Myeloperoxidase Modulates Inflammation in Generalized Pustular Psoriasis and Additional Rare Pustular Skin Diseases

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Generalized pustular psoriasis (GPP) is a severe multi-systemic inflammatory disease characterized by neutrophilic pustulosis and triggered by pro-inflammatory IL-36 cytokines in skin. While 19%–41% of affected individuals harbor bi-allelic mutations in *IL36RN*, the genetic cause is not known in most cases. To identify and characterize new pathways involved in the pathogenesis of GPP, we performed whole-exome sequencing in 31 individuals with GPP and demonstrated effects of mutations in *MPO* encoding the neutrophilic enzyme myeloperoxidase (MPO). We discovered eight *MPO* mutations resulting in MPO -deficiency in neutrophils and monocytes. *MPO* mutations, primarily those resulting in complete MPO deficiency, cumulatively associated with GPP ($p = 1.85E{-}08$; OR = 6.47). The number of mutant *MPO* alleles significantly differed between 82 affected individuals and >4,900 control subjects ($p = 1.04E{-}09$); this effect was stronger when including *IL36RN* mutations ($1.48E{-}13$) and correlated with a younger age of onset ($p = 0.0018$). The activity of four proteases, previously implicated as activating enzymes of IL-36 precursors, correlated with MPO deficiency. Phorbol-myristate-acetate-induced formation of neutrophil extracellular traps (NETs) was reduced in affected cells ($p = 0.015$), and phagocytosis assays in MPO-deficient mice and human cells revealed altered neutrophil function and impaired clearance of neutrophils by monocytes (efferocytosis) allowing prolonged neutrophil persistence in inflammatory skin. *MPO* mutations contribute significantly to GPP's pathogenesis. We implicate MPO as an inflammatory modulator in humans that regulates protease activity and NET formation and modifies efferocytosis. Our findings indicate possible implications for the application of MPO inhibitors in cardiovascular diseases. MPO and affected pathways represent attractive targets for inducing resolution of inflammation in neutrophil-mediated skin diseases.

Introduction

Neutrophil function can be affected genetically, by mutations in key genes involved in neutrophil function, or acquired, e.g., due to certain drugs.[1] Generalized pustular psoriasis (GPP [MIM: 614204]) is a genetic condition characterized by altered neutrophil function leading to sterile neutrophil pustules. GPP can manifest in acute, sometimes life-threatening flares of multi-systemic inflammation or as a more constant, progressive form of disease. It has been proposed to belong to a group of entities named auto-inflammatory keratinization diseases.[2] *IL36RN* (GenBank: NM_173170.1), encoding the antagonist of the IL-36 receptor (IL-36R), has been described as a susceptibility gene[3,4] (*IL36RN* [MIM: 605507]) with bi-allelic mutations identified in 19%–41% of affected individuals.[5,6] An impaired IL-36R-antagonist (IL-36Ra) leads to activated IL-36R that induces MAPK and NF-κB in keratinocytes and results in production of pro-inflammatory cytokines, thereby triggering skin inflammation.[3] Bi-allelic *IL36RN*

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mutations have been described in persons affected by GPP with a widely varying age of onset: 0–52 years in individuals homozygous for the most common European mutation, c.338C>T (p.Ser113Leu),[7] or in single control individuals, suggesting incomplete penetrance.[8] Frequency differences of individuals with a single *IL36RN* mutation between affected individuals and control subjects also suggest a functional role of those *IL36RN* mutations in a more complex inheritance model.[7]

Affected individuals with GPP (n = 7) were treated with an IL-36R antibody and showed marked reduction in disease activity.[9] This was the case even in individuals without an *IL36RN* mutations, suggesting that the IL-36 pathway in general is relevant in GPP. IL-36 cytokines are expressed as inactive precursors and require proteolytic processing for activation by neutrophil proteases (e.g., cathepsin G [CTSG], elastase [NE], and proteinase 3 [PR3]) and monocytic protease cathepsin S (CTSS).[10,11] The proteolytic process has been shown to be protease and substrate specific, e.g., NE activates IL-36β, but to a very limited extent IL-36α and IL-36γ.[10]

As the majority of individuals affected by GPP do not have mutations in *IL36RN* or any other known susceptibility gene,[7] we aimed to use whole-exome sequencing (WES) to identify associated genes. We also included two further neutrophilic pustulosis forms—acute generalized exanthematous pustulosis (AGEP) and acrodermatitis continua suppurativa Hallopeau (ACH)—due to the known clinical and genetic overlap with GPP (AGEP, ACH, GPP [MIM: 614204]).[12,13]

Material and Methods

Group of Affected Individuals

The study group comprised 74 individuals affected by GPP, two by AGEP, and six by ACH, the majority of whom were described previously as was the recruitment strategy.[7] All GPP- and ACH-affected individuals fulfilled the ERASPEN consensus criteria,[14] while for the AGEP-affected individuals, we validated their diagnoses according to the scoring system[15] used by RegiSCAR study groups and modified by Paulmann and Mockenhaupt.[16] Origin and clinical characteristics of individuals are presented in Tables S1, S2, and S3 and Supplemental Note. The study was approved by the ethical committee of the Friedrich-Alexander-Universität of Erlangen-Nürnberg. Written informed consent was obtained from each affected and control individual before enrolment. We conducted investigations according to Declaration of Helsinki principles.

Mpo$^{-/-}$ Mice

We maintained 8- to 10-week-old female and male wild-type mice (C57BL/6J) and *Mpo*$^{-/-}$ mice (B6.129X1-Mpotm1Lus/J) in a SPF facility with identical housing conditions controlling for the same environment and temperature at a 12 h light/dark cycle, with free access to water and regular rodent chow. The study was approved by the ethics committees of the veterinary office of Erlangen (Germany) and performed according to guidelines of laboratory animal care and use. Mice were obtained from Jackson Laboratory.

We isolated murine peripheral monocytes and neutrophils from heparinized blood using EasySep Mouse Neutrophil Enrichment Kit (Stemcell) and EasySep Mouse Monocyte Isolation Kit (Stemcell) according to the manufacturer's protocol. We used 1–2E05 monocytes and 5–10E05 neutrophils from 5–6 animals per strain per one efferocytosis assay.

WES and Targeted Sequencing of *MPO*

We performed WES of 31 singletons with GPP who did not harbor *IL36RN* mutations as described before.[5] We obtained an average coverage of 142.3 ± 31.3 (standard deviation; median: 131.7) and an average percentage of the target covered 20× of 87.4% ± 10.7% (median: 91.3%). We selected variants with a coverage of ≥20× and identified ≤2× in a group of 706 independent individuals sequenced in-house for projects involving non-immunological diseases.

