess the clinical significance of S100A12 as a diagnostic biomarker of JIA and prognostic indicator of increasing disease activity within a 3-month observation.

2. Materials and Methods

The study involved 80 patients diagnosed with JIA who were admitted to the Department of Pediatric Cardiology and Rheumatology, Medical University of Lodz, Poland, between January 2017 and February 2018. In 53 cases, blood samples were collected in two time points (with an average interval of 102.4 ± 26.0 days) in order to evaluate dynamics of serum S100A12 concentrations. Patients diagnosed with systemic JIA were excluded from the study because of its distinct pathogenesis. Results from the study group were compared to the control group containing 29 age- and sex-matched individuals who were referred to the Department due to functional cardiovascular system dysfunction.

Patients' records were comprehensively reviewed in order to build a database containing age at diagnosis and age at evaluation; JIA subtype according to International League of Associations for Rheumatology (ILAR) classification [2]; reason of admission (fresh diagnosis of JIA, disease flare, continuation of biological treatment, and check-up visit); active joint count; JADAS27 value and disease activity level determined using cut-off levels proposed by Consolaro et al. [25, 26] (patients with enthesitis-related arthritis were assessed with criteria for oligo- and polyarticular JIAs, depending on the active joint count); current therapy, including disease-modifying antirheumatic drugs, intraarticular and systemic GCS, and biological agents.

Table 1: General characteristics of the study group.

	1st time point ($n = 80$)	2nd time point ($n = 53$)
Female, *n* (%)	54 (67.5%)	36 (67.9%)
Age at diagnosis (years)	7.75 ± 4.27	7.57 ± 4.41
Age at evaluation (years)	10.40 ± 4.38	10.28 ± 4.58
Reason of admission, *n* (%)		
Fresh diagnosis of JIA	16 (20.0%)	—
Disease flare	26 (32.5%)	11 (20.8%)
Continuation of biological treatment	9 (11.25%)	12 (22.6%)
Check-up visit	29 (36.25%)	30 (56.6%)
JIA subtypes, *n* (%)		
Oligoarticular JIA	54 (67.5%)	35 (66.0%)
RF-negative polyarticular JIA	11 (13.75%)	9 (17.0%)
RF-positive polyarticular JIA	1 (1.25%)	1 (1.9%)
ERA	14 (17.5%)	8 (15.1%)
Clinical and laboratory features		
Fever on admission, *n* (%)	9 (11.3%)	0 (0.0%)
CRP > 5 mg/l, *n* (%)	13 (16.3%)	6 (11.3%)
ESR > 20 mm/h, *n* (%)	16 (20.0%)	4 (7.5%)
Disease activity level (according to JADAS27), *n* (%)		
Clinically inactive disease	19 (23.75%)	16 (30.2%)
Minimal disease activity	7 (8.75%)	12 (22.65%)
Parent-acceptable symptom state	5 (6.25%)	6 (11.3%)
High disease activity	49 (61.25%)	19 (35.85%)

JIA: juvenile idiopathic arthritis; RF: rheumatoid factor; ERA: enthesitis-related arthritis; JADAS27: juvenile arthritis disease activity score 27-joint reduced count.

After obtaining blood samples, the following laboratory tests were ordered: complete blood count, ESR, C reactive protein (CRP), and "classic" serological markers (RF, ACPA, and ANA). Collected blood samples were also stored in −80°C in order to determine serum concentrations of S100A12 protein using ELISA Kit SEB080Hu (Cloud-Clone, China).

All statistical calculations were carried out using Statistica 13.1 software (Statsoft Polska, Krakow, Poland). The values were expressed as mean ± standard deviation (SD). The Shapiro-Wilk test was performed to assess the normality of continuous variables. Spearman's rank correlation coefficients were calculated for variables not normally distributed. The Mann-Whitney *U* test and Kruskall-Wallis test were utilized for group comparisons. The receiver operating characteristic (ROC) curve was drawn for S100A12, and the area under the curve (AUC) was computed to assess its diagnostic and prognostic significance. 95% confidence intervals (CI) were calculated for sensitivity, specificity, and AUC. *P* values lower than 0.05 were considered significant.

The study was approved by the Bioethics Committee of the Medical University of Lodz (approval no. RNN/31/17/KE).

