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LETTER TO THE EDITOR

Neutrophils from patients with acquired neutropenia exhibit alterations in serine protease immunostaining and activity

The diversity of circulating neutrophils in the context of different forms of neutropenia remains poorly understood. In this study, we investigated potential alterations between neutrophils isolated from the blood of patients with chronic idiopathic neutropenia (CIN) and those from healthy donors, with a focus on neutrophil serine proteases (NSPs), namely neutrophil elastase (NE) and cathepsin G (CG). These enzymes have previously been implicated in neutrophil dysfunction and cell death.[1–4]

Among NSPs, NE is arguably the most extensively studied, as autosomal-dominant mutations in the NE-encoding gene (*ELANE*) account for the majority of cases of severe congenital neutropenia (SCN) and cyclic neutropenia (CN). Both conditions are characterized by defective granulopoiesis in the bone marrow (BM) and significantly lowered counts of mature neutrophils in the blood.[3]

Levels of either NE and its controlling inhibitor, secretory leucocyte protease inhibitor (SLPI) can change in neutropenia. Previous reports have documented severely decreased levels of NE and/or SLPI in myeloid cells and plasma of SCN patients, and to a lesser extent in individuals with CN.[5–7] However, in a more recent study, upregulated expression of NE in differentiating neutrophils was noted in association with neutropenia, possibly reflecting different mechanisms underlying discordant NE-mediated SCN pathogenicity.[8]

Given the profound alterations at the gene and/or transcription levels of NE and SLPI in neutropenia, these molecules, as well as other functionally related NSPs, are potential targets of diagnostic and therapeutic interest in this disorder. However, in contrast to hereditary SCN and CN, much less is known about the status of NSPs and SLPI in neutrophils in chronic neutropenia, caused by an acquired deficit of neutrophils.

We hypothesized that, similar to SCN, patients with acquired neutropenia, including CIN, might display not only lower neutrophil counts in the blood but also phenotypic and/or functional changes in circulating neutrophils, including altered ability of NSPs to process their protein targets.

To characterize NSP levels and/or activity in neutrophils in CIN, a total of 34 patients and 36 healthy donors (HD) from the University Hospital of Heraklion, Crete, Greece, were enrolled in the studies. The criteria for CIN characterization have been previously published.[9,10] CIN in adults is characterized by prolonged, unexplained neutropenia that does not meet the diagnostic criteria for any underlying disease, following thorough clinical and laboratory investigations. These investigations include negative anti-neutrophil antibody testing, inconclusive BM aspiration/biopsy and normal cytogenetics.[9] Immune and genetic descriptions of CIN are provided in the Supplementary Information, while the characteristics of CIN patients and healthy donors are presented in Table S1 and Figure S1. None of the CIN individuals was on G-CSF or immunosuppressive therapies. CIN is a benign type of neutropenia with the absence of recurrent severe infections, and no therapy is needed in these patients.

Flow cytometry revealed that CIN neutrophils exhibited significantly stronger staining for NE and CG, along with lower detection of SLPI, the inhibitor of both proteases, compared to HD neutrophils (Figure 1). The specificity of immunodetection of these antigens in circulating leucocytes by flow cytometry is demonstrated in Figure S2.

