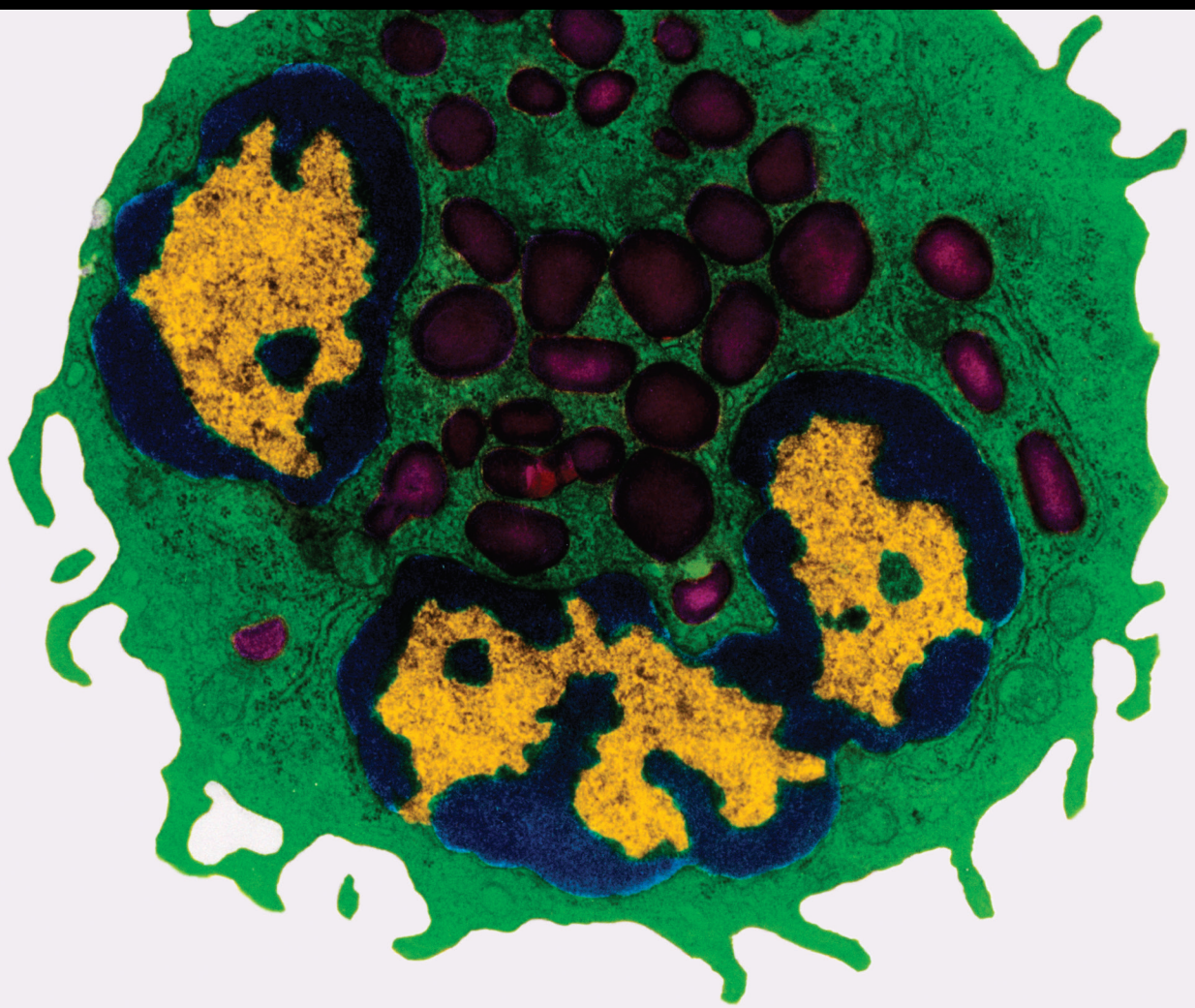
Mediators of Inflammation

Phagocytes: Protective and Pathological Roles in Infectious, Chronic, and Autoimmune Diseases

Lead Guest Editor: Juliana Vago
Guest Editors: Michelle Sugimoto, Izabela Galvão, and Luciana Pádua Tavares



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Research Article

Abnormal Macrophage Polarization in Patients with Myelodysplastic Syndrome

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Background. This study is aimed at assessing the subsets of bone marrow macrophages in patients with myelodysplastic syndrome (MDS) and exploring the role of macrophages in the pathogenesis of MDS. *Methods*. Thirty-eight newly diagnosed MDS patients were enrolled in the Department of Hematology of General Hospital of Tianjin Medical University from June 2015 to June 2016. Bone marrow monocytes and macrophage subsets (M1/M2) were detected in patients with MDS and normal controls by flow cytometry. M1 macrophages were cultured *in vitro*, and the expression of IL-1β and TNF-α mRNA was measured using real-time polymerase chain reaction. *Results*. Compared with the normal control group, the proportion of bone marrow monocytes was higher ($2.11 \pm 0.93\%$ vs. $3.66 \pm 3.38\%$), and the mean fluorescence intensity of surface molecule CD14 was lower in the higher-risk (HR) MDS group (639.05 ± 359.78 vs. 458.26 ± 306.72, $p < 0.05$). The ratio of M2 macrophages to monocytes was higher in patients with HR-MDS ($1.82 \pm 2.47\%$ vs. $3.93 \pm 3.81\%$, $p < 0.05$). The ratio of M1 to M2 macrophages was lower in the HR-MDS group (3.50 ± 3.22 vs. 1.80 ± 0.88, $p < 0.05$). The expression of IL-1β and TNF-α mRNA in M1 macrophages was significantly lower in the MDS group ($p < 0.05$). *Conclusions*. Patients with MDS had abnormal macrophage polarization, which may be involved in the alteration of bone marrow microenvironments.

1. Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of malignant and clonogenic diseases that originate from hematopoietic stem cells. The main features are abnormal hematopoiesis (myeloid cell development abnormalities) and ineffective hematopoiesis (one line or multilineage). Approximately 30% of patients develop acute myeloid leukemia (AML) during the course of the disease. The pathogenesis of MDS is associated with genetic mutations, epigenetic changes, and bone marrow microenvironments [1, 2].

The monocyte macrophage system is mainly composed of monocytes and macrophages. Its main function is to remove pathogens or waste materials from the blood and tissues, and it also plays a key role in the induction and regulation of the adaptive immune response [3]. However, recent studies have suggested that monocytes and macrophages are part of the bone marrow microenvironment related to homing, mobilization, senescence of hematopoietic stem cells, and the formation of erythropoiesis [4]. Macrophages are differentiated from monocytes. The polarization of macrophages is usually divided into two categories: classical polarizing I macrophages (M1) and type II macrophages (M2) as a substitute for polarization [5]. Classically activated M1 polarized macrophages have antitumor activity and might induce tumor tissue destruction. Tumor progression is related to the transition from the M1 to M2 phenotype. In the late stage of tumor progression, macrophages usually have an M2 phenotype, with low IL-12 expression, high IL-10 expression, low tumoricidal activity, and promotion of tissue remodeling and angiogenesis [6].

Our previous studies showed that the number of monocytes in the peripheral blood of MDS patients increased, but the ability to differentiate into macrophages and the phagocytic function decreased [7]. The macrophages in the bone marrow are a part of the bone marrow

microenvironment. Different macrophage polarization states play important roles in the differentiation of hematopoietic stem cells. We speculate that the M1/M2 polarization of bone marrow macrophages in patients with MDS may be an important factor in the pathogenesis and progression of MDS.

In this study, we evaluated M1 and M2 macrophages from the bone marrow of MDS and the culture of M1 macrophages *in vitro*.

2. Methods

2.1. Patient Characteristics. The MDS group enrolled 38 newly diagnosed MDS patients in the Department of Hematology of General Hospital of Tianjin Medical University from June 2015 to June 2016, including 20 males and 18 females, with a median age of 58 (range, 21-79) years. According to the International Prognostic Score System (IPSS), the patients were divided into the lower-risk (LR) MDS group (15 cases) and the higher-risk (HR) MDS group (23 cases) (detail in Table 1). The control group consisted of 21 healthy controls (11 males and 10 females) with a median age of 38 (23–65) years. This study was approved by the Ethics Committee of the General Hospital of Tianjin Medical University (IRB2021-WZ-052). Informed written consent was obtained from all patients and controls or their guardians according to the Helsinki Declaration.

2.2. Flow Cytometric Method. Bone marrow samples were obtained by standard bone marrow puncture using sterile heparin anticoagulant tubes. Bone marrow samples were filtered using flow cytometry tubes. CD14-FITC (Cat No.: 555397), CD68-PE (Cat No.: 565595), CD64-APC (Cat No.: 561189), CD40-PEcy7 (Cat No.: 561215), CD206-PE (Cat No.: 555954), CD163-PEcy7 (Cat No.: 556018), and isotype control antibodies (BD Biosciences, USA) were added to the tubes. The samples were then stained for 15 min in the dark at room temperature. After red blood cell lysis, the cells were washed with PBS. Finally, the cells were detected using a FACSCalibur flow cytometer (BD Biosciences, USA). Data analysis was performed using the Cell Quest software (Becton Dickinson, version 3.1).

Macrophages were defined as $CD14^+CD68^+$ cells. M1 macrophages were defined as $CD64^+CD40^+$ macrophages. M2 macrophages were defined as $CD206^+CD163^+$ macrophages (detail in Supplemental Figure 1).

