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Transcriptomes of MPO-deficient patients with generalized pustular psoriasis reveals expansion of CD4+ cytotoxic T cells and an involvement of the complement system

Short title: Transcriptomes of MPO-deficient patients with generalized pustular psoriasis

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Key words: Psoriasis, CD4+ cytotoxic T lymphocytes, classical pathway of complement, T cells, transcriptomes, single cell RNA-sequencing
Abstract

Generalized pustular psoriasis (GPP) is a severe psoriatic subtype characterized by epidermal neutrophil infiltration. Although variants in \textit{IL36RN} and \textit{MPO} have been shown to affect immune cells, a systematic analysis of neutrophils and peripheral blood mononuclear cells (PBMCs) subsets and their differential gene expression dependent on \textit{MPO} genotypes was not performed yet.

We assessed transcriptomes of MPO-deficient patients using single cell RNA-sequencing (scRNAseq) of PBMCs and RNA-sequencing of neutrophils in stable disease state. Cell type annotation by multimodal reference mapping of scRNAseq data was verified by flow cytometry of surface and intracellular markers; proportions of CD4\(^+\) cytotoxic T-lymphocytes (CTLs) and other CD4\(^+\) effector cells were increased in GPP, while frequencies of naïve CD4\(^+\) T cells were significantly lower. The expression of \textit{FGFBP2} marking CD4\(^+\) CTLs and CD8\(^+\) effector memory T-cells (TEMs) was elevated in GPP patients with disease-contributing variants compared to non-carriers (p=0.0015). In neutrophils, differentially expressed genes (DEGs) were significantly enriched in genes of the classical complement activation pathway.

Future studies assessing affected cell-types and pathways will show their contribution to GPP’s pathogenesis, and indicate whether findings can be transferred to the acute epidermal situation and whether depletion or inactivation of CD4\(^+\) CTLs may be a reasonable therapeutic approach.
Introduction

Psoriasis is a common autoimmune disease affecting an estimated 2-3% of the population in developed countries, while generalized pustular psoriasis (GPP) belongs to the rarer pustular subtypes. GPP is a multisystemic disease, often occurring in acute, potentially life-threatening episodes requiring hospitalization. Histologically, sterile epidermal pustules filled with neutrophils are characteristic for GPP. Variants identified in the gene encoding the receptor antagonist of interleukin-36 (IL-36Ra, gene *IL36RN*) have been identified as disease-causing (Marrakchi et al., 2011, Onoufriadis et al., 2011). The IL-36 receptor (IL-36R) and the precursors of its ligands IL-36α, -β and -γ are mainly expressed in keratinocytes (Marrakchi et al., 2011, Towne et al., 2004), while expression of the IL-36R has been described on bone-marrow derived cells: both murine and human dendritic cells as well as human neutrophils (Hay et al., 2018, Vigne et al., 2011). IL-36 signaling leads to activation of nuclear factor-κB and mitogen-activated protein kinases, resulting in production of pro-inflammatory cytokines, e.g. IL-8 (Carrier et al., 2011, Johnston et al., 2017, Marrakchi et al., 2011, Towne et al., 2004). In patients with *IL36RN* variants, the IL-36 pathway is unbalanced in favor of production of pro-inflammatory cytokines in keratinocytes. Therapies targeting IL-36R show convincing response in patients with and without *IL36RN* variants (Bachelez et al., 2019), highlighting the central role of this pathway in GPP.

The gene myeloperoxidase (*MPO*) has been reported as a second major disease gene (Haskamp et al., 2020, Vergnano et al., 2020), and as significant subsets of GPP patients carry variants in more than a single gene, oligogenic inheritance has been assumed (Mossner et al., 2018). Neutrophils and monocytes are the main source of MPO (Bos et
al., 1978). Functional studies of MPO-deficient patients suggest an impaired removal of dying neutrophils by monocytes, an altered IL-36 signaling due to an increased expression of IL-36 activating proteases and reduced apoptosis of neutrophils (Haskamp et al., 2020, Vergnano et al., 2020). Moreover, previous publications indicated a role of certain rare variants in CARD14 and SERPINA3 in GPP’s pathogenesis (Frey et al., 2020, Jordan et al., 2012, Mossner et al., 2018, Sugiura et al., 2014), while an association with more frequent variants in AP1S3 has been reported in British patients (Setta-Kaffetzi et al., 2014).

