DOI: 10.1002/JLB.4COVCRA0820-481RRR

JLB JOURNAL OF LEUKOCYTE BIOLOGY

ARTICLE

Neutrophils mediate Th17 promotion in COVID-19 patients

Zuzana Parackova[1] | Marketa Bloomfield[1,2] | Adam Klocperk[1] | Anna Sediva[1]

[1]Department of Immunology, 2nd Faculty of Medicine Charles University, V Uvalu, University Hospital in Motol, Prague, Czech Republic

[2]Department of Pediatrics, Thomayer's Hospital and First Faculty of Medicine, Charles University, Prague, Czech Republic

Correspondence
Zuzana Paračková, Department of Immunology, 2nd Faculty of Medicine Charles University, Faculty Hospital in Motol, V Uvalu 84, Prague 5, 15006, Czech Republic.
Email: zuzana.parackova@fnmotol.cz

Abstract

From the beginning of 2020, an urgent need to understand the pathophysiology of SARS-CoV-2 disease (COVID-19), much of which is due to dysbalanced immune responses, resonates across the world. COVID-19-associated neutrophilia, increased neutrophil-to-lymphocyte ratio, aberrant neutrophil activation, and infiltration of neutrophils into lungs suggest that neutrophils are important players in the disease immunopathology. The main objective of this study was to assess the phenotypic and functional characteristics of neutrophils in COVID-19 patients, with particular focus on the interaction between neutrophils and T cells. We hypothesize that the altered functional characteristics of COVID-19 patient-derived neutrophils result in skewed Th1/Th17 adaptive immune response, thus contributing to disease pathology. The expansion of G-MDSC and immature forms of neutrophils was shown in the COVID-19 patients. In the COVID-19 neutrophil/T cell cocultures, neutrophils caused a strong polarity shift toward Th17, and, conversely, a reduction of IFNγ-producing Th1 cells. The Th17 promotion was NOS dependent. Neutrophils, the known modulators of adaptive immunity, skew the polarization of T cells toward the Th17 promotion and Th1 suppression in COVID-19 patients, contributing to the discoordinated orchestration of immune response against SARS-CoV-2. As IL-17 and other Th17-related cytokines have previously been shown to correlate with the disease severity, we suggest that targeting neutrophils and/or Th17 represents a potentially beneficial therapeutic strategy for severe COVID-19 patients.

KEYWORDS
COVID-19, SARS-CoV-2, neutrophils, Th17, IL-17, G-MDSC, immature neutrophils

1 | INTRODUCTION

The current global research efforts concerning the newly emerged human coronavirus SARS-CoV-2 are harvesting data on various aspects of the host–pathogen interaction at a historically unprecedented pace. Disbalanced immune responses to the virus have been documented to underlie much of the "COVID-19" pathophysiology, although the specific mechanisms are not yet fully understood. Initially, the attention was focused mainly on the role of CD4+ and CD8+ T cells and NKs, followed by the phenomenon of uncontrolled hypercytokineamia.[1–3] Most recently, both the Th17 lymphocytes and neutrophils have been implicated in the immune-related damage associated with COVID-19.[4] In fact, the clinical severity of the disease was shown to correlate with excessive serum levels of IL-17 and other cytokines involved in Th17 promotion and maintenance,[2,5,6] similar to infections with other coronaviruses, for example, MERS-CoV and SARS-CoV.[7] Neutrophils, particularly their immature forms with strong proinflammatory properties, have been shown to be expanded in SARS-CoV-2,[4] exhibiting augmented production of various inflammatory cytokines, enhanced NETosis[8–10] and infiltrating lungs. As a distinct cross-talk between Th17 and neutrophils exists,[11] we set out to analyze the nature of their interaction in SARS-CoV-2.

Abbreviations: Arg-1, arginase-1; COVID-19, coronavirus disease 2019; GCN2, general control nondepressible 2; G-MDSC, granulocytic myeloid-derived suppressor cells; HD, healthy donor; IFNγ, interferon γ; IL-17, interleukine 17; MERS, Middle East respiratory syndrome coronavirus; mTOR, the mammalian target of rapamycin; NET, neutrophil extracellular traps; Neu, neutrophil; NO, nitric oxide; NOS, NO synthase; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SARS-CoV-2, Severe acute respiratory syndrome virus 2; Th17, T helper lymphocytes 17.