2.3. M1 Macrophage Cell Culture In Vitro. Peripheral blood mononuclear cells (PBMCs) were separated from fresh heparinized blood samples (5 mL) using Ficoll Solution (Suolaibao, China). The PBMCs were seeded at 3 million cells/mL in sterile RPMI 1640 (Invitrogen, CA, USA) and cultured for 7 days with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Huabei Pharmacy, Shijiazhuang, China), interferon-gamma (Sigma, USA), and lipopolysaccharide (Sigma, USA). On day 7, macrophages were collected from the bottom of the culture dishes.

2.4. Real-Time Polymerase Chain Reaction (qPCR). Total RNA from macrophages was extracted using TRIzol (Takara Bio, CA, USA), and cDNA was generated using a reverse transcriptase kit (Takara Bio, CA, USA). Gene expression was quantified by qPCR (SYBR® Premix Ex Taq II, Takara Bio, China). The primer sequences were as follows: IL-1β forward 5′-GATCACTGAACTGCACGCTCC-3′ and reverse 5′-ACTTGTTGCTCCATATCCTGT-3′, TNF-alpha forward 5′-GGAGAAGGGTGACCGACTCA-3′ and reverse 5′-CTGCCCAGACTCGGCAA-3′, and GAPDH forward 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse 5′-TGGTGAAGACGCCAGTGGA-3′. The relative quantification (RQ) of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method: $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{patients} - (Ct_{target} - Ct_{GAPDH})_{controls}$.

2.5. Statistical Analysis. The results were analyzed using the GraphPad Prism 8.0 program (GraphPad Software, Inc., San Diego, CA). Data with normal distribution were presented as means ± SD, and multiple group comparisons were performed using one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. The Quantity of Monocytes Was Increased in the Bone Marrow of Patients with HR-MDS. The proportion of bone marrow monocytes was 2.11 ± 0.93% in the control group, 1.96 ± 1.53% in the LR-MDS group, and 3.66 ± 3.38% in the HR-MDS group. There was no significant difference in the proportion of bone marrow monocytes between the normal control group and the LR group, and the proportion of bone marrow monocytes was significantly higher in the HR group than in the control group ($p < 0.05$). The proportion of bone marrow monocytes in the HR group was higher than that in the LR group, and the difference was statistically significant ($p < 0.05$; Figure 1).

The median fluorescence intensity (MFI) of $CD14^+$ cells from the bone marrow in the control group, LR-MDS group, and HR-MDS group was 639.05 ± 359.78, 501.43 ± 374.44, and 458.26 ± 306.72, respectively. There was no significant difference in the MFI of $CD14^+$ cells between the normal control group and the LR group, and the MFI of $CD14^+$ cells was significantly lower in the HR group than in the control group ($p < 0.05$). The difference between the LR and HR groups was not statistically significant (Figure 1).

3.2. The Number of M2 Macrophages Was Increased in the Bone Marrow of HR-MDS Patients. The proportion of M1 macrophages in the bone marrow monocytes was 6.41 ± 7.09% in the control group, 8.08 ± 10.31% in the LR-MDS group, and 7.80 ± 9.41% in the HR-MDS group. There were no statistically significant differences among the three groups.

The proportion of M2 macrophages in the bone marrow monocyte was 1.82 ± 2.47% in the control group, 3.18 ± 3.79% in the LR-MDS group, and 3.93 ± 3.81% in the HR-MDS group. The proportion in the HR-MDS group was significantly higher than that in the control group, and the difference was statistically significant ($p < 0.05$).

Table 1: The characteristics of myelodysplastic syndrome patients.

Case	Sex	Age	Diagnosis	Cytogenetics	IPSS
1	Male	21	RARS	46,XY	Low
2	Male	63	RCMD	46,XY	Low
3	Female	38	RAEB2	46,XY	Int-2
4	Male	46	RCMD	46,XY,-2,-12,+mar,19+,9P+	Int-2
5	Female	57	RAEB2	46,XX	Int-2
6	Male	58	RAEB2	46,XY	Int-2
7	Male	59	RAEB2	46,XY	Int-2
8	Male	59	RAEB2	46,XY	Int-2
9	Female	59	RAEB1	46,XY,13q+	Int-2
10	Male	62	RAEB2	46,XY	Int-2
11	Female	64	RAEB2	46,XX	Int-2
12	Male	65	RCMD	46,XY,del17q31	Int-2
13	Male	67	RAEB2	46,XY	Int-2
14	Female	69	RAEB2	46,XX	Int-2
15	Female	70	RAEB2	46,XX	Int-2
16	Male	76	RAEB2	No result	Int-2
17	Female	79	RAEB2	46,XX	Int-2
18	Male	42	RARS	46,XY,del20q11	Int-1
19	Female	47	RARS	46,XX	Int-1
20	Female	49	RARS	46,XX	Int-1
21	Male	50	RAEB1	46,XX	Int-1
22	Male	50	RCMD	47,XY,+8/46,XY	Int-1
23	Female	51	RAEB1	46,XX	Int-1
24	Male	57	RAEB1	46,XY	Int-1
25	Male	58	RAEB1	46,XY	Int-1
26	Female	62	5q-	5q-	Int-1
27	Male	62	RA	46,XY	Int-1
28	Female	64	RAEB1	46,XX	Int-1
29	Female	74	RARS	46,XX	Int-1
30	Female	74	RCMD	46,XX	Int-1
31	Male	27	RAEB2	3p+,-18,+mar	High
32	Female	29	RAEB2	20q-,5q-,7q-	High
33	Male	30	RAEB2	47,XY,+8/46,XY	High
34	Male	60	RAEB2	45,XY,-7	High
35	Male	68	RAEB2	46,XY,+8/45,XY+8,-6,-7	High
36	Female	76	RAEB2	del5q33,del5q31,del7q311,del7q3	High
37	Female	77	RAEB2	45,XX,-5,-2,45,XX,+mar,-5,3P-	High
38	Female	79	RAEB2	45,XX,-7	High

The ratio of M1 to M2 macrophages was 3.50 ± 3.22 in the control group, 1.68 ± 0.78 in the LR-MDS group, and 1.80 ± 0.88 in the HR-MDS group. The ratio of M1 to M2 macrophages in the control group was significantly higher than that in the LR-MDS and HR-MDS groups ($p < 0.05$). There was no significant difference in the ratio of M1 to M2 macrophages between the HR-MDS and LR-MDS groups (Figure 2).

3.3. The Expression of IL-1β and TNF-Alpha mRNA of M1 Macrophages In Vitro Was Decreased. The level of IL-1β mRNA was 2.07 ± 1.66 in the control group, 0.5 ± 0.6 in the LR-MDS group, and 0.98 ± 0.72 in the HR-MDS group. Compared with the control group, the expressions of IL-1β mRNA in the LR-MDS and the HR-MDS groups were lower, and the difference was statistically significant ($p < 0.05$), while the difference between the LR-MDS and HR-MDS groups has no statistical significance.

The level of TNF-alpha mRNA was 1.20 ± 0.75 in the control group, 0.55 ± 0.33 in the LR-MDS group, and 0.85 ± 0.36 in the HR-MDS group. Compared with the control group, the expressions of TNF-alpha mRNA in the LR-