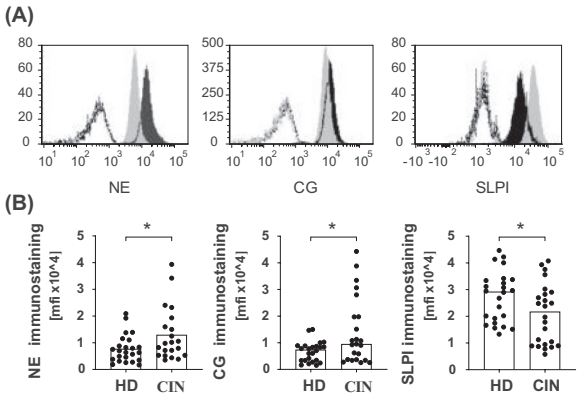

FIGURE 1 Neutrophils were isolated from the blood of the indicated donors, surface stained for CD16 followed by intracellular staining for neutrophil elastase (NE), cathepsin G (CG) or secretory leucocyte protease inhibitor (SLPI). (A) Representative flow cytometry histogram plots of chronic idiopathic neutropenia (CIN) donors (black) and healthy donors (HD) controls (grey) are shown. Open histograms indicate the respective staining controls. (B) Symbols indicate MFI values in individual HD and CIN donors and bars indicate the median value for each dataset. Statistically significant differences between HD and CIN participants are indicated by asterisk. *$p < 0.05$ by the Mann–Whitney test. Details of cell isolation and staining can be found in the Supplementary Information.

The activity of NE and CG to process specific synthetic substrates was also significantly increased in lysates from CIN neutrophils compared to HD (Figure 2). However, this increase in activity was not observed for other primary granule-stored enzymes, such as serine protease 3 (PR3), which is not regulated by SLPI, and myeloperoxidase (MPO) (Figure 2). Although these data suggest that increased NE and CG activity in CIN neutrophils may result from lower levels of their controlling inhibitor, association analysis revealed that these activities were not negatively correlated with SLPI immunostaining in CIN patients. However, in the HD group, NE activity was negatively correlated with SLPI immunostaining ($r = -0.7$, $p < 0.001$), indicating potentially reduced control of NE in CIN neutrophils (Figure S3).

While our findings align with the concept of circulating pathology-associated neutrophil diversity in neutropenia, they reveal distinct patterns of neutrophil alterations in CIN compared to SCN. In contrast to the pronounced downregulation or absence of NE, CG and MPO proteins observed in G-CSF-mobilized SCN neutrophils, as previously reported through immunofluorescence staining and Western blot analysis,[5] our findings indicate increased immunostaining and enhanced enzymatic activity of NE and CG in neutrophils from CIN patients compared to healthy donors. These observations not only suggest the acquisition of full serine-protease-dependent effector capabilities by neutrophils in CIN but also imply a potentially activated phenotype of these cells.

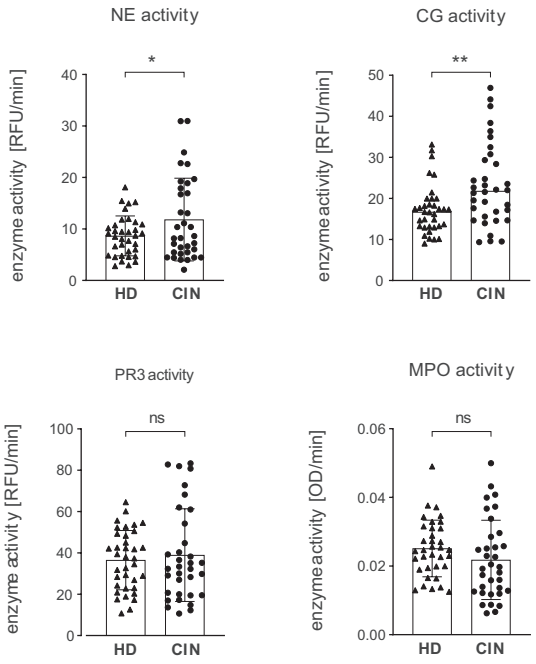

FIGURE 2 Neutrophils were isolated from the blood of indicated donors. The activity of the indicated enzymes was measured by fluorimetry, using the FRET substrates for NE, CG and PR3 or colorimetric substrate for MPO. Symbols indicate individual donors and bars indicate the mean (HNE, PR3, MPO) or median (CG) value for each dataset. $^{*}p < 0.05$, $^{**}p < 0.01$ by unpaired *t*-test with Welch's correction or the Mann–Whitney test. ns, not significant; CG, cathepsin G; MPO, myeloperoxidase; NE, neutrophil elastase; OD, optical density; RFU, relative fluorescence units. Methodological details can be found in the Supplementary Information.