3. Results

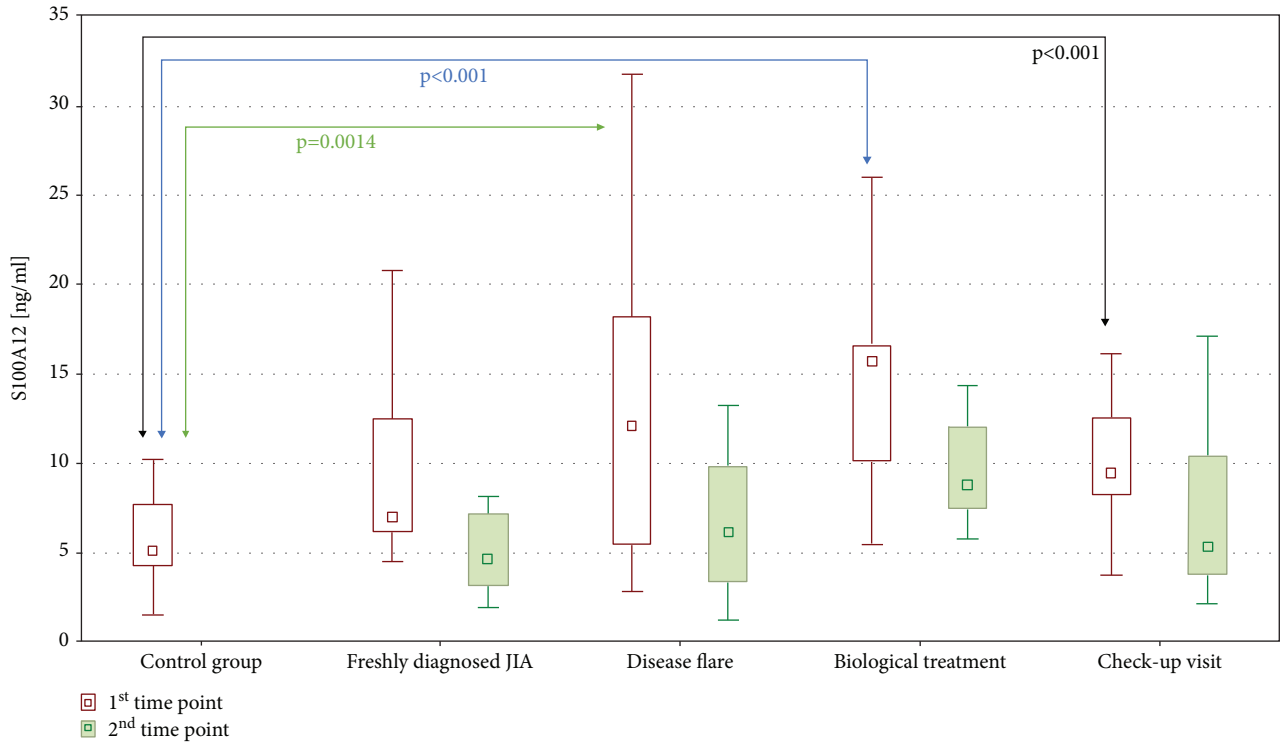
Characteristics of the study group are presented in Table 1. Serum S100A12 levels were significantly elevated in JIA patients at first time point (11.67 ± 6.59 ng/ml, $P < 0.001$) when compared to the control group (6.01 ± 2.33 ng/ml). Concentrations measured in the study group at second time point were also higher (7.45 ± 4.81 ng/ml) than in healthy individuals, but the difference did not reach statistical significance ($P = 0.40$). S100A12 values were independent from age at diagnosis ($P = 0.899$), age at the study baseline ($P = 0.768$), and sex ($P = 0.534$) of patients. They were also not related to "classic" serological markers. Moreover, levels of the protein did not differ ($P = 0.121$) between assessed subtypes of JIA (RF-positive polyarthritis was excluded from this part of analysis due to the small sample size).

Serum S100A12 concentrations were increased in JIA patients regardless of the reason of admission (Figure 1(a)). The highest values of S100A12 were observed in patients with disease flare, but the differences between subgroups of JIA patients were not statistically significant. S100A12 concentrations were significantly correlated with CRP ($r = 0.473$, $P < 0.001$) and ESR ($r = 0.353$, $P < 0.001$) values. In terms of the disease activity level (based on JADAS27), the most increased S100A12 concentrations were noted in patients with high disease activity (Figure 1(b)). However, that difference was not significant as well.