Our analysis focused on variants affecting the same genes. As mutations in *IL36RN* have been identified on both alleles, we selected for genes with homozygous variants in at least two individuals. We ignored potentially compound-heterozygous variants due to the challenge of analyzing parents in a late manifesting disease in which parents were often already deceased. Due to the experience with the *IL36RN* mutation c.338C>T (p.Ser113Leu) (minor allele frequency [MAF] in non-Finnish European individuals of gnomAD of 0.44%), we used a MAF of ≤0.5%. We excluded candidate variants located in regions of segmental duplications and considered the NGS reads in the IGV browser to obtain evidence of a real variant. We identified two genes with two homozygous mutations (Table S4) and performed targeted sequencing analysis of the *MPO* coding sequence in further 80 individuals with pustular disease (mainly GPP, also AGEP and ACH). The remaining 29 WES (n = 31 [affected individuals] − 2 [affected individuals homozygous for *MPO* variants]) were re-analyzed for heterozygous and homozygous variants in *MPO* with a minor allele frequency of ≤2% in European populations, while coding sequence of MPO was sequenced by Sanger in further 51 affected individuals. We confirmed all detected variants in the subset of 80 individuals harboring *MPO* variants by independent Sanger sequencing as described previously and using GenBank: NM_000250.2 as a reference sequence.[7] We also assessed the 12 coding exons for copy number changes using two self-established quantitative multiplex ligation-dependent probe amplification (MLPA) tests according to the manufacturer's recommendations (MRC-Holland) performed as described previously.[7]

Cumulative frequencies of functionally relevant *MPO* variants were compared to cumulative frequencies in non-Finnish European individuals of gnomAD[17] using Fisher's exact test. To obtain all functionally relevant *MPO* variants, we selected for variants with a MAF of <2%. We considered truncating variants (frameshift, stop mutations) and those at splice acceptor sites (−1/−2) or splice donor sites (+1/+2) to cause complete enzyme deficiency in homozygous state (additional 37 mutations, Table S5). For missense variants in *MPO*, we performed a literature search using NCBI's PubMed and the combination of the keywords "MPO AND mutat* AND deficien*." We assessed the publications and considered missense variants with a functional test indicating impaired protein function as relevant mutations. Thereby, we included c.518A>G (p.Tyr173Cys), c.1495C>T (p.Arg499Cys), c.1501G>A (p.Gly501Ser), c.1715T>G (p.Leu572Trp),

c.1907C>T (p.Thr636Met), and c.1927T>C (p.Trp643Arg) as six additional missense mutations (Table S5).

We analyzed *MPO* mutations under dominant and recessive modes of inheritance using cumulative numbers of all mutations, and cumulative numbers of mutations leading to either complete or partial MPO deficiency in homozygous state (Table S6). For the recessive model, homozygous and compound heterozygous individuals were considered. For the dominant model, zygosity was disregarded.

Frequencies of the two mutations identified in individuals of Moroccan or Turkish descent were analyzed in the overall Great Middle Eastern variome and its corresponding subgroups of 99 Northwestern Africans and 164 individuals from the Turkish peninsula[18] and in 226 WES/Clinical Exome Sequences of unrelated individuals of Morrocan (n = 100) and Turkish (n = 126) origin that had been carried out for diseases other than psoriasis.

Structural Prediction of *MPO* Variants

The effect of the amino acid substitutions p.Arg548Trp and p.Arg590Cys was evaluated with wAnnovar[19] and based on the high-resolution MPO crystal structure (PDB code: 1CXP).[20] Swiss-Model[21] was used to model the exchanges and RasMol[22] was applied for structure analysis and visualization.

Analyses of MPO Deficiency

A blood smear of EDTA blood of the individual homozygous for c.265_275dup and a healthy donor was performed on microscope slides according to standard procedure. The slide was air-dried for 10 min before being placed in a fixative solution for 30 s followed by a 2 min rinse with distilled water. The slide was then immersed for 30 min in a 37°C warm water bath containing Trizmal 6.3 Dilute Buffer and Peroxidase Indicator Reagent (Sigma Diagnostics) at a workstation protected from light. After rinsing again, acid hematoxylline solution (Sigma Diagnostics) was applied for 10 min; after additional rinsing and 30 min air drying, the slide was used for microscopy.

To analyze the degree of MPO deficiency in affected individuals, we used a test[23] provided in differential blood count on an Advia 120 (Siemens Healthineers). Cells were stained with 4Chloro-Naphtol, and H_2O_2 was added as a substrate. The fluorescence intensity of these cells changed according to their MPO activity. The MPXI ("Mittlerer [German: average] PeroXidase Index") is based on these data, and a value >10 is considered as MPO-deficient according to the manufacturer.

To determine MPO activity alternatively, we used a commercially available ELISA kit (HycultBiotech), based on a systematic comparative study.[24] We prepared 2E06 cells per experiment in 100 μL PBS buffer and incubated them for 1:30 h at 37°C and 5% CO_2. After 1 h of incubation, we washed wells three times and added 50 μL of 0.03% H_2O_2 solution per well. We also added 50 μL of 1:1,000 200 μM ADHP (10-Acetyl-3,7-dihydroxyphenoxazine) (AAT Bioquest) and measured the fluorescence intensity at Ex/Em 535/590.

We performed a Kruskal Wallis test to examine whether MPO activity is different in individuals of different numbers of mutant *MPO* alleles.

Mutagenesis Experiments for *MPO* Variants

We obtained an MPO expression vector from Sino Biological and created mutants using specific PCR primers. We transfected HEK cells with the different expression vectors. After 3 days, we harvested the supernatant and measured it for MPO activity by ELISA. To ensure comparable transfection rates, we co-transfected cells with an OFP expression vector and analyzed them by FACS. We set the mean of the wild-type activity of three independent experiments to 1 and compared it to mutants' activities.

Number of Mutant Alleles in *MPO* or *MPO* and *IL36RN* as a Predictor of Pustular Psoriasis and Association Analyses

We analyzed 2,433 WES generated in-house with the same technology (Illumina) sequenced for non-inflammatory diseases and 2,504 individuals included in the 1000 Genomes project (phase 3)[25] for mutations in *MPO* and *IL36RN*, the latter previously detected in our group of affected individuals.[7] To assess all functionally relevant variants in *MPO* and *IL36RN*, we evaluated all variants as described above for *MPO*. We included variants with a MAF of <2%, considered truncating and splicing variants to be functional, and validated the remaining ones using NCBI's Pubmed and the combination of the keywords "IL36RN AND mutat* AND deficien*." To test whether mutations in (1) *MPO* or (2) *MPO* and *IL36RN* predict affection status, we performed a Firth's logistic regression analysis. We fitted a linear model (age/mutations) and determined p values by ANOVA to examine correlation between dosage of mutant alleles and age of onset. In order to take the major effect of bi-allelic *IL36RN* mutations on GPP's pathogenesis into account, we omitted individuals with bi-allelic *IL36RN* mutations from the combined analysis of *MPO* and *IL36RN*.