Despite the fact that NE, CG, PR3 and MPO are localized in the same type of granules in neutrophils and often collaborate in their physiologic function,[11] only NE and CG were found to be altered in CIN neutrophils in terms of immunostaining and proteolytic activity, suggesting a link between these changes and defects in neutrophil survival.

In the context of neutropenia, it is notable that both NE and CG have been implicated in neutrophil death programmes, albeit at different stages of neutrophil maturation and/or based on different mechanisms. SCN-causative NE variants induce the death of neutrophil precursors due to a maturation block at the promyelocyte stage of neutrophil development by evoking unfolded protein responses.[3] These types of NE mutations can be accompanied by a reduction of NE levels and/or the generation of proteolytically defective NE in developing neutrophils, but whether either altered levels of this enzyme or its proteolytic activity have a role in neutrophil precursor cell death is less clear.[12]

Alternatively, NE-misfolding mutations linked not to a reduction, but upregulation of NE transcript and protein levels, can affect neutrophil production in SCN in association with or in response to acute oxidative stress.[8] It is likely that upregulated NE levels in these SCN patients may also result in elevated NE proteolytic activity unless NE capacity to cleave its targets is ameliorated by specific anti-proteases. Therefore, the overall outcome involving NE in dysfunctional neutrophils in CIN and some SCN patients might be similar.

CG can drive apoptosis in neutrophils or their precursors.[1,4] Additionally, CG has been implicated in other forms of neutrophil cell death, such as programmed necrosis with cell lysis and the release of proinflammatory cytokines,[2] which could be relevant to acquired forms of neutropenia often accompanied by chronic inflammatory conditions.[9]

The involvement of CG in inducing neutrophil cell death was observed when the protease relocated from primary granules into the cytosol and was not inhibited by cytosolic inhibitors, due to their genetic deficiency.[1] Similar phenomenon of unbalanced and/or mislocalized proteolytic activity of CG may account for modified functional features of neutrophils in CIN.

Although the exact involvement of NSPs and their inhibitors in different forms of neutropenia requires further mechanistic studies, our findings suggest that changes in NE and CG immunostaining and activity in neutrophils may serve as indicators or contributing factors to the development of CIN.

AUTHOR CONTRIBUTIONS

Conceptualization: JC and JSM; *Formal analysis*: AM, JSM and IM; *Funding acquisition*: JC and HAP; *Investigation*: AM, JSM, IM and CMP; *Methodology*: JSM and IM; *Resources*: IM, BK and HAP; *Supervision*: JC, JSM and HAP; *Writing—original draft*: JC and JSM; *Writing—review and editing*: AM, JSM, IM, CMP, BK, HAP and JC.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

All procedures were performed according to the ethical standards of the institutional and national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Bioethics Committee of the University of Crete (#158/04-11-2022).

PATIENT CONSENT STATEMENT

Human blood samples were collected from fully informed and consented individuals.

Angelika Mazur[1]
Joanna Skrzeczynska-Moncznik[1]
Irene Mavroudi[2,3]
Christina Maria Perraki[2,3]
Brice Korkmaz[4,5]
Helen A. Papadaki[2,3]
Joanna Cichy[1]

[1]*Department of Immunology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland*
[2]*Hemopoiesis Research Laboratory, School of Medicine, University of Crete, Heraklion, Greece*
[3]*Department of Hematology, University Hospital of Heraklion, Heraklion, Greece*
[4]*INSERM, Respiratory Disease Research Centre, U1100, Tours, France*
[5]*University of Tours, Tours, France*

Correspondence
Joanna Cichy, Department of Immunology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 30-387 Krakow, Poland.
Email: joanna.cichy@uj.edu.pl

ORCID

Helen A. Papadaki https://orcid.org/0000-0002-5146-9699
Joanna Cichy https://orcid.org/0000-0002-0552-8344

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.