2 | RESULTS AND DISCUSSION

In our previous study, we showed that peripheral neutrophils of severe SARS-CoV-2 patients (CoV2-Neu) are expanded, display marked hyperresponsiveness, as well as enhanced degranulation and

Received: 3 August 2020 | Revised: 4 November 2020 | Accepted: 7 November 2020

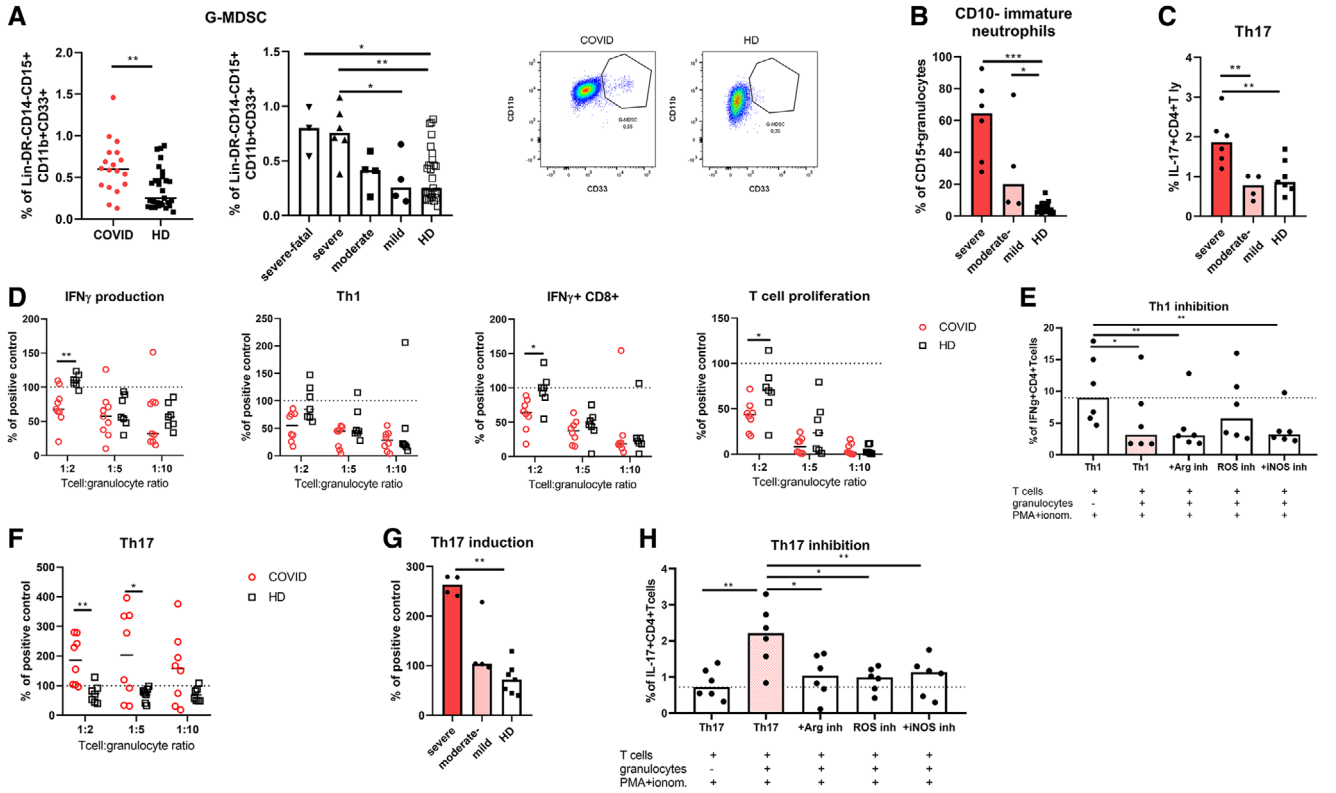

FIGURE 1 **T cell polarization by neutrophils.** (**A**) Increased frequencies of G-MDSC (CD3-CD19-CD20-CD56-HLA-DR+CD14-CD15+CD11b+CD33+cells) in 18 COVID-19 patients detected in whole blood and compared to 29 healthy donors (HDs) by flow cytometry. (**B**) Increased percentage of CD10- immature neutrophils (CD15+CD66b+CD10-) in 6 COVID-19 patients with severe symptoms and 4 COVID-19 patients with moderate or mild symptoms detected in the whole blood and compared with 29 HDs detected by flow cytometry. (**C**) Augmented Th17 (IL-17+CD4+CD3+ T cells upon PMA and ionomycin stimulation) in COVID-19 patients ($n = 6$ with severe and $n = 4$ with moderate or mild symptoms) and compared with 7 HDs detected by flow cytometry. (**D**) Percentage of IFNγ+CD3+, Th1 (IFNγ+CD4+) and IFNγ+CD8+ T cells detected by flow cytometry after overnight autologous cocultivation of isolated CD3+ T cells with neutrophils at various ratios. Proliferating Ki67+CD4+ were detected by flow cytometry after 5 days of cocultivation in DynaBeads presence. (**E**) Effect of Arg-1 (5 μg/ml), NOS (5 μg/ml), and ROS (superoxide dismutase 400 U/ml and catalase 2000 U/ml) inhibitors on Th1 frequencies in Tcell/neutrophils cocultures (ratio 1:2) detected by flow cytometry after overnight incubation. (**F**) Th17 (IL-17+CD4+) frequencies after overnight cocultivation of CD3+ T cells with neutrophils at various ratios detected by flow cytometry in COVID-19 ($n = 8$) and HD ($n = 7$). (**G**) Induced Th17 frequencies were diversified according to the severity of the disease (patients with severe symptoms $n = 4$, moderate and mild symptoms $n = 3$). (**H**) Th17 frequencies in Tcell/neutrophils cocultures (ratio 1:2) in the presence of inhibitors of Arg-1 (5 μg/ml), NOS (5 μg/ml), and ROS (superoxide dismutase 400 U/ml and catalase 2000 U/ml) were detected by flow cytometry after overnight incubation. Statistical analysis was performed using the Wilcoxon paired or Mann–Whitney unpaired *t*-test. Values of $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) were considered statistically significant