Screening of Healthy Donors

We performed several experiments on fresh blood samples of single available individuals affected by GPP and voluntary healthy donors. All consenting GPP individuals were female; therefore, we selected a group of age-matched healthy female donors. Those donors were sequenced for exons and flanking introns of *MPO* and *IL36RN* by Sanger to exclude potential individuals with functional variants; we confirmed their MPXI to be in the normal range.

Isolation of Neutrophils and RT-PCR

We isolated neutrophils from human whole blood using a commercially available kit (Miltenyi Biotec). We depleted erythrocytes with an erythrocyte depletion kit (Miltenyi Biotec) and obtained RNA using RNeasy Micro Kit (QIAGEN). We performed RT-PCRs using cDNA of a heterozygous and homozygous individual of c.2031−2A>C, of two further individuals not carrying c.2031−2A>C, and of two healthy donors using primers located in exons 10 and 12 and analyzed them as previously described.[26] RT-PCR products not corresponding to the wild-type's size were extracted with a gel extraction kit (QIAGEN) and sequenced by Sanger.

Cell Separations from Peripheral Blood

We isolated polymorphonuclear leukocytes/neutrophil granulocytes and peripheral blood mononuclear cells (PBMCs) from fresh EDTA blood by density-gradient centrifugation on Lymphoflot (BioRad). We gained $CD14^+$ monocytes from PBMCs using EasySep Human CD14 Positive Selection Kit II (Stemcell).

Analyses of Proteases' Activities

We determined the activity of CTSG using a commercially available assay (Abcam). After centrifugation (500 × *g* for 5 min), we

lysed 1E06 cells in 100 μL assay buffer. After 10 min incubation on ice, we centrifuged lysates at 16,000 × *g* for 10 min. We used this lysate for assays of serine protease activities in neutrophils. We added 40 μL substrate solution to 50 μL of lysate. After the specific cleavage of the artificial substrate by CTSG, we measured the absorbance of the resulting dye group p-NA (4-Nitroaniline) at 405 nm for 30 min (SpectraMax M3, Molecular Devices). For NE activity, we used a fluorometric assay (Cayman, Abcam). To control for background signals, we used 5 μL lysate with 95 μL of assay buffer. We read the fluorescence intensities at Ex/Em 485 nm/525 nm. To assess PR3 activity, we diluted 20 μL of lysate with 80 μL PBS and mixed it with 20 μL of 15 μM substrate ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO2)-NH2 as described previously. To investigate Cathepsin S activity, we used a commercially available assay (Biovision, K144) according to the manufacturer's protocol on lysates of 1.5E06 monocytes, generated as described above.

Formation of Neutrophil Extracellular Traps (NETs) by Plate Reader-Based Quantification

2.5E05 isolated human neutrophils suspended in 200 μL RPMI medium 1640 (GIBCO) were pipetted into each well of a 96-well flat bottom plate (Cellstar, Greiner Bio-one). 5 μM Sytox Green (Thermo Fisher Scientific) containing either phorbol myristate acetate (PMA) (100 ng/mL; Sigma), A23187 (5 μM, Sigma), or vehicle (RPMI medium) were added. The plate was tightly sealed and analyzed in an infinite 200 pro plate reader (TECAN) for 240 min at 37°C and 5% CO_2. Relative fluorescence units were normalized to the starting values and the respective vehicle control.

Flow Cytometry-Based Efferocytosis Assay of Murine and Human Cells

To track peripheral neutrophil granulocytes, we labeled them with CPD (ebioscience) following the manufacturer's protocol and co-cultured them with monocytes (ratio of neutrophils to monocytes 5:1) in sterile FACS tubes for 24 h (RPMI+10%FCS, 37°C, 5% CO_2). To distinguish between phagocytosed, CPD-positive neutrophils cells and free neutrophils, we counterstained monocytes with anti-CD11b-FITC antibody. We determined absolute numbers of remaining neutrophils by flow cytometric analysis of CD11b-/CPD+ cells via 123count eBeads (ebioscience) (Figure S1). We analyzed all experiments by flow cytometry (FACS Canto II, BD Biosciences).

CD47 Staining

To measure CD47 expression on neutrophil granulocytes, we stained 1E05 neutrophil granulocytes with a monoclonal anti-CD47 antibody (1:300, BD Bioscience) for 30 min at 4°C. We washed cells twice with PBS and analyzed them immediately using flow cytometry.

Statistical Analyses

We performed statistical analyses using R.[27] We calculated Spearman's correlation coefficient R and a p value according to Pearson's correlation to assess the correlation of MPO activity with the activities of proteases. We performed Welch t tests to determine differences in formation of NETs, in efferocytosis, and in CD47 staining on neutrophil granulocytes between MPO-deficient affected individuals and healthy donors.

Single-Cell RNA-Seq of PBMCs and Analysis of Subtype of Monocytic Cells

We isolated PBMCs from whole blood using BD Vacutainers (BD) according to the manufacturers' instructions. Libraries were prepared using the Chromium controller (10X Genomics) in conjunction with the single-cell 3′ v2 kit according to the manufacturers' instructions. Libraries were sequenced on an Illumina HiSeq 2500 sequencer to a depth of 160 million reads per sample. We performed primary data analysis as previously described[28] and used Seurat (v.3)[29] for QC and data analysis. We filtered cells with regard to the number of features (200 lower limit and 1,800–2,500, depending on the sample, upper limit) and percentage of mitochondrial RNA (<8.5–10). We obtained 1,143 and 3,966 cells from a affected individuals with total and almost total (respectively) MPO deficiency and 5,457 and 5,219 cells from healthy donors 1 and 2, respectively. Datasets were integrated using the functions "FindIntegrationAnchors" and "IntegrateData" with dims = 1:20. Resolution of 0.2 resulted in 14 clusters in the UMAP (Uniform Manifold Approximation and Projection) plot (Figure S2). We used 42 as random seed and identified Cluster 2 as $CD14^+$ monocytes, which was composed as follows: 153 and 509 cells of total and almost total MPO-deficient individuals, respectively, and 652 and 1,049 cells of healthy donors 1 and 2, respectively. We calculated differentially expressed genes with "FindMarkers" and performed pathway analyses with Ingenuity Pathway Analysis from QIAGEN (05.04.2019) using an adjusted p value (≤0.05) as cutoff.

