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 gene3,4 (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), or in single control individuals, suggesting incomplete penetrance. 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.

Affected individuals with GPP (n = 7) were treated with an IL-36R antibody and showed marked reduction in disease activity. This was the case even in individuals without an IL36RN mutation, 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). 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.

As the majority of individuals affected by GPP do not have mutations in IL36RN or any other known susceptibility gene, 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]).

**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 the recruitment strategy. All GPP- and ACH-affected individuals fulfilled the ERASPEN consensus criteria, while for the AGEP-affected individuals, we validated their diagnosis according to the scoring system used by RegiSCAR study groups and modified by Paulmann and Muckenhaup. 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 committee 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. 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. 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.

Cumulative frequencies of functionally relevant MPO variants were compared to cumulative frequencies in non-Finnish European individuals of gnomAD 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 (frame-shift, 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 muta* AND deficient*.” 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), and c.1495C>T (p.Ala499Val) (Table S5).
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\(^2\) and in 226 WES/Clinical Exome Sequences of unrelated individuals of Moroccan (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\(^1\) and based on the high-resolution MPO crystal structure (PDB code: 1CXP).\(^2\) Swiss-Model\(^2\) was used to model the exchanges and RasMol\(^2\) 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 hematoxyline 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\(^2\) provided in differential blood count on an Advia 120 (Siemens Healthines). Cells were stained with 4Chloro-Naphtol, and H\(_2\)O\(_2\) 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.\(^2\) We prepared 2006 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\(_2\)O\(_2\) solution per well. We also added 50 µL of 1:1,000 200 µM ADHP (10-Acetyl-3,7-dihydroxyphenoxazine) (AAAT Bioquest) and measured the fluorescence intensity at Ex/Em S35/S90.

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)\(^2\) 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 RNasy 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.\(^2\) 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 Easy-Sep Human CD14 Positive Selection Kit II (Stemcell).

**Analyses of Proteases’ Activities**

We determined the activity of CTS G 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 buffer. We read the fluorescence intensities at Ex/Em 485 nm/525 nm. To track peripheral neutrophil granulocytes, we labeled them with 123count eBeads (ebioscience) (Figure S1). 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 (MPO [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 H₂O₂ to highly reactive radicals HOCl/HOBr. 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
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/C02A 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—use of an alternative splice-acceptor site at –109 in intron 11 resulting in a translational stop 72 amino acids later (p.Phe678Trpfs*
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)* | 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
*Using 110,052 as no. of controls’ alleles.17

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>Å [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 monocyteic 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.14,35 Our data suggest a comparable mechanism in human neutrophils, and to the best of our knowledge previously undescribed for PR3 and monocyctic 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

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 were significantly higher than in control subjects (1000 Genomes project and in-house control subjects) and 82 affected individuals. When considering all 82 affected individuals with 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.

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escalate inflammation by cleavage of IL-36 precursors to the very active pro-inflammatory IL-36 cytokines (Figure 5A). 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). Allo- genic 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 ago39,40 but was not recognized as a general risk factor, probably due to GPP’s low prevalence (1–2:1,000,000 individuals41). 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 degranulation10 or as proteases
bound to neutrophil extracellular traps (NETs). We confirmed previous findings of a reduced ability to form NETs in the PMA-induced pathway in MPO-deficient neutrophils (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, 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. 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 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.
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.

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 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.

Impaired efferocytosis has previously been shown to be involved in other auto-inflammatory disorders—periodontitis and rheumatoid arthritis—in mice and human. 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 mononuclear 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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