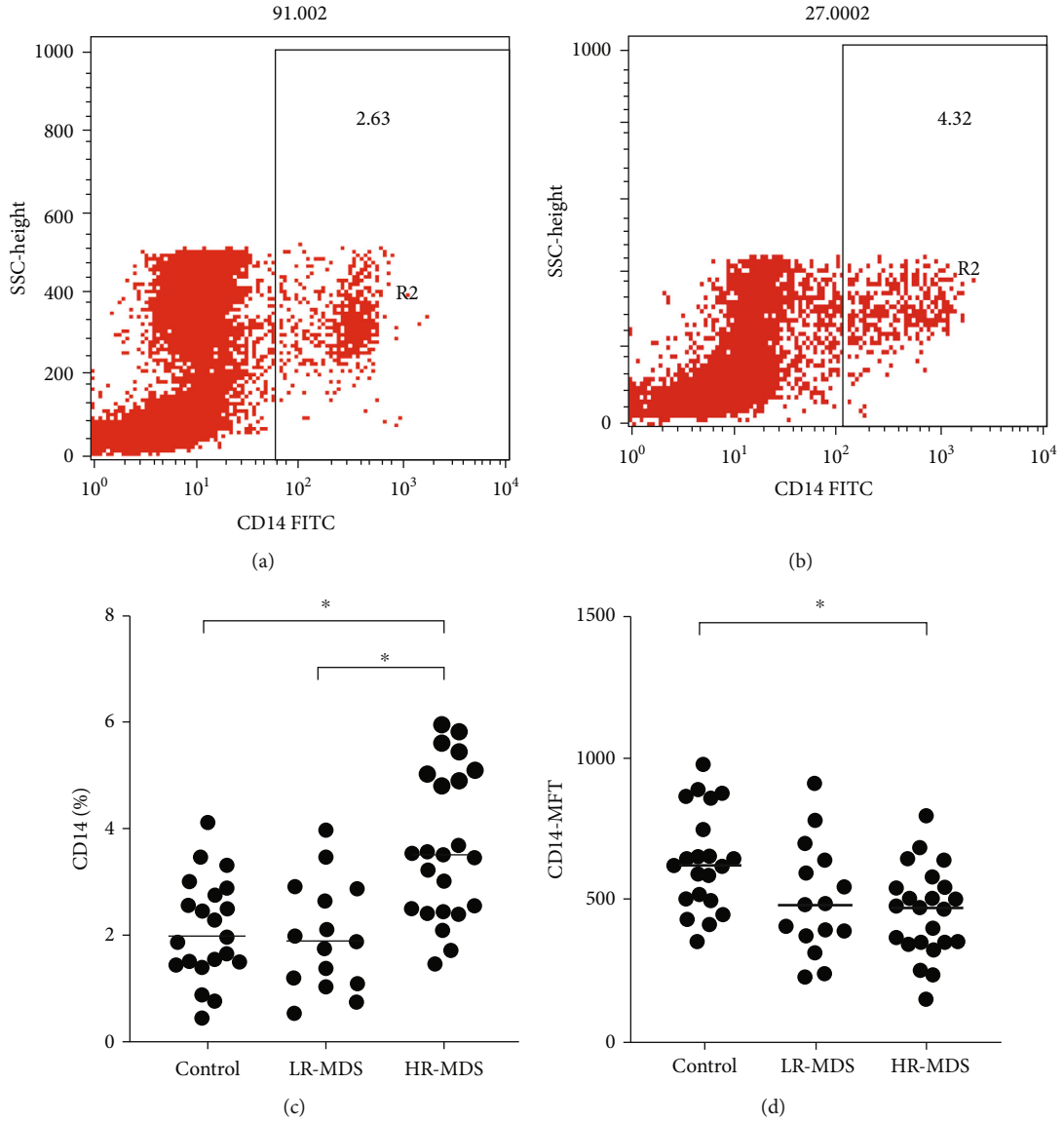

Figure 1: The quantity of monocytes in bone marrow of patients with MDS. (a) Representative dot plots from flow cytometric (FACS) analyses showing the $CD14^+$ cell frequency among bone marrow mononuclear cells obtained from healthy controls. (b) Representative dot plots from FACS analyses showing the $CD14^+$ cell frequency among bone marrow mononuclear cells obtained from MDS patients. (c) The proportion of $CD14^+$ cells from bone marrow of MDS patients and controls. (d) The median fluorescence intensity (MFI) of $CD14^+$ cells from bone marrow of MDS patients and controls. $^*p < 0.05$.

MDS and HR-MDS groups were lower, and the differences were statistically significant ($p < 0.05$) (Figure 3).

4. Discussion

Bone marrow macrophages play an important role in maintaining the homeostasis of the hematopoietic stem cell niche. Removing macrophages can release hematopoietic stem cells into the peripheral blood [8]. $CD14^+$ monocytes/macrophages could increase the expansion of erythroid progenitor cells and increase the number of $CD34^+$ HSPCs through coculture [9].We previously found that the proportion of peripheral blood monocytes in patients with MDS increased, and the phagocytic ability of differentiated macrophages decreased [7]. In the present study, we found that the proportion of monocytes in the bone marrow of patients with HR-MDS was significantly higher than that of the control group, and the MFI of cell surface antigen CD14 was also significantly different from that observed in the control group. As the disease progressed, the number of abnormal monocytes increased in the BM of the patients. Monocytes showed abnormal maturation and differentiation.

Tissue macrophages and inflammatory macrophages are derived from monocytes in the peripheral blood or from the embryonic origin of tissue macrophages, which have strong plasticity [10]. To adapt to changes in the microenvironment,

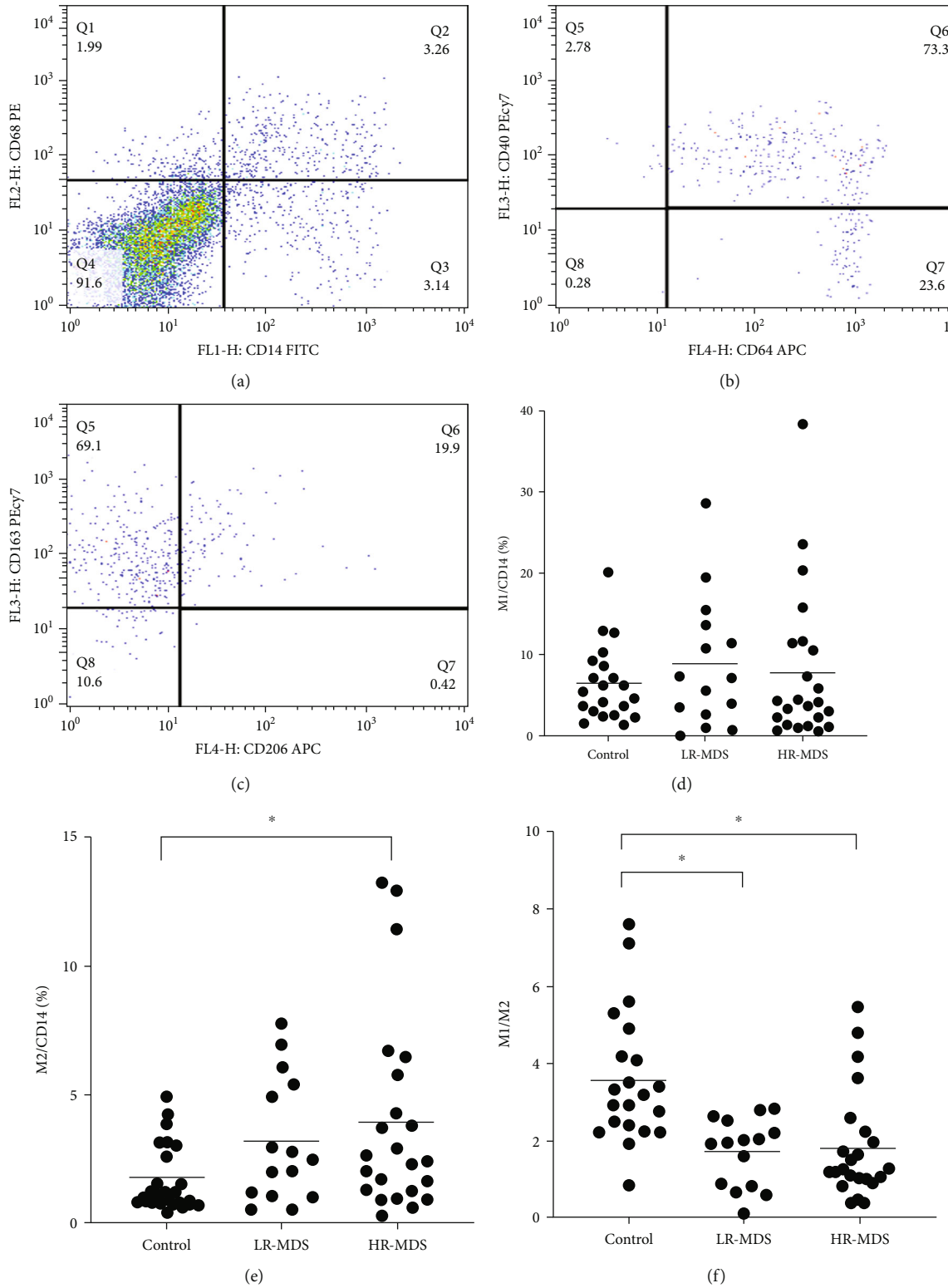

Figure 2: The percentage of macrophages in bone marrow of patients with MDS. (a) Representative dot plots from flow cytometric (FACS) analyses showing the macrophage ($CD14^+CD68^+$ cells) frequency among bone marrow mononuclear cells. (b) Representative dot plots from FACS analyses showing the M1 macrophage ($CD64^+CD40^+$ macrophages) frequency among bone marrow mononuclear cells. (c) Representative dot plots from FACS analyses showing the M2 macrophage ($CD206^+CD163^+$ macrophages) frequency among bone marrow mononuclear cells. (d) The frequency of M1 macrophages from bone marrow of MDS patients and controls. (e) The frequency of M2 macrophages from bone marrow of MDS patients and controls. (f) The ratio of M1/M2 macrophages from bone marrow of MDS patients and controls. $^*p < 0.05$.