Results

To elucidate further pathways that are relevant in the majority of individuals with GPP for whom pathogenic mutations have not been identified, we performed WES in 31 affected individuals who did not harbor *IL36RN* mutations. Under an autosomal-recessive model of inheritance, we identified *MPO* as a candidate gene based on two homozygous rare deleterious variants. Sequences of *MPO* (WES in 29 individuals, targeted Sanger sequencing in 51 individuals) revealed six further variants in another 13 independent GPP-affected, 1 ACH-affected, and 1 AGEP-affected individual (Table S1). Overall, we identified 4 individuals with bi-allelic variants and 13 additional individuals with one *MPO* variant. All persons with two variants had a diagnosis of GPP (Table S1, Supplemental Note). Five of the eight variants were previously classified as mutations causing MPO deficiency[30–32] (MPOD [MIM: 254600]), while three variants—c.265_275dup (p.Ser94Alafs*24), c.1642C>T (p.Arg548Trp), and c.1768C>T (p.Arg590Cys)—have to the best of our knowledge not been previously linked to impaired protein function. *MPO* encodes the neutrophilic heme-containing enzyme myeloperoxidase that oxidizes Cl^-/Br^- with H_2O_2 to highly reactive radicals HOCl/HOBr.[33]

Prediction for the homozygous frameshift variant c.265_275dup (p.Ser94Alafs*24) in exon 5 of 12 was nonsense-mediated mRNA decay and therefore lack of MPO synthesis. Protein prediction algorithms suggested harmful (deleterious or damaging) effects of the

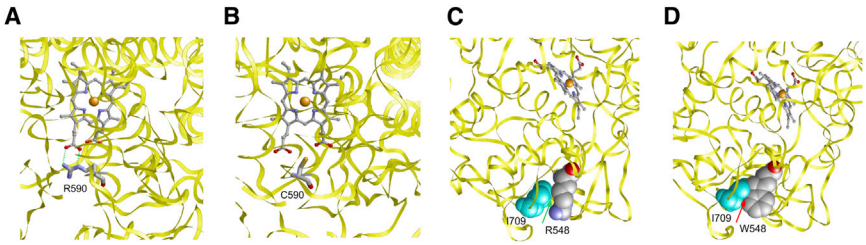

Figure 1. Structural Modeling of p.Arg590Cys and p.Arg548Trp on the MPO Structure

The backbone of the MPO protein is shown as a yellow ribbon.

(A) Arg590 forms two hydrogen bonds (green dotted lines) to a propionate group of the heme. Arg590 and the heme group are shown in stick presentation with the central iron atom depicted as an orange ball.

(B) In the p.Arg590Cys variant, the shorter cysteine side chain cannot form hydrogen bonds with the heme, expected to negatively affect catalytic activity.

(C) Arg548 forms hydrophobic interactions (green arrow) with Ile709. Both residues are shown in space-filled presentation (Ile709 in cyan).

(D) In the p.Arg548Trp variant, there exist steric clashes (red arrow) between Trp548 and Ile709 side chains, expected to cause a decrease in the overall protein stability.

c.1642C>T (p.Arg548Trp) and c.1768C>T (p.Arg590Cys). Structural modeling of p.Arg590Cys indicated a direct interaction of the wild-type residue Arg590 with heme and formation of hydrogen bonds to the propionate carboxyl group of ring D that is missing in mutant Cys590 due to a shorter and uncharged side chain (Figures 1A and 1B). The loss of hydrogen bonds probably led to reduced fixation of heme within the MPO molecule with the consequence of compromised catalytic activity. In case of p.Arg548Trp, structural modeling suggested that the wild-type residue Arg548 forms hydrophobic interactions with the adjacent Ile709; in contrast, the bulkier Trp548 cannot be sterically accommodated and forms clashes with Ile709 (Figures 1C and 1D), expected to cause a decrease in overall protein stability.

We performed a series of experiments to analyze effects of coding variants on MPO activity. We observed a lack of MPO staining in neutrophils in the individual homozygous for c.265_275dup (p.Ser94Alafs*24) (Figure 2A), while determination of MPO activity—in HEK cells transiently transfected with MPO mutant constructs and/or by fluorometric assays on neutrophils obtained from affected individuals—demonstrated loss-of-function in all further variants (Table S5, Figures 2C and 2D). We confirmed the degree of impairment of previously reported mutations to be partial or complete; the other identified variants all cause complete MPO deficiency. In a homozygous individual, we determined the enzymatic impairment of splice-acceptor mutation c.2031−2A>C as being caused by preferential inclusion of intron 11 resulting in premature protein truncation (Figure 2B). Our study does not emphasize a previously described mechanism[31]—use of an alternative splice-acceptor site at −109 in intron 11 resulting in a translational stop 72 amino acids later (p.Phe678Trpfs*

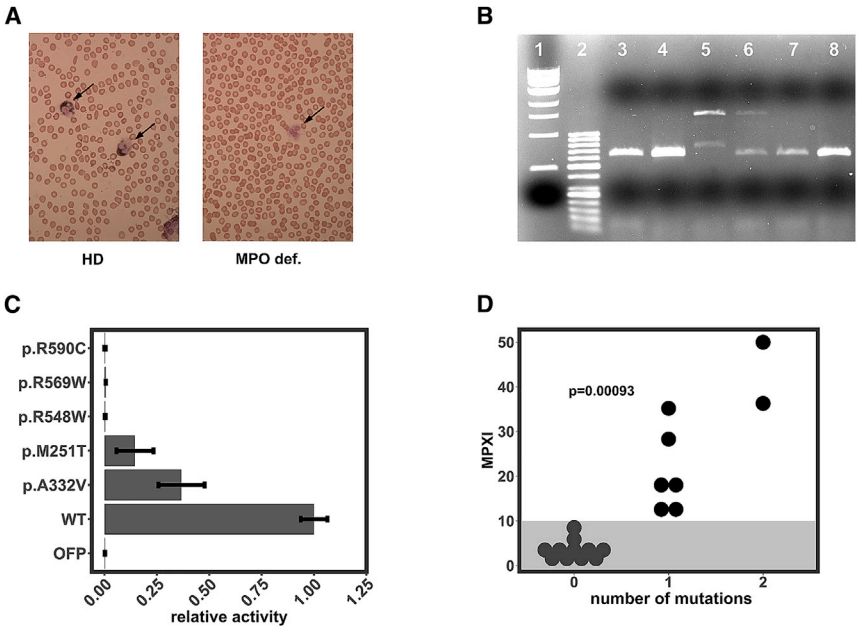

Figure 2. Assessments of MPO -Deficiency due to Eight *MPO* Mutations in Independent Experiments

(A) MPO staining in neutrophils of a healthy donor (left) and the MPO-deficient individual carrying c.265_275dup (p.Ser94Alafs*24) homozygously (right). Neutrophils are highlighted by arrows. Larger cells with blue-violet staining in healthy donor correspond to neutrophils and indicate functional MPO, while MPO could not be stained in neutrophils obtained from an affected individual.

