RAPID COMMUNICATION

Spontaneous NETosis and type I IFN signaling activation in resting neutrophils of chronic granulomatous disease patients with *CYBB* mutations

Mutations in *CYBB*, encoding gp91phox subunit of NADPH oxidase in phagocytes, impair the respiratory burst of neutrophils and result in X-linked chronic granulomatous disease (CGD). While inflammatory response and NETosis are important modalities employed by neutrophils for pathogen clearance, variants in these cell functions in CGD neutrophils (CGD-PMN) could possibly explain the insufficient defense and accumulation of phagocytes in the sites of infection. To decipher the intrinsic features of CGD-PMN, neutrophils from X-linked CGD patients with *CYBB* mutations and age-matched healthy donors (HD-PMN) were compared. Our study found an enhanced spontaneous neutrophil extracellular trap (NET) formation with histone hypercitrullination in resting CGD-PMN. RNA sequencing (RNA-seq) analysis and qPCR validation further revealed a prominent activated type I interferon (IFN) gene signature. This suggested that in CGD patients an inefficient clearance of pathogen caused a chronic stimulation to provoke a spontaneous type I IFN-mediated neutrophil activation and NET formation which were never seen in resting neutrophils of healthy controls.

To understand the effects of the genetic mutation on enzyme expression and neutrophil function in CGD, ten unrelated patients with *CYBB* mutations were enrolled, and the clinical information and laboratory genetic analysis were collected (Table S1). In this study, due to the rarity and severity of the disease, the limitations of clinical practice prevented us from collecting enough blood samples from one patient for all experimental purposes. Thus, samples used for RNA-seq and protein expression detection did not come from the same patients. As one of the main subunits of flavocytochrome b$_{558}$ which is the transmembrane component of NADPH oxidase, gp91phox was detected at mRNA and protein levels in HD-PMN but not in CGD-PMN (Fig. 1A–D). As a validation in other phagocytes, missing of gp91phox expression was also observed in monocytes (Fig. S1). As expected, CGD-PMN without gp91phox exhibited poor ROS production upon PMA stimulation (Fig. 1E, F), which was in accordance with CGD patients previously reported.[1]

To our surprise, spontaneous NETosis was found in resting CGD-PMN but not HD-PMN (Fig. 1G). Immunofluorescence staining revealed that histone-hypercitrullinated NETs characteristic of polymorphonuclear appearance with intact nuclear membranes. By contrast, upon PMA stimulation, both CGD-PMN and HD-PMN initialized strong NETosis, which appeared to rupture and release long stretches of extensively decondensed chromatin into the extracellular space, forming web-like chromatin structures. It has been known that the generation of ROS is a prerequisite for NETosis. Now that patients have a defect ROS production due to *CYBB* mutation, a spontaneous NET formation must have been induced through other ROS resources. Accumulating evidence revealed that mitochondrion is another major site of ROS generation, and mitochondrial ROS synthesis could be sufficient to generate NETs in systemic lupus erythematosus and CGD.[2] Indeed, through MitoSOX Red staining, a specific dye for mitochondrial ROS, we proved that mitochondrial superoxide production was higher in CGD-PMN (Fig. 1I). Different from typical suicidal NETosis, our confocal microscope further revealed that the spontaneous NETs in CGD-PMN were phenotypically prone to vital pathway in which integrity of cell membrane was preserved to allow the release of NETs via vesicular exportation. Immunofluorescence results using JC-1 indicated that CGD-PMN had a decreased mitochondrial membrane potential (Fig. 1H). The NET formation was abrogated after treating

Abbreviations

CGD	chronic granulomatous disease
HD	healthy donors
NADPH	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
SLE	systemic lupus erythematosus
CYBB	cytochrome b_{558} beta chain
NET	Neutrophil extracellular trap
IFN	interferon
ISGs	interferon-stimulated genes
HBSS	Hank's Balanced Salt solution
PMA	Phorbol 12-myristate 13-acetate
citH3	citrullinated histone H3
DAPI	4',6-diamidino-2-phenylindole
DEGs	differentially expressed genes
GO	Gene Ontology
FPKM	fragments per kilobase of exon model per million mapped fragments
PCA	principal component analysis
BP	biological process
CC	cellular component
MF	molecular function
GSEA	gene set enrichment analysis
OAS1	2′-5′-Oligoadenylate Synthetase 1