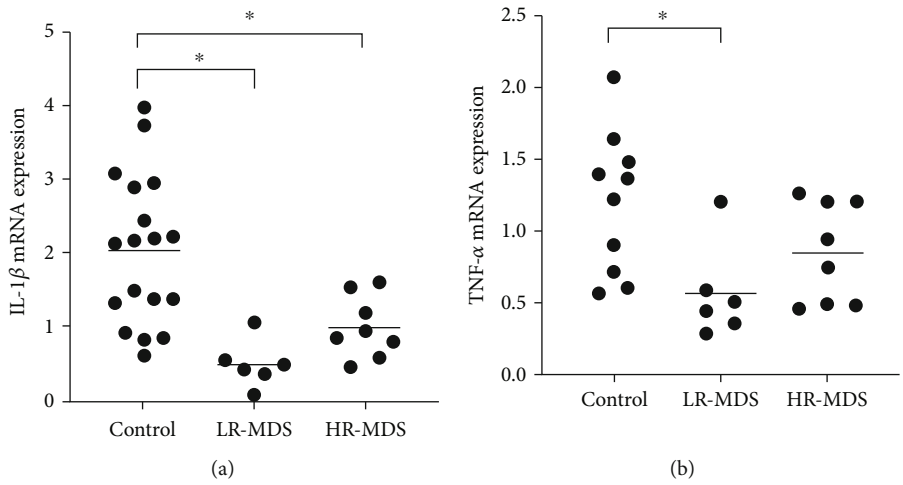

Figure 3: The expression of IL-1β and TNF-α mRNA in M1 macrophages in vitro. (a) The expression of IL-1β mRNA in M1 macrophages. (b) The expression of TNF-α mRNA in M1 macrophages. $^{*}p < 0.05$.

macrophages can polarize into different types [11]. The functions, cytokines, and surface markers of polarized macrophages are different. Macrophage polarization is generally divided into two categories: classical polarization of type I macrophages (M1) and alternative polarization of type II macrophages (M2) [12]. Studies have found that M1 macrophages are usually induced by IFN-γ, LPS, and toll-like receptor agonists. These macrophages secrete proinflammatory factors such as IL-6, IL-12, IL-1β, and TNF-α and highly express MHC class I and MHC class II molecules that recognize tumor-specific antigens. Therefore, M1 macrophages play important roles in the inflammatory response and antitumor immune response. In contrast, M2 macrophages play important roles in anti-inflammatory activity and tumor growth. M2 macrophages are further divided into four subtypes: M2A, M2B, M2C, and M2D [6]. Studies have shown that tumor-associated macrophages (TAMs) are similar to M2 macrophages, and the M2D subtype is considered to be tumor-associated macrophages [13]. Sica and Mantovani [6] found that the phenotype of TAM macrophages was M2, for example, the IL-12^{low} IL-10^{high} in an advanced stage of tumors. Other researchers believe that such macrophages are conducive to tumor growth, survival, and angiogenesis [6, 14–16].

In this study, we compared the proportion of macrophages, the ratio of M1 to M2, and the expression of macrophage surface molecules between patients with MDS and the control group. We found that the ratio of M2 macrophages to monocytes was higher in patients with MDS. The ratio of M1 to M2 macrophages was lower in the MDS group. There was no significant difference in the proportion of M1 macrophages between MDS patients and the control group. The results showed that with the development of MDS, the macrophages in the bone marrow further polarized to the M2 subtype and not to the M1 subtype, and the antitumor effect of macrophages was insufficient.

In this study, we found that the expression of IL-1β and TNF-α mRNA in M1 macrophages of patients with MDS was significantly lower than that in the control. Dumont et al. [17] found that macrophages stimulated by LPS highly expressed IL-1β and TNF-α and inhibited the proliferation of colon cancer cells. Klimp et al. [18] also confirmed that macrophages stimulated by LPS and IFN-γ could kill tumor cells by secreting TNF. Studies have shown that TNF-α promotes the apoptosis of MDS progenitor cells [19], and the concentration of TNF-α in the bone marrow supernatant and plasma of MDS patients was increased, and the expression of TNF receptor and TNF-α mRNA was increased in mononuclear cells of MDS. As a proinflammatory factor, IL-1β has various effects on hematopoiesis. IL-1β at physiological concentrations can promote the secretion of GM-CSF and other colony-stimulating factors and promote hematopoiesis [20]. Allampallam et al. [21] found that the mononuclear cells of MDS also expressed IL-1β. Basiorka et al. [22] found that MDS HSPC overexpressed inflammatory protein and activated the NLRP3 complex, thus activating cysteinase 1, secreting IL-1β, and promoting cell death. Therefore, we found that the expression of IL-1β and TNF-α mRNA decreased by culturing macrophages from MDS patients *in vitro* and stimulating them to differentiate into M1 using LPS and IFN-γ treatment. We speculated that the inflammatory factors secreted by M1 macrophages in the MDS group were decreased, and M1 macrophages in patients with MDS had insufficient antitumor function, and their proinflammatory and antitumor effects were weakened, which may be related to the occurrence and progression of MDS.

The increase in M2 polarization in the bone marrow of patients with MDS is beneficial for the proliferation of MDS clonal cells. Repolarization of M2 cells to the M1 phenotype is a method of cancer immunotherapy, which can effectively restore the response of the innate and adaptive immune systems, leading to tumor regression [23]. Demethylation drugs, decitabine and azacytidine, are the standard treatments for relatively high-risk MDS. Demethylation drugs combined with histone deacetylase inhibitors or PD1/PDL1 could increase M1 macrophages and activate type I interferon [24, 25]. Therefore, using a combination of drugs that can promote M1 polarization may be an interesting direction for the treatment of MDS.

Our study has some limitations, such as whether the induced M1 macrophages express the surface markers of M1 cells, such as iNOS and STAT-1, and the levels of TNF-α and IL-1β secreted by these M1 macrophages.

In conclusion, we found that the polarization of bone marrow macrophages in patients with MDS was abnormal, M1 macrophages were relatively reduced, and IL-1β and TNF were decreased. This may be a manifestation of an abnormal bone marrow microenvironment in patients with MDS. Regulation of macrophage polarization may be one of the directions of MDS targeted therapy.

Data Availability

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

Conflicts of Interest

All authors declare no conflict of interest.

Authors' Contributions

Gaochao Zhang, Liyan Yang, and Yu Han contributed equally to this work. G.Z., L.Y., and Y.H. performed research and analyzed the data; H.W. designed studies, ensured the correct analysis of the data, and drafted the manuscript; H.N., L.Y., L.X., and Z.S. assisted in research design, oversaw data collection, and contributed to the writing of the manuscript. All authors carefully revised the manuscript and finally approved the manuscript.

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Supplementary Materials

Supplemental Figure 1: the gating strategy for macrophages. (A) Bone marrow mononucleated cells were gated with SSC and FSC. (B) Monocytes were gated with CD14. (C) Macrophages were defined with $CD14^+CD68^+$ cells. (D) M1 macrophages were defined with $CD14^+CD68^+CD40^+CD64^+$ cells. (E) M2 macrophages were defined with $CD14^+CD68^{+-}$ $CD163^+CD206^+$ cells. *(Supplementary Materials)*

References

[1] D. A. Arber, A. Orazi, R. Hasserjian et al., "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia," *Blood*, vol. 127, no. 20, pp. 2391–2405, 2016.

[2] M. Cazzola, "Myelodysplastic syndromes," *The New England Journal of Medicine*, vol. 383, no. 14, pp. 1358–1374, 2020.

[3] A. Shapouri-Moghaddam, S. Mohammadian, H. Vazini et al., "Macrophage plasticity, polarization, and function in health and disease," *Journal of Cellular Physiology*, vol. 233, no. 9, pp. 6425–6440, 2018.

[4] S. Pinho and P. S. Frenette, "Haematopoietic stem cell activity and interactions with the niche," *Nature Reviews. Molecular Cell Biology*, vol. 20, no. 5, pp. 303–320, 2019.

[5] P. J. Murray, "Macrophage polarization," *Annual Review of Physiology*, vol. 79, no. 1, pp. 541–566, 2017.

[6] A. Sica and A. Mantovani, "Macrophage plasticity and polarization: in vivo veritas," *The Journal of Clinical Investigation*, vol. 122, no. 3, pp. 787–795, 2012.

[7] Y. Han, H. Wang, and Z. Shao, "Monocyte-derived macrophages are impaired in myelodysplastic syndrome," *Journal of Immunology Research*, vol. 2016, Article ID 5479013, 7 pages, 2016.

[8] I. G. Winkler, N. A. Sims, A. R. Pettit et al., "Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs," *Blood*, vol. 116, no. 23, pp. 4815–4828, 2010.

[9] E. Heideveld, F. Masiello, M. Marra et al., "CD14+ cells from peripheral blood positively regulate hematopoietic stem and progenitor cell survival resulting in increased erythroid yield," *Haematologica*, vol. 100, no. 11, pp. 1396–1406, 2015.

[10] L. C. Davies, S. J. Jenkins, J. E. Allen, and P. R. Taylor, "Tissue-resident macrophages," *Nature Immunology*, vol. 14, no. 10, pp. 986–995, 2013.

[11] P. J. Murray and T. A. Wynn, "Protective and pathogenic functions of macrophage subsets," *Nature Reviews. Immunology*, vol. 11, no. 11, pp. 723–737, 2011.

[12] T. Chanmee, P. Ontong, K. Konno, and N. Itano, "Tumor-associated macrophages as major players in the tumor microenvironment," *Cancers*, vol. 6, no. 3, pp. 1670–1690, 2014.

[13] N.-B. Hao, M.-H. Lü, Y.-H. Fan, Y.-L. Cao, Z.-R. Zhang, and S.-M. Yang, "Macrophages in tumor microenvironments and the progression of tumors," *Clinical & Developmental Immunology*, vol. 2012, article 948098, pp. 1–11, 2012.

[14] A. Mantovani, F. Marchesi, A. Malesci, L. Laghi, and P. Allavena, "Tumour-associated macrophages as treatment targets in oncology," *Nature Reviews. Clinical Oncology*, vol. 14, no. 7, pp. 399–416, 2017.

[15] A. Mantovani and A. Sica, "Macrophages, innate immunity and cancer: balance, tolerance, and diversity," *Current Opinion in Immunology*, vol. 22, no. 2, pp. 231–237, 2010.

[16] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews. Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[17] P. Dumont, A. Berton, N. Nagy et al., "Expression of galectin-3 in the tumor immune response in colon cancer," *Laboratory investigation*, vol. 88, no. 8, pp. 896–906, 2008.