(B) Splicing analysis of c.2031−2A>C in a homozygous (lane [l.] 5) and heterozygous (l.6) individual as well as four healthy donors (l.3–4) and two affected individuals without *MPO* mutation (l.7) or with missense mutation in *MPO* (l.8). In the homozygous individual, two PCR products were identified: r.[2030_2031ins [2031−109_2031−1],2030_2031ins [2030+1_2031−1] (insertion of intron 11 or insertion of 109 intronic base pairs). l.1: 1 kb ladder, l.2: 0.5 kb ladder.

(C) Degree of MPO activity in HEK cells transiently transfected with wild-type or five *MPO* mutant constructs.

(D) Correlation of *MPO* mutations with MPO deficiency assessed by fluorometric assay in 19 individuals; MPXI, correlates inversely with MPO activity; MPXI-values < 10 indicate normal range (gray).

Table 1. Cumulative Frequencies of All *MPO* Mutations and Groups of All Mutations Resulting in Partial or Complete MPO Deficiency and Evidence for Association

Degree of MPO Deficiency by Rare Mutations	Cumulative Frequency in 82 Affected Individuals (Proportion of Rare Alleles)	Cumulative Frequency in NFE of gnomAD (Proportion of Rare Alleles)[a]	p Value/OR [95% CI]
Partial and complete	12.8% (21/164)	5.3% (5,858/110,052)	1.87E−04/2.61 [1.57–4.15]
Complete	9.8% (16/164)	1.6% (1,809/110,052)	1.85E−08/6.47 [3.60–10.89]
Partial	3.0% (5/164)	3.7% (4,049/110,052)	0.84/0.82 [0.26–1.96]

Σ, sum; NFE, non-Finnish European individuals of gnomAD
[a]Using 110,052 as no. of controls' alleles.[17]

72)—as we observed it only to a minor degree. Independent of the underlying mechanism, our studies demonstrate that c.2031−2A>C causes total MPO deficiency in homozygous state.

Due to the high number of individuals with one heterozygous *MPO* mutation, we assessed whether intragenic copy number changes contribute to MPO deficiency, but did not identify a single intragenic deletion or duplication. MPO activity was significantly different with regard to the number of *MPO* mutations (p = 0.00093), and homozygous or compound heterozygous individuals had a more pronounced deficiency than heterozygotes (Figure 2D). Mutation frequencies of functionally relevant MPO variants (missense, truncating, and splicing variants) in affected individuals were significantly higher than in control subjects (12.8% versus 5.3%) (p = 1.87E−04; OR = 2.61; Tables 1 and S5). This association was even more pronounced when we considered only mutations resulting in complete enzymatic deficiency: 9.8% in affected individuals versus 1.6% in control subjects (p = 1.85E−08; OR = 6.47), strongly indicating that MPO deficiency contributes to GPP susceptibility. As gnomAD is enriched in individuals of European origin, we aimed to exclude that the mutations c.265_275dup (p.Ser94Alafs*24) and c.1768C>T (p.Arg590Cys), identified homozygously in single individuals of Moroccan and Turkish origin, respectively (Table S2), are frequent in those populations. By analyzing 398 Northwest African alleles and 580 Turkish alleles, we did not identify a single individual carrying either of the two mutations.

Using models of different modes of inheritance, we obtained evidence for larger effects under the recessive mode of inheritance with an OR of 50.32 for all mutations or an OR of 93.0 for mutations leading to complete MPO deficiency in homozygous state (Table S6). Although effects under the dominant model provided a relevant range of effects in the same two mutational categories, our data indicate that one *MPO* mutation does not sufficiently explain disease susceptibility.

Phenotypic analyses in subgroups of individuals with and without *MPO* mutations revealed that individuals with *MPO* mutations had more frequently concomitant palmoplantar pustular psoriasis, tongue involvement, and positive family history for inflammatory skin and joint disease, while age of onset, course of disease, and concomitant diseases were comparable (Table S3).

Unexpectedly, we found 5 of the 17 individuals with *MPO* mutations to be also heterozygous or even homozygous for *IL36RN* mutations (Tables S7B and S7C), which raised the question of a possible gene dosage effect. We considered 21 functional relevant variants in *IL36RN* (c.−27−2A>T, c.80T>C [p.Leu27Pro], c.104A>G [p.Lys35Arg], c.110_111dup [p.Lys38LeufsTer42], c.115+1G>T, c.121G>T [p.Glu41Ter], c.142C>T [p.Arg48Trp], c.146G>A [p.Trp49Ter], c.201C>A [p.Cys67Ter], c.220C>T [p.Gln74Ter], c.227C>T [p.Pro76Leu], c.243+1G>A, c.280G>A [p.Glu94Lys], c.304C>T [p.Arg102Trp], c.335dup [p.Ser113ValfsTer14], c.338C>T [p.Ser113Leu], c.338C>A [p.Ser113Ter], c.348C>A [p.Tyr116Ter], c.368C>T [p.Thr123Met], c.457C>T [p.Gln153Ter], c.*49+1G>A) and 54 in *MPO* (Table S5) in 4,937 control subjects (the 1000 Genomes project and in-house control subjects) and 82 affected individuals. When comparing all 82 affected individuals with 4,937 control individuals (the 1000 Genomes project and in-house control subjects) for number of mutant alleles in *MPO,* we confirmed that affected individuals carry significantly more mutations (p = 1.04E−09, Figure 3A, Table S7A). This correlation was even stronger when we included heterozygous *IL36RN* mutations (1.48E−13, Figure 3B, Table S7B). The dosage of mutant alleles also correlated with age of onset (p = 0.0018, Figure 3C). Of note, affected individuals with homozygous or compound heterozygous *MPO* mutations had a nominally lower age of onset than the subgroup of individuals with both *IL36RN* and *MPO* mutations. Due to the smaller subgroups, these findings need replication in independent study groups.