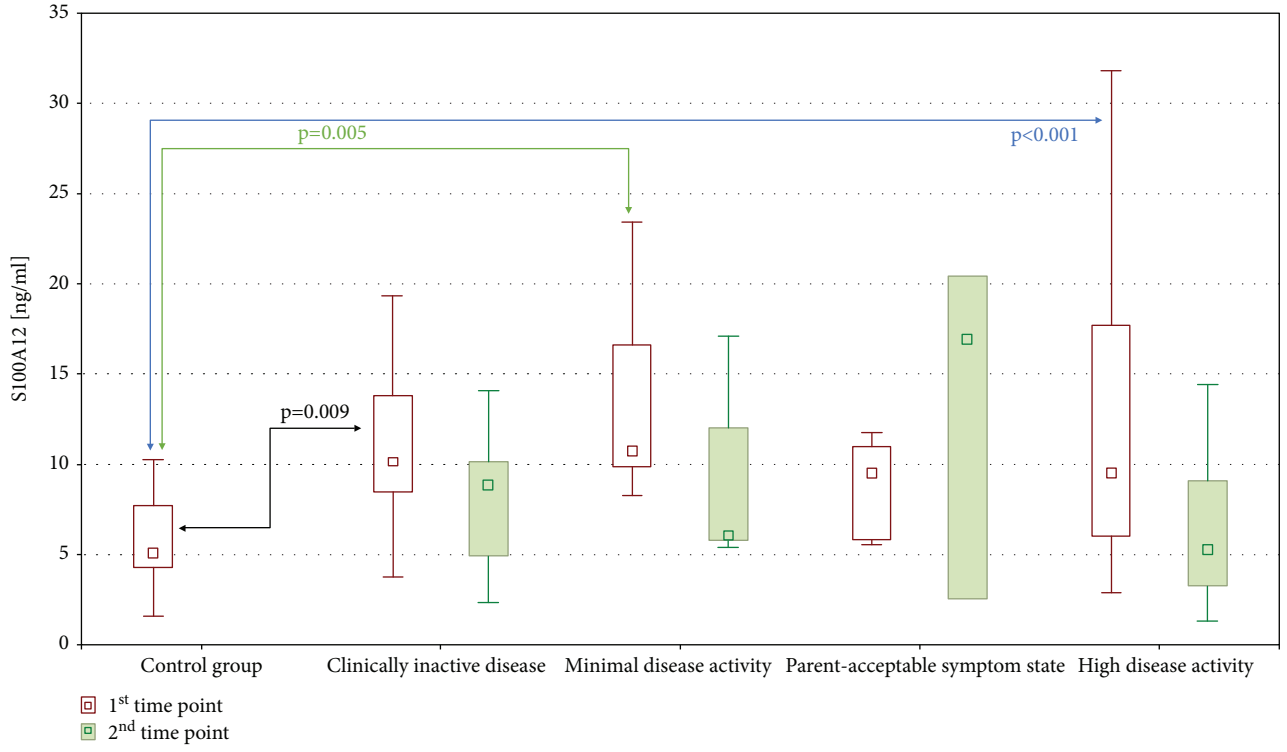
Apart from disease-modifying antirheumatic drugs (all patients in the study group were treated with methotrexate), forty-three JIA patients (53.8%) were using systemic GCS at first time point. Moreover, in twenty-four children (30.0%), intraarticular GCS were necessary during the first hospitalization within the study. However, S100A12 levels did not differ significantly (Figure 2) depending on usage of systemic or intraarticular GCS ($P = 0.766$ and $P = 1.00$, respectively). Nine patients were also treated with biological agents, but the significant difference in S100A12 levels was not observed ($P = 0.0853$).

Considering values noted at second time point, S100A12 levels increased in 14 patients (average change: 3.63 ± 3.50 ng/ml). Concurrent elevation of JADAS27 was observed in 2 (14.3%) patients. Concentrations of the marker decreased in 39 patients (average change: 7.23 ± 5.49 ng/ml). Simultaneous decline of JADAS27 was noted in 26 (66.7%) children. Differences in S100A12 levels between both time points were statistically significant ($P < 0.001$).