[18] A. H. Klimp, H. Hollema, C. Kempinga, A. van der Zee, E. G. de Vries, and T. Daemen, "Expression of cyclooxygenase-2 and inducible nitric oxide synthase in human ovarian tumors and tumor-associated macrophages," *Cancer Research*, vol. 61, no. 19, pp. 7305–7309, 2001.

[19] C. I. Maratheftis, E. Andreakos, H. M. Moutsopoulos, and M. Voulgarelis, "Toll-like receptor-4 is up-regulated in hematopoietic progenitor cells and contributes to increased apoptosis in myelodysplastic syndromes," *Clinical cancer research*, vol. 13, no. 4, pp. 1154–1160, 2007.

[20] C. Orelio, M. Peeters, E. Haak, K. van der Horn, and E. Dzierzak, "Interleukin-1 regulates hematopoietic progenitor

and stem cells in the midgestation mouse fetal liver," *Haematologica*, vol. 94, no. 4, pp. 462–469, 2009.

[21] K. Allampallam, V. Shetty, S. Mundle et al., "Biological significance of proliferation, apoptosis, cytokines, and monocyte/-macrophage cells in bone marrow biopsies of 145 patients with myelodysplastic syndrome," *International Journal of Hematology*, vol. 75, no. 3, pp. 289–297, 2002.

[22] A. A. Basiorka, M. G. KL, E. A. Eksioglu et al., "The NLRP3 inflammasome functions as a driver of the myelodysplastic syndrome phenotype," *Blood*, vol. 128, no. 25, pp. 2960–2975, 2016.

[23] Z. Lu, J. Zou, S. Li et al., "Epigenetic therapy inhibits metastases by disrupting premetastatic niches," *Nature*, vol. 579, no. 7798, pp. 284–290, 2020.

[24] M. L. Stone, K. B. Chiappinelli, H. Li et al., "Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 51, pp. E10981–E10990, 2017.

[25] M. Najafi, N. Hashemi Goradel, B. Farhood et al., "Macrophage polarity in cancer: a review," *Journal of Cellular Biochemistry*, vol. 120, no. 3, pp. 2756–2765, 2019.

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Review Article

COVID-19 and Neutrophils: The Relationship between Hyperinflammation and Neutrophil Extracellular Traps

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Coronavirus disease 2019 (COVID-19) is a virus-induced respiratory disease that may progress to acute respiratory distress syndrome (ARDS) and is triggered by immunopathological mechanisms that cause excessive inflammation and leukocyte dysfunction. Neutrophils play a critical function in the clearance of bacteria with specific mechanisms to combat viruses. The aim of this review is to highlight the current advances in the pathways of neutrophilic inflammation against viral infection over the past ten years, focusing on the production of neutrophil extracellular traps (NETs) and its impact on severe lung diseases, such as COVID-19. We focused on studies regarding hyperinflammation, cytokine storms, neutrophil function, and viral infections. We discuss how the neutrophil's role could influence COVID-19 symptoms in the interaction between hyperinflammation (overproduction of NETs and cytokines) and the clearance function of neutrophils to eliminate the viral infection. We also propose a more in-depth investigation into the neutrophil response mechanism targeting NETosis in the different phases of COVID-19.

1. Introduction

The World Health Organization (WHO) established the coronavirus disease 2019 (COVID-19) as a pandemic on March 11, 2020. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a member of the coronavirus family, a class of enveloped viruses with a positive-sense single-stranded RNA genome. This virus can cross species barriers and induce illnesses ranging from the usual cold to severe interstitial pneumonia, respiratory failure, and septic shock [1]. While there is a global effort in the development of vaccines and improvement of diagnostic methods [2, 3] and therapies that relieve the symptoms and prognosis of COVID-19 patients under severe infection [4], there remain gaps in our understanding of the pathophysiology of COVID-19 related to innate immunity.

In a scenario where patients with severe COVID-19 could develop dysfunction of the immune response that aggravates the hyperinflammation [5, 6], it is hypothesized that neutrophils can amplify pathological damage or control other cell subsets depending on the infection features. Therefore, to use the potential of NETs with minimal damage to the hosts, there must be a right balance of NET formation and reduction of the amount of NETs that accumulate in tissues [7].

Notwithstanding the rapid progress in the field, there are many critical unknown features of neutrophils in fighting viral infections. We highlighted the current progress in the pathways of neutrophilic inflammation in viral infection, with a focus on the release of NETs and its influence on lung disease. The knowledge summarized in this study should benefit researchers in integrating neutrophil biology to design new and more efficient virus-targeted interventions concerning COVID-19.

2. Hyperinflammation

Although a well-regulated innate immune process is the first protection action against viral infections [8], in severe COVID-19 condition occurs hyperinflammation ("cytokine

storm") that might lead to the acute respiratory distress syndrome (ARDS) [6, 9].

Cytokines play a relevant function in immunopathology during virus infections. The host-viral interactions are established via host identification of pathogen-associated molecular patterns (PAMPs) of the virus [10]. This identification occurs through host pattern recognition receptors (PRRs) manifested on innate immune cells (e.g., neutrophils, dendritic cells, epithelial cells, and macrophages) [11], and the recognition of PAMPs and viral danger-associated molecular patterns (DAMPs) by conserved PRRs marks the first line of defense against pathogens, involving toll-like receptors (TLRs) [11].

TLR stimulation activates the nuclear factor-κB (NF-κB) signaling cascade, causing the production of inflammatory markers from monocytes (interleukin- (IL-) 1, tumor necrosis factor-alpha (TNF-α), and IL-6) to control virus infections [8] by direct antiviral pathways and the recruitment of other leukocytes [10]. Moreover, the exacerbated oxidative stress induced by elevated concentrations of cytokines, along with reduced concentrations of interferon α and interferon β (IFN-α, IFN-β), influences the severity of COVID-19 [12].

Several mediators control the release of chemoattractants and neutrophil activity [10], and studies have demonstrated that higher values of proinflammatory markers are related to extensive lung damage and pulmonary inflammation in MERS-CoV [13] and ARDS infection [14]. COVID-19 in the severe state exhibits a cytokine storm with elevated plasma levels of chemokine ligand 2 (CCL2), IFNγ, IFNγ-inducible protein 10, G-CSF, chemokine C-C motif ligand 3 (CCL3), IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-17, and TNF-α [12, 15]. Nucleotide-binding oligomerization domain- (NOD-) like receptor and increased plasma levels of chemokines and cytokines in COVID-19 patients relate to the severity of the disease rather than did those nonsevere patients [5]. In this sense, Huang et al. [15] found that patients in the intensive care unit (ICU) with laboratory-confirmed COVID-19 infection had higher plasma levels of IL-2, IL-7, IL-10, interferon-inducible protein 10, granulocyte colony-stimulating factor, CCL2, CCL3, and TNF-α when compared with non-ICU patients [15].

3. Neutrophils: The First Cell Recruitment

Neutrophils are innate immune cells with a brief lifespan after leaving the bone marrow and exist in a quiescent, primed, or active state. These leukocytes are the leading players in innate immunity since they are among the first innate leukocytes recruited during infections [16]. The primary function of neutrophil is clearance of pathogens and debris through phagocytosis [17]. They also have a distinct array of other immune roles, such as the liberation of NETs for viral infection inactivation [18] and cytokine production to restrict virus replication [16].

The release of neutrophil-chemoattractive elements and the resulting recruitment of neutrophils are a global host response to viral infection [19]. In this scenario, the neutrophil cell membrane also expresses a complex array of receptors and adhesion molecules for various ligands, including immunoglobulins, membrane molecules on other cells, and cytokines [20].

In addition to the trafficking to infection places to phagocytize viruses, the neutrophils can initiate, enlarge, and/or repress adaptive immune effector processes by promoting bidirectional cross-talk with T cells [21, 22]. Following the acute inflammation arising from immunological processes, such as viral infections, neutrophils with decreased expression of CD62L weaken T cell migration via the CXCL11 chemokine gradient by releasing H_2O_2 into an immunological synapse [23]. Thus, neutrophils that uncovered viral antigens can home to draining lymph nodes, acting as antigen-presenting cells (APC) [24]. Hufford et al. [25] evidenced that neutrophils expressing viral antigen as an outcome of direct infection by influenza A virus (IAV) display the most potent APC activity and that viral antigen-presenting neutrophils infiltrating the IAV-infected lungs act as APC for effector CD8(+) T lymphocytes in the infected lungs [25]. Neutrophils recruit the T cell molecular mechanism during the influenza virus infection and associate to CXCL12 reservoirs left behind. CD8+ T cells follow the chemoattractant trail left behind by neutrophil uropods to the influenza virus infection site [26].

Decreased cell number or impaired leukocyte function can play a part in advance of mild to severe clinical disease conditions [16]. Regarding the new coronavirus, the neutrophil-to-lymphocyte ratio (NLR), a well-known marker of infection and systemic inflammation, has evidenced an enhanced inflammatory response in COVID-19 patients [5]. Since the ARDS is the primary cause of mortality in patients with COVID-19, the elevated NLR values suggest a poor prognosis in COVID-19 disease [27], especially severe COVID-19 compared to mild patients. Sun et al. [28] studied 116 patients with COVID-19 and showed a higher NLR [28]. The authors compared severe COVID-19 patients admitted to the ICU with others or severe patients not admitted to the ICU. They reported that COVID-19 patients have the lowest count of lymphocytes and the highest neutrophil count and NLR [28]. Wang et al. [29] also showed that several COVID-19 patients have a rising neutrophil count and a falling lymphocyte count during the severe phase [29]. Similarly, Barnes et al. [30] found extensive neutrophil infiltration in pulmonary capillaries from a COVID-19 patient [30]. Nevertheless, even though severe cases of COVID-19 appear to be related to increased NLR levels [5], whether NLR could be an independent predictor of mortality in COVID-19 patients still requires investigation.