OAS2	2′-5′-Oligoadenylate Synthetase 2
OAS3	2′-5′-Oligoadenylate Synthetase 3
OASL	2′-5′-Oligoadenylate Synthetase Like
IFI6	interferon alpha inducible protein 6
IFI27	interferon alpha inducible protein 27
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFIT3	interferon-induced protein with tetratricopeptide repeats 3
IFITM3	interferon-induced transmembrane protein 3
IFI44	interferon-induced protein 44
IFI44L	interferon-induced protein 44 like
MX1	MX dynamin like GTPase 1
MX2	MX dynamin like GTPase 2
ISG15	ISG15 ubiquitin like modifier
ISG20	ISG20 ubiquitin like modifier
RSAD2	radical S-adenosyl methionine domain containing 2
BST2	bone marrow stromal cell antigen 2
STAT1	signal transducer and activator of transcription 1
STAT2	signal transducer and activator of transcription 2
IRF7	interferon regulatory factor 7

CGD-PMN with MitoTEMPO, a mitochondria-specific superoxide scavenger, suggesting that spontaneous NETosis in CGD-PMN relied on mitochondrial ROS production (Fig. 1G). The dominant mitochondrial ROS production-induced vital NETosis in CGD-PMN might account for delayed pathogen clearance and accumulated neutrophils in the inflammatory site leading to tissue granulomatosis.[3] In sum, our study demonstrated the dependence of mitochondrial ROS synthesis and its effects on inducing spontaneous NETs in the absence of NADPH oxidase activity in CGD patients.

To explore if there are differences between HD-PMN and CGD-PMN at the molecular level, HD-PMN ($n = 6$) and CGD-PMN ($n = 3$) were isolated and subjected to high-throughput RNA-seq analysis. We found that the general gene expression distribution of the total fragments per kilobase of exon model per million mapped fragments (FPKM) values showed similarity, whereas the individual FPKM values were different amongst the 6 HD-PMN and 3 CGD-PMN (Fig. S2A). As expected, the indicated 6 HD-PMN or 3 CGD-PMN respectively manifested closer clustering characteristics in gene expression pattern as confirmed by the heatmap diagram (Fig. S2B) and principal component analysis (Fig. S2C). The distinguishable landscape of gene expression pattern between CGD-PMN and HD-PMN revealed a total number of 433 up-regulated and 280 down-regulated genes ($P < 0.05$, $|\log_2 FC| \geqq 1$) (Fig. 1J). Gene Ontology (GO) analysis for biological process (BP) using the screened differentially expressed genes (DEGs) revealed that immunologically relevant signaling pathways, including defense response to the virus and response to IFN, were enriched in CGD-PMN (Fig. 1K; Fig. S2D). In accordance with GO analysis, gene set enrichment analysis (GSEA) also indicated that pathways including defense response to the virus (GO: 0051607) and type I IFN signaling