To link MPO deficiency to the IL-36-pathway, previously implicated in disease, we measured activity of MPO and of neutrophil serine proteases NE, CTSG, and PR3 and of monocytic protease CTSS in affected individuals and observed a strong inverse correlation (R = −0.80–0.94, Figure 4A). Previously, diminished reactive radicals in $Mpo^{-/-}$ mice have been attributed to reduced oxidation of two neutrophil serine proteases and hence increased activity of these proteases.[34,35] Our data suggest a comparable mechanism in human neutrophils, and to the best of our knowledge previously undescribed for PR3 and monocytic protease CTSS in general. When considering GPP as a primary skin disease triggered by activation of IL-36 in keratinocytes, an increased activity of serine proteases might be essential, as serine proteases have been shown to

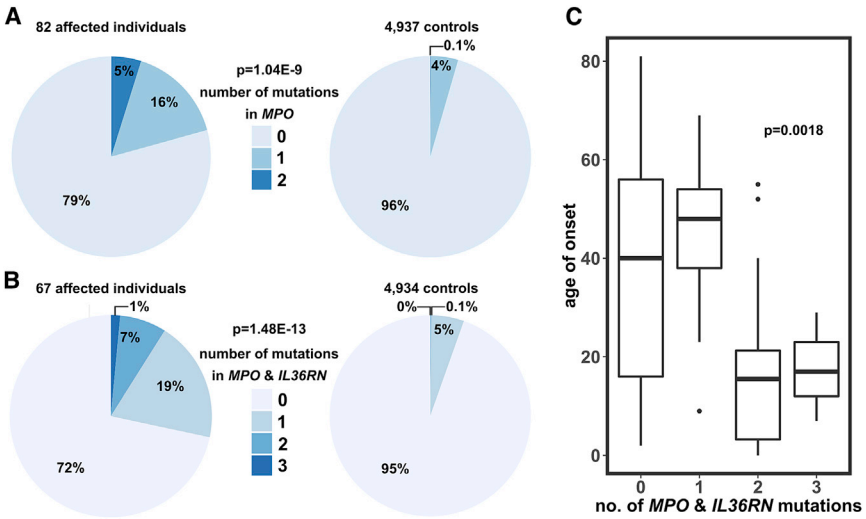

Figure 3. Number of Mutant Alleles in GPP, AGEP, and ACH

(A and B) Number of mutant alleles in *MPO* (A) and in *MPO* and *IL36RN* (B) as predictors of affection status. Pie charts illustrate proportions of individuals with 0–2 *MPO* mutations or proportions of individuals with 0–3 mutations in *MPO* and *IL36RN* in affected individuals and control subjects (in-house exomes, the 1000 Genomes project), omitting individuals with two *IL36RN* mutations in (B).

(C) Distribution of number of mutant alleles (x axis) in correlation with age of onset in 77 affected individuals (y axis).

escalate inflammation by cleavage of IL-36 precursors to the very active pro-inflammatory IL-36 cytokines (Figure 5A).[10,11] Moreover, we confirmed previous studies that identified a reduced formation of neutrophil extracellular traps (NETs) in neutrophils of MPO-deficient individuals following induction by PMA (Figures 4B–4D).[36,37]

Since persistence of neutrophils in inflamed skin is a hallmark of GPP, we investigated whether this is related to reduced phagocytosis of neutrophils by monocytes (efferocytosis). Residual neutrophils in $Mpo^{-/-}$ mice as well as human MPO-deficient cells were indeed significantly increased (Figures 4E and 4F, $p = 0.02$, $p = 0.0046$, respectively), confirming altered phagocytosis of neutrophils of affected individuals (Figure 5C). Allogenic combinations of MPO-deficient neutrophils and healthy donors' monocytes and vice versa indicated altered functionality of both cell types, neutrophils and monocytes (Figure 4G). An increased CD47-staining on neutrophils of affected individuals (Figure 6A)—a molecule that has been accused as a "don't eat me" signal in oncology—suggested a neutrophil-related pathway contributing to reduced phagocytosis. The variable staining, though, pointed to an involvement of additional neutrophilic pathways relevant for phagocytosis. As mRNA of peripheral neutrophils is very limited and *MPO* is also expressed in monocytes, we analyzed differential gene expression in monocytes of MPO-deficient affected individuals using single-cell RNA-seq. When analyzing pathways of differentially expressed genes (DEGs), we identified downregulation of several phagocytosis-related pathways (Figure 6B). Among those phagocytosis-related DEGs, we detected *SPI1* (oncogene SPI1 [MIM: 165170]) and *CYBA* (cytochrome b (−245), alpha subunit [MIM: 608508]) to be downregulated in MPO-deficient individuals 1.93-fold and 2.63-fold, respectively (Figure 6C), representing candidates involved in efferocytosis-related pathways.

In combination, these data indicate that altered neutrophil function together with impaired clearance of neutrophils by monocytes (efferocytosis) might explain prolonged persistence of neutrophils in skin of affected individuals and reduced ability to resolve inflammation.

Discussion

MPO deficiency has not yet been consistently associated with disease in human, contrary to $Mpo^{-/-}$ mice, which have been described to be more susceptible to candida infection.[38] Interestingly, MPO- deficiency has been reported in single persons with GPP decades ago[39,40] but was not recognized as a general risk factor, probably due to GPP's low prevalence (1–2:1,000,000 individuals[41]). These early cases are in agreement with our findings and independently support MPO deficiency as a relevant risk factor in GPP's pathogenesis.

Cardiologic research has assigned MPO a central role in atherosclerosis and several other cardiovascular diseases. Unlike in GPP, MPO deficiency in $Mpo^{-/-}$ mice and other experiments modeling human cardiovascular diseases showed a beneficial effect in most cardiovascular manifestations;[42,43] therefore, MPO inhibitors are being developed and tested in animal models. Our data would implicate that application of those drugs in human should be performed under careful monitoring for potential occurrence of neutrophilic pustulosis of the skin.

The genetic data of our study indicate that GPP has an oligogenic inheritance pattern, as suggested previously,[5,7] and that *MPO* mutations—especially those leading to complete enzyme deficiency—explain part of the reduced penetrance and the variable age of onset in GPP. While this study suggests a role of *MPO* mutations in palmoplantar pustular psoriasis, tongue affection, and a more general contribution to familiarity of psoriatic inflammatory diseases, genotype-phenotype correlations should be evaluated in independent study groups and extended phenotypes.