4. Neutrophil Extracellular Traps (NETs) and Viral Infection

Neutrophils can develop a sophisticated network of DNA called NETs through NETosis, a liberation of web-like structures of nucleic acids wrapped with histones that detain viral particles [31]. Upon discovery, the researchers believed that the production of NETs defended only against fungi and bacteria [32]. However, the NETosis process plays an important function in the response to viral diseases [33], thereby

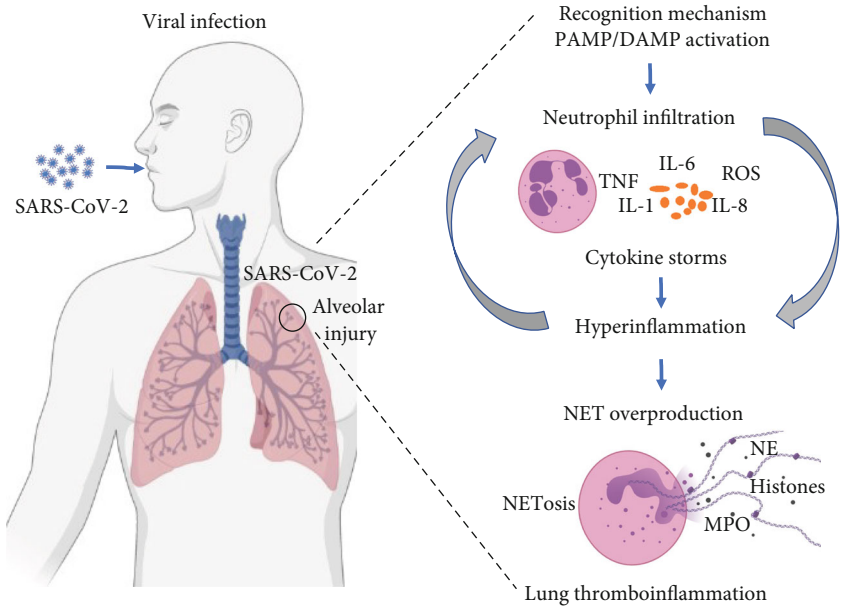

Figure 1: The interaction hypothesis between neutrophil and hyperinflammation in COVID-19. After the host-viral interaction, the virus signaling leads to a cascade of interactions between the virus recognition mechanism, neutrophil activation, and inflammatory stimuli. The NETosis process can protect the host during the virus response or exacerbate lung hyperinflammation in COVID-19 patients. The figure is made with BioRender (https://app.biorender.com/). Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; PAMP: pathogen-associated molecular pattern; DAMP: danger-associated molecular pattern; TNF: tumor necrosis factor; IL-6: interleukin-6; IL-1: interleukin-1; IL-8: interleukin-8; ROS: reactive oxygen species; NE: neutrophil elastase; MPO: myeloperoxidase.

protecting the host during the virus response by trapping and eliminating distinct pathogens [31].

The formation of NETs is a controlled process, even though the related signals remain unknown. NETosis is conditional on the production of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [34]. There is evidence of NETosis produced in a ROS-independent mechanism [35]. In general, the NETosis process includes the release of nuclear chromatin lined with effector proteins and peptidyl arginine deiminase type IV (PAD4) activation [36]. After stimulation, the neutrophil nuclear envelope disintegrates to enable the mixing of chromatin with granular proteins [37]. Myeloperoxidase (MPO) and neutrophil elastase (NE) stimulate chromatin condensation and deteriorate histones [38]. In the presence of histone hypercitrullination, PAD4 mediates chromatin decondensation, and the DNA-protein complexes are released extracellularly as NETs [37]. Therefore, differently from apoptosis or necrosis, both the granular membrane and nuclear membrane deteriorate during NETosis, whereas plasma membrane integrity remains [36].

The overproduction of NETs induces lung tissue damage by NETosis-related enzymes such as NE and MPO [39]. Uncontrolled NET production correlates with disease gravity and lung injury extension. For instance, NETosis markers are related to bacterial burden and local inflammation in the lung [40] and patients with pneumonia-associated ARDS have neutrophils in a "primed" condition to generate NETs [41].

During chronic obstructive pulmonary disease aggravation, the production of NETs increases in people with acute respiratory failure [39] and in ARDS patients [40, 42]. The elevated NET production, as noted in patients with severe IAV infection [43], increased injury to the pulmonary endothelial and epithelial cells [44], directing to severe pneumonia. Zhu et al. [43] also noted that the production of NETs positively correlates with multiple organ dysfunction syndromes [43].

The inflammatory process is a trigger for thrombotic complications usually noted in COVID-19 patients, and the immunothrombotic dysregulation seems to be an important key marker for the disease severity [45]. Skendros et al. [46] found that complement activation potentiates the platelet/-NET/tissue factor/thrombin axis during SARS-CoV-2 infection [46]. In contrast, Nicolai et al. [47] noted that, in COVID-19, inflammatory microvascular thrombi are found in the kidney, lung, and heart, containing NETs related to the fibrin and platelets. In blood, Nicolai et al. also show that COVID-19 patients have neutrophil-platelet aggregates and a different platelet and neutrophil activation pattern, which alters with the disease severity [47]. Middleton et al. [48] also found that plasma MPO-DNA complexes increased in COVID-19 and that the elevated NET formation correlates with COVID-19-related ARDS. Together, these findings suggest the timely application of therapeutic strategies that can disrupt the vicious cycle of COVID-19 immunothrombosis/-thromboinflammation by targeting neutrophil activation and NET formation.

In addition to the physical containment promoted by NETosis [33], NETs contain DNA, modified extracellular histones, proteases, and cytotoxic enzymes that allow neutrophils to centralize lethal proteins at infection sites [7]. The mechanisms of NETs' release in the viral response seem to

Table 1: Interventional studies registered at the ClinicalTrials.gov database relating the treatment of COVID-19 with NET inhibitors.

NCT identifier	Status	Location	Study type	Condition or disease	Intervention and phase	Primary outcome	Estimated completion date
NCT04409925	Not yet recruiting	Canada	Nonrandomized pilot study	COVID-19	Dornase Alfa Phase: 1	(1) Rate of all adverse events	January 2021
NCT04359654	Not yet recruiting	United Kingdom	Randomized clinical trial	COVID-19 Hypoxia	Drug: Dornase Alfa Phase: 2	(1) Change in inflammation (C-reactive protein)	November 2020
NCT04445285	Recruiting	United States	Randomized clinical trial	COVID-19	Dornase Alfa Phase: 2	(1) All-cause mortality (2) Systemic therapeutic response	February 2021
NCT04432987	Recruiting	Turkey	Randomized clinical trial	COVID-19	Dornase Alfa Phase: 2	(1) Clinical improvement and inflammatory markers in blood (2) Intubation or extubation	September 2020
NCT04402944	Not yet recruiting	United States	Randomized clinical trial	COVID-19	Dornase Alfa Phase: 2	(1) Ventilator-free days	December 2021
NCT04322565	Recruiting	Italy	Randomized clinical trial	COVID-19 Pneumonia	Colchicine Phase: 2	(1) Clinical improvement (2) Hospital discharge	December 2020
NCT04326790	Recruiting	Greece	Randomized clinical trial	COVID-19	Colchicine Phase: 2	(1) Time to clinical deterioration (2) Concentration of cardiac troponin	September 2020
NCT04402970	Recruiting	United States	Nonrandomized clinical trial	COVID-19 ARDS	Dornase Alfa Phase: 3	(1) Improvement in partial pressure of O_2 to fraction of inspired O_2 ratio	May 2022
NCT04355364	Recruiting	France	Randomized clinical trial	COVID-19 ARDS	Dornase Alfa Phase: 3	(1) Occurrence of at least one grade improvement (ARDS scale severity)	August 2020
NCT04322682	Recruiting	United States	Randomized clinical trial	COVID-19	Colchicine Phase: 3	(1) Number of participants who die or require hospitalization	December 2020
NCT04328480	Recruiting	Argentina	Randomized clinical trial	COVID-19	Colchicine Phase: 3	(1) Number of participants who die (all-cause mortality)	August 2020

Retrieved October 30, 2020, from https://www.https://www.clinicaltrials.gov/ct2/home. Abbreviations: NCT: National Clinical Trial; O_2: oxygen; ARDS: acute respiratory distress syndrome.

involve neutrophil NE production attributed to the change of macrophage role by the cleavage of TLRs [49]. A range of stimuli, including toxic factors, viruses, and proinflammatory cytokines, such as TNF-α and IL-8, can lead neutrophils to release NETs [7, 33]. Mechanisms that determine strain specificity to induce NETosis formation during viral infection are still unknown.