proinflammatory cytokine production.[4] Here, we analyzed the composition of SARS-CoV-2 neutrophils (CoV2-Neu) pool in detail and found it to be enriched with granulocyte myeloid-derived suppressor cells (G-MDSCs, defined as HLA-DR+CD14-CD15+CD11b+CD33+) (Fig. 1A and Supplementary Fig. S1) and immature forms of CD10- neutrophils (Fig. 1B). Both of these cell subpopulations possess immunomodulatory properties. The expansion of both G-MDSCs and immature neutrophils was more pronounced in the severe COVID-19 cases than in healthy donors (HDs). Also, we determined the overall peripheral blood frequencies of Th17 cells (CD4+ T cells producing IL-17A) (Fig. 1C and Supplementary Figs. S2 and S3) and found them to be expanded in patients with severe COVID-19.

Neutrophils have been shown to exert versatile functions in adaptive immune responses, therefore we explored the role of CoV2-Neu and their products in T cell induction in autologous cocultures. First, we examined IFNγ production by COVID-19 T cells (CoV2-T) in the absence or presence of neutrophils. The presence of CoV2-Neu significantly reduced the production of IFNγ by autologous CD3+ lymphocytes, compared with the HDs neutrophils (Fig. 1D). This reduction was more profound in CD8+CD3+ than in CD4+CD3+ T lymphocytes (Fig. 1D) and was markedly stronger in cocultures with patient-derived neutrophils compared with the HDs. CoV2-Neu also diminished the CoV2-T cell proliferation rate (Fig. 1D). Thus, we demonstrate that the CoV2-Neu exert a suppressive effect on IFNγ+ T lymphocytes. Performing the same experiments in the presence of neutrophil inhibitory compounds, we found that arginase-1 inhibitor and NOS inhibitor failed to rescue the IFNγ production (Fig. 1E). The inhibition of reactive oxygen species (ROS) induction led to partial restoration of IFNγ production, however the effect was statistically insignificant.