Upon activation, neutrophils release proteases, which activate precursors of IL-36 cytokines either as soluble molecules following degranulation[10] or as proteases

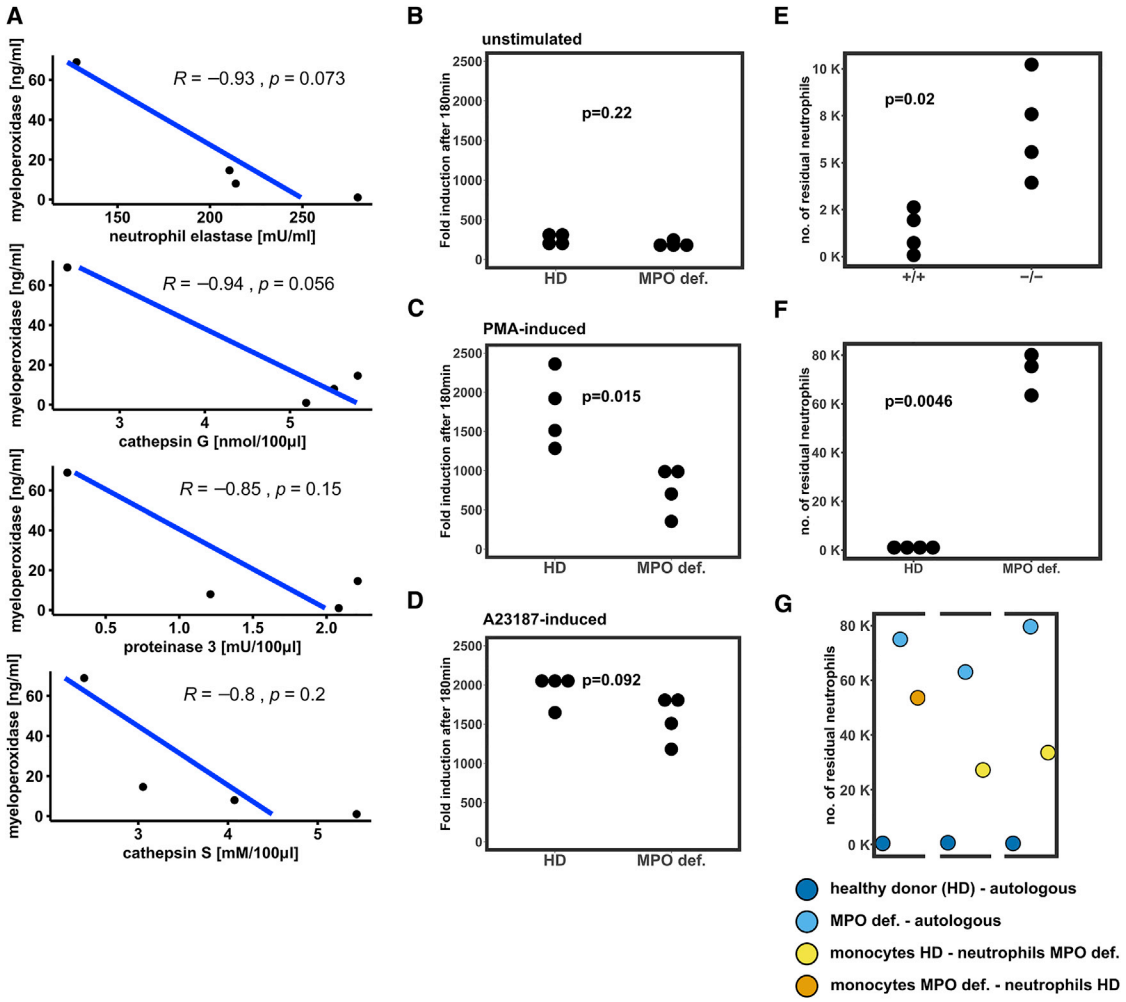

Figure 4. Activity of Three Neutrophil Serine Proteases and Monocytic Protease CTSS in MPO Deficiency, Formation of Neutrophil Extracellular Traps, and Phagocytosis of Neutrophils by Monocytes in Mice and Human

(A) Inverse correlation of MPO activity with activity of serine proteases in four affected individuals. x axis, activity of protease; y axis, MPO activity.

(B–D) Formation of neutrophil extracellular traps (B) without stimulation, (C) after induction with PMA, and (D) with A23187, shown as fold change after incubation for 180 min and normalized to starting point and vehicle control.

(E–G) Number of residual cells (y axis; K = 1,000) reflecting neutrophils not phagocytosed by monocytes in (E) murine $Mpo^{-/-}$ and wild-type cells, (F) human cells of three MPO-deficient individuals with GPP (MPO-def.) and four healthy donors, and (G) allogenic incubations of MPO-deficient individuals' and healthy donors' neutrophils and monocytes in comparison to autologous incubations; each of the three image sections shows two autologous combinations of the same healthy donor and of the MPO-deficient individual and their allogenic combination.

bound to neutrophil extracellular traps (NETs).[44] We confirmed previous findings of a reduced ability to form NETs in the PMA-induced pathway in MPO-deficient neutrophils[36,37] (Figure 5B). The reduced NET formation might argue in favor of the soluble proteases to be more prominent in the MPO-related, acutely inflamed epidermis than proteases bound to NETs. More generally, NET formation has been shown to also have anti-inflammatory effects,[45,46] while their exact formation in acute human disease episodes *in vivo* is difficult to model. NE, but not the further neutrophilic proteases, has also been shown to cleave IL-36Ra precursors to the more active antagonist *in vitro*, thereby counterbalancing pro-inflammatory IL-36 cytokines.[47] Our data indicate an elevated activity of three neutrophilic serine proteases and of the monocytic protease CTSS which might allow activation of IL-36α, IL-36β, and IL-36γ cytokines and therefore shift the balance to predominant pro-inflammatory IL-36 signals (Figure 5A). We propose a link between the IL-36 pathway and neutrophil function via an increased activity of neutrophil serine proteases, thereby indicating an interaction between neutrophils and keratinocytes in GPP's pathogenesis.

Furthermore, the additional altered functionality of neutrophils and monocytes offers a hypothesis for a mechanism allowing persistence of harmful neutrophils in skin, thereby hindering resolution of inflammation. The combination of two different mechanisms—activity of proteases

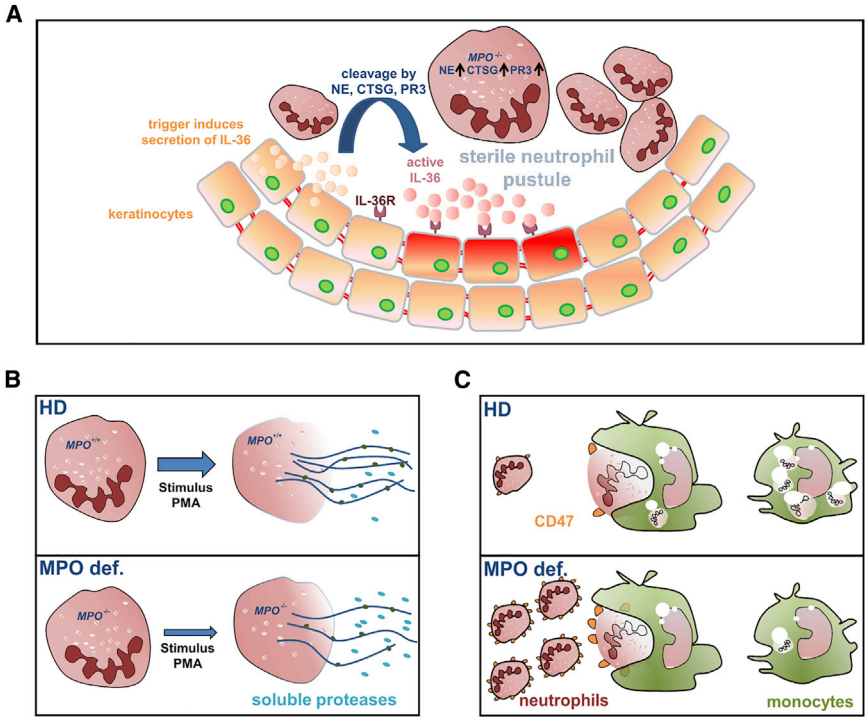

Figure 5. Model of MPO Deficiency in Different Pathways

(A) In MPO-deficient neutrophils, the activity of serine proteases in neutrophils is increased. Those proteases are known activating enzymes of precursors of IL-36α, IL-36β, and IL-36γ leading to the characteristic pro-inflammatory imbalance of the IL-36 pathway in generalized pustular psoriasis.