Lung inflammation is the leading cause of the life-threatening respiratory complication at the severe levels of COVID-19 [50]. Veras et al. [51] investigated the potentially detrimental function of NETs in the pathophysiology of 32 hospitalized severe COVID-19 patients and found that the levels of NETs increase in tracheal aspirate and plasma from patients with COVID-19 and their neutrophils naturally produced more significant concentrations of NETs [51]. The authors also reported NETs in the lung tissue specimens from autopsies of COVID-19 patients. *In vitro*, they noted that viable SARS-CoV-2 cause NET production by healthy neutrophils through a PAD-4-dependent manner and that NETs produced by SARS-CoV-2-activated neutrophils instigated lung epithelial cell death [51]. Zuo et al. [52] also investigated sera from COVID-19 patients and found higher cell-free DNA, myeloperoxidase-DNA (MPO-DNA), and citrullinated histone H3 (Cit-H3) [52]. *In vitro*, they also noted that sera from COVID-19 patients trigger NET release from control neutrophils [52].

Although the literature does not report direct evidence linking NETs and SARS-CoV2 clearance, virus entrapping by NETs was already found in syncytial respiratory virus infection [53] or influenza [54]. Furthermore, in virus infection, NETs are efficient to block viruses at the infection site, entrapping them in a DNA web [22]. Therefore, the NETosis process induced by the virus could operate as a double-edged sword: on the one hand, there are essential and efficient mechanisms for trapping the virus [55], and on the other, there are highly intense immunological and inflammatory processes triggered by NET release causing damage to the organism [7]. These interactions could influence the COVID-19 symptoms in the relationship between hyperinflammation (overproduction of NETs and cytokine storm) and the function of neutrophils to destroy the viral infection (Figure 1).

5. Concluding Remarks and Future Directions

The exacerbated NET formation can drive to a cascade of inflammatory reactions that destroys surrounding tissues, favors microthrombosis, contributes to the progress of cancer cell metastasis, and results in permanent damage to the pulmonary, cardiovascular, and renal systems [56]. Whether by coincidence or a cause-and-effect relationship, these organs are affected in the severe state of the COVID-19

disease [57, 58]. The uncontrolled and poorly acknowledged host response regarding the cytokine storm is one of the major causes of severe COVID-19 conditions [12]. In this pandemic scenario, there is a compelling need to investigate the mechanisms associated with hyperinflammation process and NET production in response to COVID-19.

The NLR is an independent risk factor for severe COVID-19 [27], and neutrophilia forecasts poor outcomes in COVID-19 patients [29]. In this sense, new frontiers in NET assessment regarding COVID-19 may be expressed by analyzing NETosis directly after sputum induction or after bronchoscopy using the bronchial alveolar fluid of COVID-19 patients [42]. Since patient samples usually become accessible at the hospital, it could investigate whether the existence of NETs is associated with the severity of COVID-19.

Treatments using NET-targeting approaches, although would not directly target the new coronavirus, could reduce the damage caused by hyperinflammation [59], thereby decreasing the disease's severity and avoiding invasive mechanical ventilation, consequently diminishing mortality. Drugs that target NETs include inhibitors of the molecules necessary for NET formation, such as gasdermin D [60], PAD4 [61], and NE [62]. Studies on treatment of inflammatory state in COVID-19 patients with NET inhibitors are still in development (please see Table 1).

Caution is needed to define which people would advantage from suppressing the neutrophil response and which would help more from a strengthened neutrophil action during viral infections. Despite prior studies linking pulmonary diseases to aberrant NET formation [3, 4], our understanding of NETosis mechanisms in viral infection is still limited.

The hyperinflammation is related to the severity of COVID-19 by influencing the pulmonary inflammation [12]. Neutrophils exhibit an intense response to virus infection, promoting bidirectional cross-talk with T cells [21]. Neutrophils also express a complex array of receptors and adhesion molecules for various ligands, including immunoglobulins and inflammatory markers [20]. In this sense, severe cases of COVID-19 appear to be related to increased NLR levels [5], and treatments using NET-targeting approaches have the potential to decrease the damage caused by hyperinflammation [40, 41]. The researchers should consider hyperinflammation in the different phases of COVID-19, neutrophil response mechanisms, and NETosis.

Data Availability

The data supporting this narrative review are from previously reported studies and datasets, which have been cited.

Conflicts of Interest

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

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References

[1] G. Kutti-Sridharan, R. Vegunta, R. Vegunta, B. P. Mohan, and V. R. P. Rokkam, "SARS-CoV2 in different body fluids, risks of transmission, and preventing COVID-19: a comprehensive evidence-based review," *International Journal of Preventive Medicine*, vol. 11, p. 97, 2020.

[2] L. L. Fernandes, V. B. Pacheco, L. Borges et al., "Saliva in the diagnosis of COVID-19: a review and new research directions," *Journal of Dental Research*, vol. 99, no. 13, pp. 1435–1443, 2020.

[3] L. L. Fernandes, L. Borges, V. B. Pacheco et al., "SARS-CoV-2 : a promising path in salivary diagnosis," *The Open Dentistry Journal*, vol. 14, no. 1, pp. 343-344, 2020.

[4] A. C. L. Fernandes, A. J. M. Vale, F. P. Guzen, F. I. Pinheiro, R. N. Cobucci, and E. P. de Azevedo, "Therapeutic options against the new coronavirus: updated clinical and laboratory evidences," *Frontiers in Medicine*, vol. 7, p. 546, 2020.

[5] C. Qin, L. Zhou, Z. Hu et al., "Dysregulation of immune response in patients with coronavirus 2019 (COVID-19) in Wuhan, China," *Clinical Infectious Diseases*, vol. 71, no. 15, pp. 762–768, 2020.

[6] Q. Ruan, K. Yang, W. Wang, L. Jiang, and J. Song, "Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China," *Intensive Care Medicine*, vol. 46, no. 5, pp. 846–848, 2020.

[7] O. Z. Cheng and N. Palaniyar, "NET balancing: a problem in inflammatory lung diseases," *Frontiers in Immunology*, vol. 4, p. 1, 2013.

[8] G. Zhu, Y. Xu, X. Cen, K. S. Nandakumar, S. Liu, and K. Cheng, "Targeting pattern-recognition receptors to discover new small molecule immune modulators," *European Journal of Medicinal Chemistry*, vol. 144, pp. 82–92, 2018.

[9] C.-K. Min, S. Cheon, N.-Y. Ha et al., "Comparative and kinetic analysis of viral shedding and immunological responses in MERS patients representing a broad spectrum of disease severity," *Scientific Reports*, vol. 6, no. 1, article 25359, 2016.

[10] R. Channappanavar and S. Perlman, "Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology," *Seminars in Immunopathology*, vol. 39, no. 5, pp. 529–539, 2017.

[11] D. Kolli, T. S. Velayutham, and A. Casola, "Host-viral interactions: role of pattern recognition receptors (PRRs) in human pneumovirus infections," *Pathogens*, vol. 2, no. 2, pp. 232–263, 2013.

[12] P. Mehta, D. F. McAuley, M. Brown, E. Sanchez, R. S. Tattersall, and J. J. Manson, "COVID-19: consider cytokine storm syndromes and immunosuppression," *The Lancet*, vol. 395, no. 10229, pp. 1033-1034, 2020.

[13] W. H. Mahallawi, O. F. Khabour, Q. Zhang, H. M. Makhdoum, and B. A. Suliman, "MERS-CoV infection in humans is associated with a pro-inflammatory Th1 and Th17 cytokine profile," *Cytokine*, vol. 104, pp. 8–13, 2018.

[14] M. Prete, E. Favoino, G. Catacchio, V. Racanelli, and F. Perosa, "SARS-CoV-2 inflammatory syndrome. Clinical features and

rationale for immunological treatment," *International Journal of Molecular Sciences*, vol. 21, no. 9, article 3377, 2020.

[15] C. Huang, Y. Wang, X. Li et al., "Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China," *The Lancet*, vol. 395, no. 10223, pp. 497–506, 2020.

[16] P. P. Lamichhane and A. E. Samarasinghe, "The role of innate leukocytes during influenza virus infection," *Journal of Immunology Research*, vol. 2019, Article ID 8028725, 17 pages, 2019.

[17] C. Rosales, "Neutrophils at the crossroads of innate and adaptive immunity," *Journal of Leukocyte Biology*, vol. 108, no. 1, pp. 377–396, 2020.

[18] F. D. Barr, C. Ochsenbauer, C. R. Wira, and M. Rodriguez-Garcia, "Neutrophil extracellular traps prevent HIV infection in the female genital tract," *Mucosal Immunology*, vol. 11, no. 5, pp. 1420–1428, 2018.

[19] J. Bordon, S. Aliberti, R. Fernandez-Botran et al., "Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective versus deleterious inflammatory response in pneumonia," *International Journal of Infectious Diseases*, vol. 17, no. 2, pp. e76–e83, 2013.

[20] C. D. Russell, S. A. Unger, M. Walton, and J. Schwarze, "The human immune response to respiratory syncytial virus infection," *Clinical Microbiology Reviews*, vol. 30, no. 2, pp. 481–502, 2017.

[21] S. Costa, D. Bevilacqua, M. A. Cassatella, and P. Scapini, "Recent advances on the crosstalk between neutrophils and B or T lymphocytes," *Immunology*, vol. 156, no. 1, pp. 23–32, 2019.

[22] V. D. Giacalone, C. Margaroli, M. A. Mall, and R. Tirouvanziam, "Neutrophil adaptations upon recruitment to the lung: new concepts and implications for homeostasis and disease," *International Journal of Molecular Sciences*, vol. 21, no. 3, p. 851, 2020.