pathway (GO: 0060337) were collectively enriched in the CGD-PMN (Fig. 1L). The expression levels of DEGs involved in these two pathways were illustrated in hierarchical clustering heatmaps (Fig. S2E). Genes of enhanced expression in both virus defense and type I IFN pathways include OAS1, OAS2, OAS3, OASL, IFI6, IFI27, IFITM3, IFI44L, MX1, MX2, ISG20, STAT1, STAT2, RSAD2, ISG15, and BST2. Most of them are active participants in IFN signaling and inflammatory responses, which agrees with the pro-inflammatory manifestation in CGD patients. The up-regulation of the above interferon-stimulated genes (ISGs) in CGD-PMN was validated by qPCR and reassured our belief that an active type I IFN gene signature existed in resting CGD-PMN (Fig. 1M; Table S2). Active type I IFN signaling is responsible for the recruitment of neutrophils to sites of infection, regulation of neutrophil function, and immunopathogenesis.[4] In fact, CGD was previously identified as a type I IFN autoimmune disease manifested by up-regulated ISGs in peripheral blood after stimulation.[5] In our study, resting CGD-PMN was shown to have increased ISG expression which suggested a pro-inflammatory status presented as a consequence of chronic stimulation due to insufficient phagocyte ROS production. In addition, elevated mitochondria-derived ROS in resting CGD-PMN could be a cause or a consequence of ISG up-regulation. This observation agreed with the previous report that a mitochondrial ROS-dependent NETosis of low-density granulocytes from individuals with CGD can promote externalization of pro-inflammatory oxidized mtDNA and subsequent activation of STING and/or TLR-dependent type I IFN synthesis.[2] Our study proved an elevated mitochondrial ROS production accompanied by a type I IFN signaling activation in resting neutrophils in CGD patients in the absence of NADPH oxidase activity. However, the key drivers of spontaneous
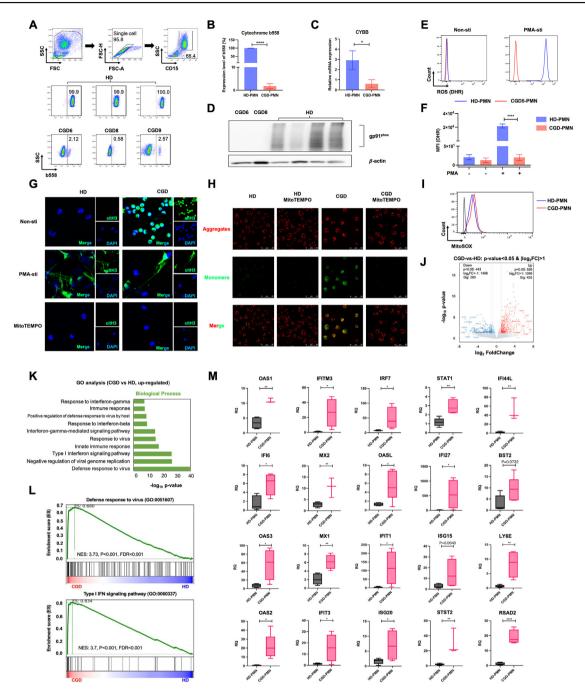
Figure 1 Functional analysis and gene expression profile of CGD-PMN. **(A, B)** Flow cytometry dot plots and positive proportions of cytochrome b$_{558}$ expression in neutrophils (CD15) from CGD patients and healthy donors. **(C)** Relative mRNA level of *CYBB* expression was detected by qPCR with GAPDH as the endogenous control. **(D)** gp91^{phox} expression in CGD-PMN and HD-PMN was detected by Western blot. **(E)** FACS histograms for ROS generation in CGD5-PMN and HD-PMN before and after stimulation with PMA (100 nM). **(F)** Mean fluorescence intensity (MFI) values of DHR123 were shown by bar diagrams. **(G)** Confocal images of NET formation in non-stimulated and PMA-stimulated neutrophils, and non-stimulated neutrophils with 100 μM MitoTEMPO. Citrullinated histone H3 (citH3) was in green and DAPI was in blue. Original magnification = 63 ×. **(H)** HD-PMN and CGD-PMN were seeded into 35-mm glass-bottom dished and stained with JC-1 (JC-1 aggregates: red; JC-1 monomers: green). Scale bars = 100 μm. **(I)** The histograms showing MitoSOX Red-based flow cytometric detection of mitochondrial ROS production. **(J)** The volcano plot of differential gene expression between CGD-PMN and HD-PMN. **(K)** GO analysis for the potential pathways of up-regulated DEGs. **(L)** Gene set enrichment analysis for the potential function of all DEGs. **(M)** qPCR results for type I IFN-related gene expression in CGD-PMN, normalized to GAPDH expression. The results for all measurements were displayed as mean ± SEM. *P* value > 0.05 indicated no statistically significant difference (ns) (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

NETosis and the role of type I IFNs underlying the NETotic phenotype of patient cells require further exploration.

The main limitation of the current study is that there are few CGD patients for whom enough neutrophils are available for research purposes, and cell number from one patient is limited, which restricted us from applying them to all the experiments (flowcytometry, immunofluorescence, Western blot, RNA-seq, qPCR, *etc*.). Besides, we cannot collect enough CGD samples to refine our data. As different patient samples were obtained at various disease stages, it is hard to know how the chronic stimulation time and the pathogen the patient encountered shape the neutrophil function.

In summary, distinguished from HD-PMN, impaired functional gp91^{phox} expression and extremely poor ROS production, but enhanced spontaneous NET formation with histone hypercitrullination in resting CGD-PMN were observed in our study. Sustained type I IFN signaling was associated with resting CGD-PMN and could contribute to the pro-inflammatory manifestation in CGD patients. However, whether these features resting CGD-PMN possessed have anything to do with inefficient pathogen clearance remains to be further explored experimentally.

Conflict of interests

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.101118.

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Zhengjing Lu [a], Wenjun Mou [a], Jianxin He [b], Xuedong Ge [c], Xiaolin Wang [a,**], Jingang Gui [a,*]

[a] *Laboratory of Tumor Immunology, Key Laboratory of Major Diseases in Children, Ministry of Education, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing 100045, China*
[b] *Department of Respiratory Medicine, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, China National Clinical Research Center for Respiratory Diseases, Beijing 100045, China*
[c] *Information Center, PLA Rocket Force Characteristic Medical Center, Beijing 100088, China*

*Corresponding author.

**Corresponding author.
E-mail addresses: wangxl19891012@163.com (X. Wang), guijingang@bch.com.cn (J. Gui)
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