S100A12 had 100.0% specificity (95% CI: 88.1%—upper limit is not applicable) and 46.3% sensitivity (95% CI: 35.0–57.8%) as a diagnostic JIA biomarker for cut-off level 10.73 ng/ml with AUC 0.787 (95% CI: 0.701–0.873). The ROC curve is illustrated in Figure 3(a). S100A12 was also assessed as a potential prognostic marker for predicting exacerbations of the disease. It was characterized with



(a)



(b)

Figure 1: S100A12 levels depending on (a) reason of admission and (b) disease activity level. *P* values were presented only for statistically significant comparisons. JIA: juvenile idiopathic arthritis.

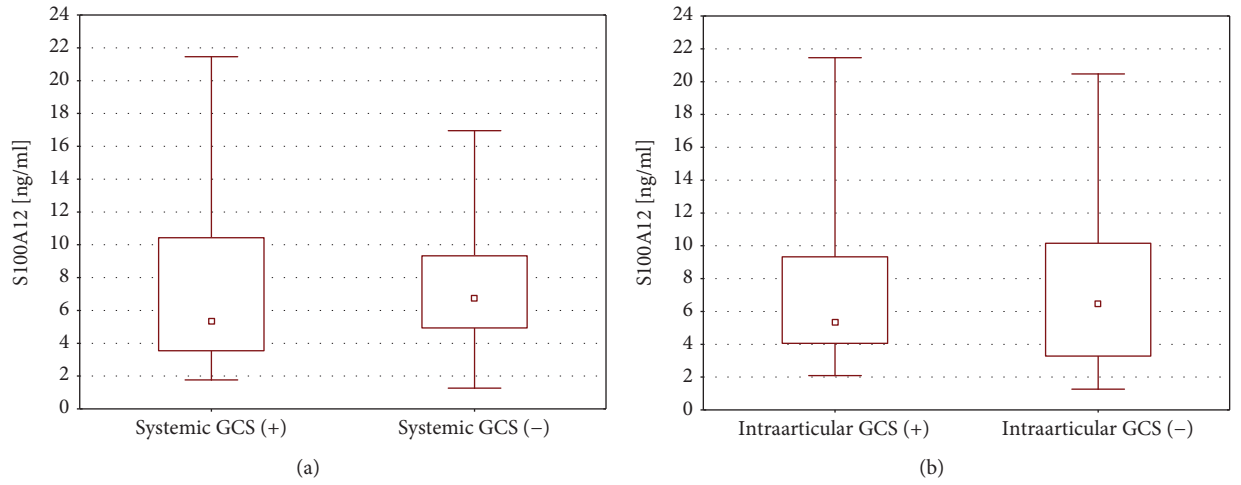


Figure 2: S100A12 levels at 2nd time point depending on (a) systemic GCS intake within the observation period and (b) intraarticular GCS injection at 1st time point. GCS: glucocorticosteroids.