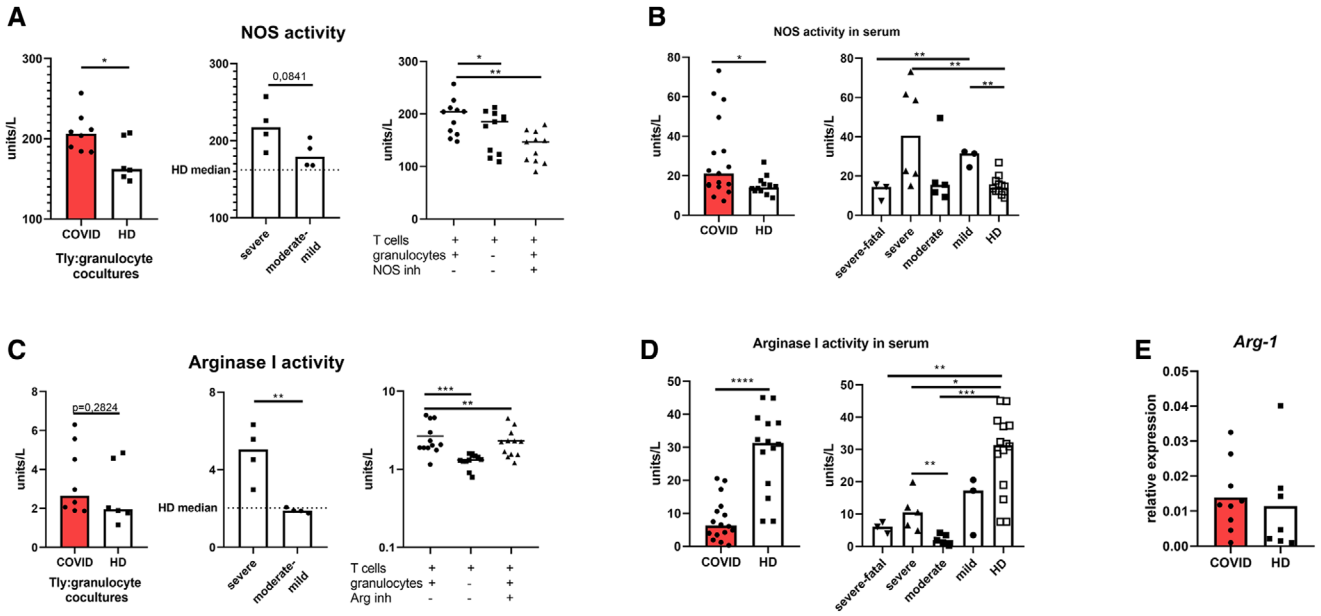

FIGURE 2 **Mechanisms of Th17 polarization**. (**A**) Elevated NOS activity in COVID-19 patients ($n = 8$) determined in T cell/neutrophils cocultures in 1:2 ratio after overnight cocultivation and compared with 6 healthy donors (HDs). Patients were diversified according to the severity of the disease. NOS activity was also analyzed in cultures with or without neutrophils and in presence of NOS inhibitor (5 μg/ml). (**B**) Increased NOS activity determined in COVID-19 patients ($n = 17$) serum and compared with HD ($n = 12$). Patients were diversified according to the severity of the disease. (**C**) Arginase-1 (Arg-1) activity analyzed in T cell/neutrophils cocultures in 1:2 ratio after overnight cocultivation in COVID-19 patients ($n = 8$) and 6 HDs. Patients were diversified according to the severity of the disease. Arg-1 activity was also investigated in cultures with or without neutrophils and in presence of Arg-1 inhibitor (5 μg/ml). (**D**) Diminished Arg-1 activity determined in COVID-19 patients ($n = 16$) serum and compared to HD ($n = 14$). Patients were diversified according to the severity of the disease. (**E**) *Arg-I* expression in 9 COVID-19 and 7 HD neutrophils analyzed by RT-PCR. The expression was normalized to *GADPH*. Statistical analysis was performed using the Wilcoxon paired or Mann–Whitney unpaired *t*-test. Values of $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) were considered statistically significant