(B) The formation of neutrophil extracellular traps (NETs; dark-blue lines) in MPO-deficient cells is reduced compared to healthy donors, which might suggest a preponderance of soluble proteases (light blue) activating IL-36 precursors compared to NET bound proteases (dark blue).

(C) Phagocytosis of neutrophils by monocytes is less efficient in MPO-deficient individuals and $Mpo^{-/-}$ mice in comparison to the wild type.

and impaired clearance of neutrophils—and an involvement of many neutrophil and monocytic pathways induced by *MPO* mutations show the complexity and entanglement of MPO-related disease pathogenesis. The link of neutrophil function to the IL-36 pathway might provide an explanation for the therapeutic response in individuals without *IL36RN* mutations.

Impaired efferocytosis has previously been shown to be involved in other auto-inflammatory disorders—periodontitis and rheumatoid arthritis—in mice and human.[48] In contrast though, these diseases have been shown to be mediated by alterations in monocytes, but not by impaired neutrophils. This study identifies an involvement of impaired efferocytosis in psoriasis. Further investigations of the efferocytosis processes in both MPO-deficient individuals and MPO-deficient mice will reveal whether alterations of phagocytosis-molecules in neutrophils and/or in

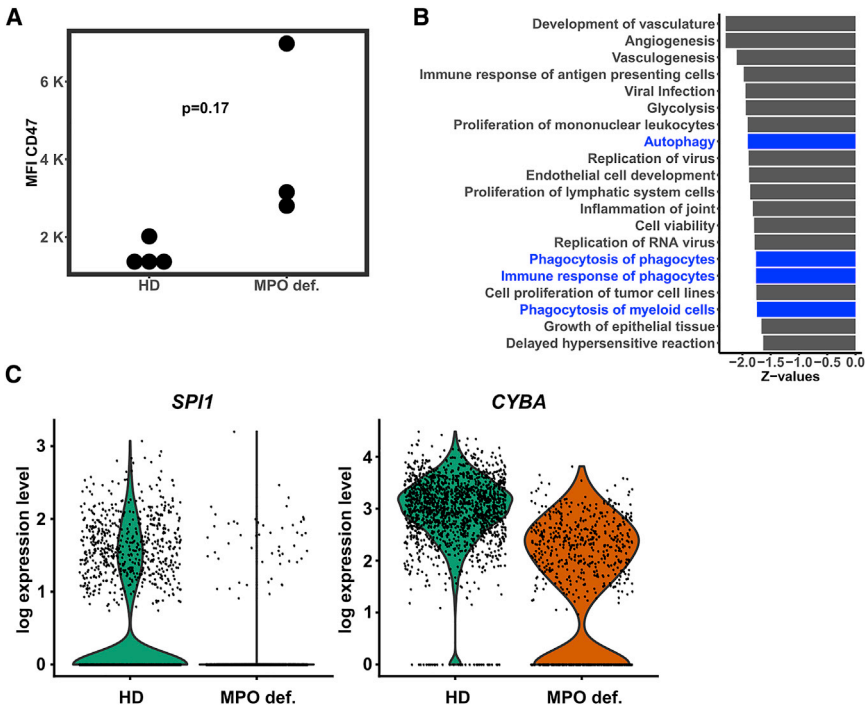

Figure 6. Protein and Transcriptional Changes in Neutrophils and Monocytes of MPO-Deficient Individuals with GPP and Healthy Donors

(A) CD47 staining on neutrophils of three MPO-deficient individuals (MPO def.) and four healthy donors.

(B) Pathways identified to be most differentially downregulated in monocytes of affected individuals; pathways relevant for phagocytosis are shown in blue.

(C) Violin plots demonstrating two differentially expressed genes of phagocytosis-related pathways (y axis, log expression level) between healthy donors and MPO-deficient individuals (x axis). Each dot corresponds to one cell, violin forms show distribution of expression levels in individuals and control subjects.

monocytes will be accessible targets considering existing therapies such as anti-CD47 antibody therapy used in various malignancies.[49]

In conclusion, our study suggests that MPO deficiency is involved in the pathogenesis of GPP via regulation of the activity of neutrophil and monocytic proteases, which in turn has been described to activate pro-inflammatory IL-36 in acute disease, and via modifications of phagocytosis of neutrophils by monocytes. These mechanisms might also be relevant in other neutrophil-driven diseases, e.g., Sweet syndrome (acute febrile neutrophilic dermatosis [AFND] [MIM: 608068]) and pyoderma gangrenosum, as MPO deficiency has been described in a single individual with pyoderma gangrenosum.[50] Overall, our study implicates MPO as a modulator of inflammation in humans and therefore MPO itself or affected pathways represent attractive targets for inducing resolution of inflammation in neutrophil-mediated skin diseases.

Data and Code Availability

Most of the relevant datasets generated and/or analyzed during the current study are available in this manuscript and its supplementary information files. Datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2020.07.001.

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Declaration of Interests

H.B. received a research grant by Morphosys and the Celgene Corporation. M.S. was an investigator, speaker, consultant, or an advisory board member for Abbvie, Amgen, Celgene, Galderma, GSK, Janssen Cilag, Leo, Lilly, MSD, Novartis, Regeneron, Pfizer, Sanofi, and UCB. W.S. received travel expenses for attending meetings and/or (speaker) honoraria from Abbvie, Almirall, Celgene, Janssen, LEO Pharma, Lilly, Novartis, Pfizer, Sanofi Genzyme, and UCB. All other authors declare no competing interests.

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Web Resources

GenBank, https://www.ncbi.nlm.nih.gov/genbank/
gnomAD v2.1.1 access date 04/17/20, https://gnomad.broadinstitute.org/
OMIM, https://www.omim.org/
PubMed, http://www.ncbi.nlm.nih.gov/PubMed/
RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do

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