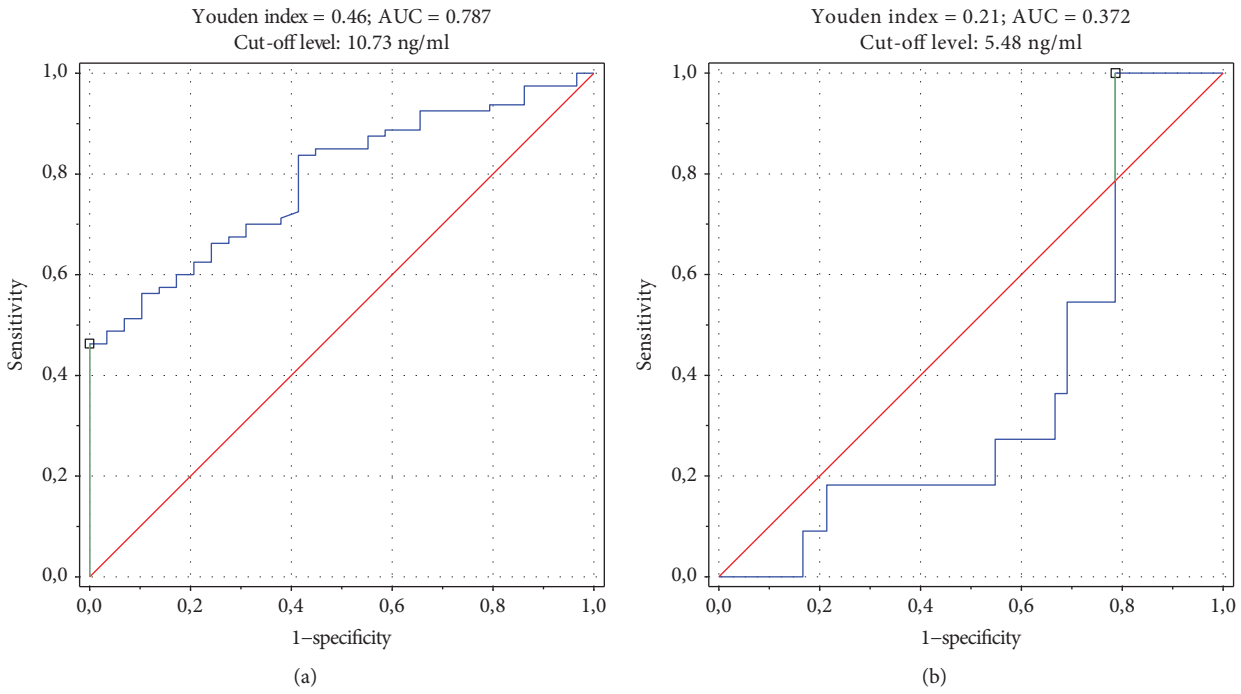


Figure 3: Receiver operating characteristic curve for S100A12 as (a) JIA diagnostic biomarker and (b) disease flare prognostic biomarker. AUC: area under the curve; JIA: juvenile idiopathic arthritis.

100.0% sensitivity (95% CI: 71.5%—upper limit is not applicable), 21.4% specificity (95% CI: 10.3–36.8%), and AUC 0.372 (95% CI: 0.203–0.542) as an indicator of disease flare within a 3-month observation (Figure 3(b)).

4. Discussion

Personalized treatment involving the "treat to target" strategy opens a new chapter in the therapeutic approach in JIA patients. Effectiveness of such therapy can be maximized by reliable assessment of disease activity, both in patients presenting symptoms and in children achieving clinical remission on medication. One of the potential diagnostic and prognostic biomarkers, S100A12 protein, was included in our study in order to evaluate its clinical significance.

Serum S100A12 levels were significantly higher in JIA patients when compared to healthy individuals. These results are consistent with findings of Foell et al. [21]

and Bojko [27]. Unlike Yamasaki et al. [22], we did not observe significant differences in S100A12 values between included JIA subtypes. However, the remarkable specificity of S100A12 (100% for cut-off value 10.73 ng/ml) suggests potential utilization of that biomarker in differential diagnosis in patients suspected of JIA.

Serum S100A12 concentrations were noticeably increased in patients with high disease activity. The hundred-percent sensitivity in predicting disease flare within a 3-month observation (cut-off value 5.48 ng/ml) supports findings of the previous study by Gerss et al. [23]. As reported by Gohar et al. [24], S100A12 values were independent from GCS intake, which makes that protein a reliable prognostic marker in patients who need to use them. In our study group, more than a half of JIA patients were taking systemic GCS at the study baseline.

Although S100A12 is postulated as an indicator of treatment response [24], decrease of its serum concentration was related to the decline in the JADAS27 value only in 66.7% of patients. Therefore, it should not be considered as an isolated marker of the effectiveness of therapy. On the other hand, Giancane et al. [28] stated that the pain should be considered as a direct cause and main indication for treatment in JIA patients. Older age at diagnosis and longer disease duration are the risk factors of lower efficacy in reduction of patients' symptoms [29]. Measuring the JADAS value on the day of the check-up visit may not be the most informative indicator of patients' well-being between the assessments, which may have influenced the evaluation of the relation between S100A12 and disease activity. Smartphone applications should be elaborated on in order to get the better feedback from the patient [28, 30].

The main limitation of the study was the considerable diversity of the study group. A reliable assessment of effectiveness of treatment and measurements of potential biomarker values should be performed in a possibly homogenous group of patients independent from factors influencing the results. Additionally, the findings from the study group should be compared with patients with arthritides other than JIA in order to evaluate the usefulness of S100A12 in differential diagnosis.

5. Conclusions

Including S100A12 protein in everyday practice of the pediatric rheumatologists might be helpful in making therapeutic decisions. Escalation of aggressiveness of therapy in the right group of patients may potentially reduce the frequency of disease flares. Setting the clinical algorithm of ordering the S100A12 test is challenging until it becomes widely accessible in clinical laboratories.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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