Next, we demonstrated a strong polarity shift toward Th17 in the patients' CoV2-T cell-granulocyte autologous cocultures, compared with the HDs (Fig. 1F and Supplementary Fig. S1A). The induction of Th17 cells was stronger with CoV2-Neu from severe COVID-19 patients compared with the CoV2-Neu from mild cases (Fig. 1G). To elucidate the possible underlying mechanism of the preferential Th17 promotion, we added inhibitors of arginase-1 (Arg-1), NOS, and ROS into the cell cultures. Arg-1 is a T cell immunosuppressive enzyme that converts L-arginine to L-ornithine and NOS is another prototypic pleiotropic enzyme with suppressive effect on T cells. Arg-1, NOS, and ROS-dependant induction of Th17 has been demonstrated in several immunopathologies, even though the exact mechanisms are elusive. In systemic lupus erythematosus, elevated Arg activity was shown and Arg-mediated up-regulation of mTOR and GCN2 kinase (general control nondepressible 2), the signaling pathways involved in Th17 induction, was suggested.[12] NO enhances the in vitro cytokine-driven Th17 induction and, in cancer patients, NO signaling is required for Th17 stability.[13] Mitochondrial ROS might induce Th17 through TGFβ production.[14,15] In our experiments, we observed that each Arg-1, NOS, and ROS inhibitor was able to independently reduce Th17 counts (Fig. 1H and Supplementary Fig. S1A). We then established that the activity of NOS was significantly elevated in CoV2-T cell-granulocytes cocultures compared with the HDs, and the NOS elevation positively correlated with the disease severity (Fig. 2A). Correspondingly, NOS activity was increased in patients' sera compared with the HDs (Fig. 2B); however, no correlation with the disease severity was detectable. Next, we analyzed the activity of Arg-1 in our experiment settings. COV2-Neu presence in the cell coculture increased Arg-1 activity in a positive correlation with the disease severity (Fig. 2C). Moreover, when Arg-1 inhibitor was added, Arg-1 activity diminished as expected (Fig. 2C) and the Th17 frequencies decreased correspondingly (Fig. 1E). Unexpectedly, Arg-1 activity was diminished in the patients' sera compared with the HDs (Fig. 2D). Although the Arg-1 activity in the supernatants and in sera varied, *Arg-1* gene expression was similar in patients' neutrophils and in HDs (Fig. 2E).

G-MDSC have been shown to promote Th17 differentiation via NOS and arginase-dependent mechanisms[12,13]; thus, the increased frequencies of both populations in SARS-CoV-2 are suggestive of their mutual interaction. While NOS activity is induced mainly by Th1 cytokines, Arg-1 is induced predominantly by Th2 cytokines.[16] Hypothetically, this dichotomous regulation may underlie the increased NOS activity and diminished Arg-1 activity in the sera of the SARS-CoV-2 patients, as viral infections are likely to induce predominantly Th1-biased environments.

IL-17A was demonstrated to augment the destruction of the lung parenchyma resulting in acute respiratory distress syndrome via the alteration of neutrophil recruitment, apoptosis, and functions. Conversely, IL-17 inhibition, operating upstream of IL-1 and IL-6, has been

successfully used in treatment of inflammatory autoimmune diseases, such as psoriasis and psoriatic arthritis (secukinumab, ixekizumab, brodalumab), likely as a result of reduced neutrophil recruitment.[5]

To our knowledge, this is the first study utilizing functional tests to elucidate the role of neutrophils in impaired T cell responses in COVID-19 and as such it provides background for future research. However, this study is not without limitations. The sample size is relatively small and not all patients were involved in all experiments due to the limited amount of blood available per sampling. The study cohorts were highly heterogeneous in age, comorbidities, and COVID-19-related risk factors. Moreover, due to the autologous experiment setting, it is not strictly definitive whether the observed Th17 promotion in COVID-19 patients was caused by the properties of patients' neutrophils or by altered functions of T cells.