[23] J. Pillay, V. M. Kamp, E. van Hoffen et al., "A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 327–336, 2012.

[24] M. V. Lukens, A. C. van de Pol, F. E. J. Coenjaerts et al., "A systemic neutrophil response precedes robust $CD8^+$ T-cell activation during natural respiratory syncytial virus infection in infants," *Journal of Virology*, vol. 84, no. 5, pp. 2374–2383, 2010.

[25] M. M. Hufford, G. Richardson, H. Zhou et al., "Influenza-infected neutrophils within the infected lungs act as antigen presenting cells for anti-viral CD8(+) T cells," *PLoS One*, vol. 7, no. 10, article e46581, 2012.

[26] K. Lim, Y.-M. Hyun, K. Lambert-Emo et al., "Neutrophil trails guide influenza-specific $CD8^+$ T cells in the airways," *Science*, vol. 349, no. 6252, article aaa 4352, 2015.

[27] F. A. Lagunas-Rangel, "Neutrophil-to-lymphocyte ratio and lymphocyte-to-C-reactive protein ratio in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis," *Journal of Medical Virology*, vol. 92, no. 10, pp. 1733-1734, 2020.

[28] S. Sun, X. Cai, H. Wang et al., "Abnormalities of peripheral blood system in patients with COVID-19 in Wenzhou, China," *Clinica Chimica Acta*, vol. 507, pp. 174–180, 2020.

[29] D. Wang, B. Hu, C. Hu et al., "Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China," *JAMA*, vol. 323, no. 11, pp. 1061–1069, 2020.

[30] B. J. Barnes, J. M. Adrover, A. Baxter-Stoltzfus et al., "Targeting potential drivers of COVID-19: neutrophil extracellular traps," *The Journal of Experimental Medicine*, vol. 217, no. 6, article e20200652, 2020.

[31] C. N. Jenne, C. H. Y. Wong, F. J. Zemp et al., "Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps," *Cell Host & Microbe*, vol. 13, no. 2, pp. 169–180, 2013.

[32] V. Brinkmann, U. Reichard, C. Goosmann et al., "Neutrophil extracellular traps kill bacteria," *Science*, vol. 303, no. 5663, pp. 1532–1535, 2004.

[33] T. Saitoh, J. Komano, Y. Saitoh et al., "Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1," *Cell Host & Microbe*, vol. 12, no. 1, pp. 109–116, 2012.

[34] V. Delgado-Rizo, M. A. Martínez-Guzmán, L. Iñiguez-Gutierrez, A. García-Orozco, A. Alvarado-Navarro, and M. Fafutis-Morris, "Neutrophil extracellular traps and its implications in inflammation: an overview," *Frontiers in Immunology*, vol. 8, p. 81, 2017.

[35] O. Tatsiy and P. P. McDonald, "Physiological stimuli induce PAD4-dependent, ROS-independent NETosis, with early and late events controlled by discrete signaling pathways," *Frontiers in Immunology*, vol. 9, article 2036, 2018.

[36] S. Yousefi, D. Stojkov, N. Germic et al., "Untangling "NETosis" from NETs," *European Journal of Immunology*, vol. 49, no. 2, pp. 221–227, 2019.

[37] T. A. Fuchs, U. Abed, C. Goosmann et al., "Novel cell death program leads to neutrophil extracellular traps," *The Journal of Cell Biology*, vol. 176, no. 2, pp. 231–241, 2007.

[38] V. Papayannopoulos, K. D. Metzler, A. Hakkim, and A. Zychlinsky, "Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps," *The Journal of Cell Biology*, vol. 191, no. 3, pp. 677–691, 2010.

[39] F. Grabcanovic-Musija, A. Obermayer, W. Stoiber et al., "Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation," *Respiratory Research*, vol. 16, no. 1, p. 59, 2015.

[40] C. Mikacenic, R. Moore, V. Dmyterko et al., "Neutrophil extracellular traps (NETs) are increased in the alveolar spaces of patients with ventilator-associated pneumonia," *Critical Care*, vol. 22, no. 1, p. 358, 2018.

[41] I. Bendib, L. de Chaisemartin, V. Granger et al., "Neutrophil extracellular traps are elevated in patients with pneumonia-related acute respiratory distress syndrome," *Anesthesiology*, vol. 130, no. 4, pp. 581–591, 2019.

[42] J. J. M. Wong, J. Y. Leong, J. H. Lee, S. Albani, and J. G. Yeo, "Insights into the immuno-pathogenesis of acute respiratory distress syndrome," *Annals of Translational Medicine*, vol. 7, no. 19, p. 504, 2019.

[43] L. Zhu, L. Liu, Y. Zhang et al., "High level of neutrophil extracellular traps correlates with poor prognosis of severe influenza A Infection," *The Journal of Infectious Diseases*, vol. 217, no. 3, pp. 428–437, 2018.

[44] T. Narasaraju, E. Yang, R. P. Samy et al., "Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis," *The American Journal of Pathology*, vol. 179, no. 1, pp. 199–210, 2011.

[45] Y. Zuo, M. Zuo, S. Yalavarthi et al., "Neutrophil extracellular traps and thrombosis in COVID-19," *Journal of Thrombosis and Thrombolysis*, vol. 1-8, 2020.

[46] P. Skendros, A. Mitsios, A. Chrysanthopoulou et al., "Complement and tissue factor-enriched neutrophil extracellular traps are key drivers in COVID-19 immunothrombosis," *The Journal of Clinical Investigation*, vol. 130, no. 11, pp. 6151–6157, 2020.

[47] L. Nicolai, A. Leunig, S. Brambs et al., "Immunothrombotic dysregulation in COVID-19 pneumonia is associated with respiratory failure and coagulopathy," *Circulation*, vol. 142, no. 12, pp. 1176–1189, 2020.

[48] E. A. Middleton, X.-Y. He, F. Denorme et al., "Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome," *Blood*, vol. 136, no. 10, pp. 1169–1179, 2020.

[49] H. Domon, K. Nagai, T. Maekawa et al., "Neutrophil elastase subverts the immune response by cleaving toll-like receptors and cytokines in pneumococcal pneumonia," *Frontiers in Immunology*, vol. 9, p. 732, 2018.

[50] W.-J. Guan, Z.-Y. Ni, Y. Hu et al., "Clinical characteristics of coronavirus disease 2019 in China," *The New England Journal of Medicine*, vol. 382, no. 18, pp. 1708–1720, 2020.

[51] F. P. Veras, M. Pontelli, C. Silva et al., "SARS-CoV-2–triggered neutrophil extracellular traps mediate COVID-19 pathology," *The Journal of Experimental Medicine*, vol. 217, no. 12, article e20201129, 2020.

[52] Y. Zuo, S. Yalavarthi, H. Shi et al., "Neutrophil extracellular traps in COVID-19," *JCI Insight*, vol. 5, no. 11, article e138999, 2020.

[53] G. A. Funchal, N. Jaeger, R. S. Czepielewski et al., "Respiratory syncytial virus fusion protein promotes TLR-4-dependent neutrophil extracellular trap formation by human neutrophils," *PLoS One*, vol. 10, no. 4, article e0124082, 2015.

[54] L. A. Perrone, J. K. Plowden, A. García-Sastre, J. M. Katz, and T. M. Tumpey, "H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice," *PLoS Pathogens*, vol. 4, no. 8, article e1000115, 2008.

[55] S. P. Muraro, G. F. De Souza, S. W. Gallo et al., "Respiratory syncytial virus induces the classical ROS-dependent NETosis through PAD-4 and necroptosis pathways activation," *Scientific Reports*, vol. 8, no. 1, article 14166, 2018.

[56] S. K. Jorch and P. Kubes, "An emerging role for neutrophil extracellular traps in noninfectious disease," *Nature Medicine*, vol. 23, no. 3, pp. 279–287, 2017.

[57] R. O. Bonow, G. C. Fonarow, P. T. O'Gara, and C. W. Yancy, "Association of coronavirus disease 2019 (COVID-19) with myocardial injury and mortality," *JAMA Cardiology*, vol. 5, no. 7, pp. 751–753, 2020.

[58] N. Chen, M. Zhou, X. Dong et al., "Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study," *The Lancet*, vol. 395, no. 10223, pp. 507–513, 2020.

[59] S. Cicco, G. Cicco, V. Racanelli, and A. Vacca, "Neutrophil extracellular traps (NETs) and damage-associated molecular patterns (DAMPs): two potential targets for COVID-19 treatment," *Mediators of Inflammation*, vol. 2020, Article ID 7527953, 25 pages, 2020.

[60] J. J. Hu, X. Liu, S. Xia et al., "FDA-approved disulfiram inhibits pyroptosis by blocking gasdermin D pore formation," *Nature Immunology*, vol. 21, no. 7, pp. 736–745, 2020.

[61] C. C. Yost, H. Schwertz, M. J. Cody et al., "Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation," *The Journal of Clinical Investigation*, vol. 126, no. 10, pp. 3783–3798, 2016.

[62] T. Tagami, R. Tosa, M. Omura et al., "Effect of a selective neutrophil elastase inhibitor on mortality and ventilator-free days in patients with increased extravascular lung water: a post hoc analysis of the PiCCO Pulmonary Edema Study," *Journal of Intensive Care*, vol. 2, no. 1, p. 67, 2014.