In summary, we provide evidence that neutrophils promote the induction of Th17 in COVID-19 patients. As both cell populations are involved in the immune-mediated damage, we suggest that targeting either neutrophils or Th17, directly and/or via their products, may be therapeutically advantageous in COVID-19.

AUTHORSHIP

Z.P. designed the study, performed experiments, analyzed data, and wrote the manuscript. M.B. interpreted the results and wrote the manuscript. A.K. provided patient information and biologic material and reviewed the manuscript. A.S. reviewed and edited the manuscript.

ACKNOWLEDGMENT

The study was supported by the Czech Ministry of Health AZV NU20-05-00320 and by GAUK 954218.

DISCLOSURES

The authors declare no conflicts of interest.

ORCID

Zuzana Parackova https://orcid.org/0000-0002-2398-532X

REFERENCES

1. Cao X. COVID-19: immunopathology and its implications for therapy. *Nat Rev Immunol*. 2020;20:269-270.
2. Xu Z, Shi L, Wang Y, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. 2020;8:420-422.
3. Tan M, Liu Y, Zhou R, et al. Immunopathological characteristics of coronavirus disease 2019 cases in Guangzhou, China. *Immunology*. 2020:261-268.
4. Parackova Z, Zentsova I, Bloomfield M, et al. Disharmonic inflammatory signatures in COVID-19: augmented neutrophils' but impaired monocytes' and dendritic cells' responsiveness. *Cells*. 2020;9:1-19.
5. Pacha O, Sallman MA, Evans SE. COVID-19: a case for inhibiting IL-17? *Nat Rev Immunol*. 2020;20:345-346.
6. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet (London, England)*. 2020;395:497-506.
7. Mahallawi WH, Khabour OF, Zhang Q, Makhdoum HM, Suliman BA. MERS-CoV infection in humans is associated with a pro-inflammatory Th1 and Th17 cytokine profile. *Cytokine*. 2018;104:8-13.
8. Middleton EA, He X-Y, Denorme F, et al. Neutrophil extracellular traps (NETs) contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. *Blood*. 2020.
9. Liu J, Liu Y, Xiang P, et al. Neutrophil-to-lymphocyte ratio predicts severe illness patients with 2019 novel coronavirus in the early stage. *medRxiv*. 2020.
10. Fornasari PM. COVID-19: neutrophils "unfriendly fire" imbalance proteolytic cascades triggering clinical worsening and viral sepsis. *Potential Role Explanation for Convalescent Plasma as "Fire Hose"*. 2020.
11. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010;115:335-343.
12. Wu H, Zhen Y, Ma Z, et al. Arginase-1-dependent promotion of TH17 differentiation and disease progression by MDSCs in systemic lupus erythematosus. *Sci Transl Med*. 2016;8:331ra40.
13. Obermajer N, Wong JL, Edwards RP, et al. Induction and stability of human Th17 cells require endogenous NOS2 and cGMP-dependent NO signaling. *J Exp Med*. 2013;210:1433-1445.
14. Zhi L, Ustyugova IV, Chen X, Zhang Q, Wu MX. Enhanced Th17 differentiation and aggravated arthritis in IEX-1–deficient mice by mitochondrial reactive oxygen species-mediated signaling. *J Immunol*. 2012;189:1639-1647.
15. Zhang D, Jin W, Wu R, et al. High glucose intake exacerbates autoimmunity through reactive-oxygen-species-mediated TGF-β cytokine activation. *Immunity*. 2019;51:671-681. e5.
16. Munder M. Arginase: an emerging key player in the mammalian immune system: review. *Br J Pharmacol*. 2009;158:638-651.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Parackova Z, Bloomfield M, Klocperk A, Sediva A. Neutrophils mediate Th17 promotion in COVID-19 patients. *J Leukoc Biol*. 2021;109:73–76. https://doi.org/10.1002/JLB.4COVCRA0820-481RRR