Independent from variants in either gene, transcriptomes in neutrophils of GPP patients outlined an involvement of the IFN-I pathway (Catapano et al., 2020). Recent studies suggest a role of several further cell types in the pathogenesis of GPP: An increased gene expression of IL-17A in skin (Johnston et al., 2017) and a reasonable response of GPP to IL-17A blockage (Imafuku et al., 2016, Saeki et al., 2015, Wilsmann-Theis et al., 2018, Yamasaki et al., 2017) indicate a role of IL-17 and more generally T cells in this rare entity. Arakawa et al. confirmed this aspect by showing that IL-36 signaling increases the proliferation of CD4+ T cells including IL-17A producing cells in GPP patients (2018). In addition, Odobasic et al. provided evidence that MPO is involved in T cell driven inflammation in knockout mice (2013). A significant subset of GPP patients are also affected by psoriasis vulgaris (Baker and Ryan, 1968, Twelves et al., 2019); and in this common subtype, T cells are known to maintain systemic inflammatory states. Overall, these studies indicate that keratinocytes, neutrophils and certain T cell subsets represent major cellular players in GPP.
A systematic method to analyze the composition of different cell types and their transcriptome is single cell RNA sequencing (scRNAseq). Only recently, multimodal reference mapping was introduced as a tool to annotate clusters in a reproducible and objective manner (Hao et al., 2020). Based on a reference data set containing the transcriptome and cell surface antigen expressions at single cell level, this method allows annotating scRNAseq data automatically and more detailed than previously available approaches, e.g. SingleR (Aran et al., 2019).

In order to assess the role of circulating immune cells systematically, we performed transcriptome analyses of peripheral blood mononuclear cells (PBMCs) at single cell level and of neutrophils in MPO-deficient GPP. We could verify altered cell proportions of CD4⁺ T cells at protein and RNA levels and observed a significant enrichment of differentially expressed genes (DEGs) of the classical complement pathway in neutrophils.

**Results**

**Cellular characterization of PBMCs by single cell RNA sequencing**

The two patients analyzed by scRNAseq carried bi-allelic *MPO* variants with GPP1 carrying an additional heterozygous *IL36RN* variant (Table S1). By using multimodal reference mapping of the 20,508 cells of all probands, we received the dimensional reduction plot (reference uniform manifold approximation and projection (refUMAP)) with annotated clusters (Figure 1a,b). In total, we could annotate 31 different cell types (Figure 1a,c). Some clusters could be easily detected by already described markers (Figure S1a). Compared to the annotation based on the expression of CD4 and CD8 in UMAP (Figure
S1b), the clusters of different CD4+ and CD8+ T cells could be differentiated more precisely (Figure 1a). While CD4+ and CD8+ effector cells were more frequent in GPP patients than in healthy donors (HD), naïve T cells and a subgroup of dendritic cells (cDC1) represented a lower proportion (Figure 1b,c, Table S2). In more detail, 92.5% of cells of the cluster CD4+ cytotoxic T cells (CTLs) were from GPP individuals (Figure 1b,1c, Table S2). Similarly, CD4+ effector memory T cells (CD4+ TEM; 71%) and CD8+ effector memory T cells (CD8+ TEM; 62%) were more frequent in GPP samples. In contrast, the clusters of naïve T cells were mainly composed of HD cells: CD4+ naïve T cells – 76.6%, and CD8+ naïve T cells – 71.8%. While the discussed cell clusters represent more frequent cell types, we also observed differences in rarer cell types, e.g. in innate lymphoid cells (ILCs) and in gamma delta T cells (gdT cells); ILCs and gdT cells were more common in GPP patients (76% and 65%, respectively).

Next, we wondered, if these results can be reproduced in unsupervised clustering based on RNA expression profiles alone. This approach resulted in 16 clusters (Figure S1c). Cells of GPP patients were detected more frequently in cluster 1 representing mainly CD4+ CTLs (35%) and CD8+ TEMs (35%) according to the reference mapping. In contrast, 75% of cells in cluster 11 (68% CD4+ naïve) were from HD (Figure S1d). The uneven distribution of GPP patients' versus HD cells in naïve T cells and effector T cells is highlighted in the UMAP using individual colors (Figure S1b, right panel).

Confirmation of cellular characterization of PBMCs at protein and RNA level

Since multimodal reference mapping is a method to our knowledge without a previous report of confirmation by an independent technique, we aimed to verify the different frequencies of CD4+ CTLs, CD4+ TEMs and CD8+ TEMs between patients and control
individuals at protein and RNA level. As the gene expression of GZMA, GZMK and FGFBP2 (encoding KSP37) in combination with the cell type markers CD4 and CD8 identified those cell clusters in our single cell analyses (Figure 1a,b, 2a), we decided to measure the amount of the intracellular proteins GZMA, GZMK and KSP37 in CD4+ and CD8+ T cells by flow cytometry. Additionally, we included CD56+ NK cells, since NK cells expressed all markers as well (Figure 2a,b).

Analyses of intracellular protein expression in three patients and four HD confirmed our scRNAseq data (Figure 2c). The three GPP patients carried different combinations of variants in MPO and/or IL36RN: GPP1: 2 MPO and 1 IL36RN variant, completely MPO-deficient; GPP2: 2 MPO variants, partially MPO-deficient; GPP3: 1 MPO variant, partially MPO-deficient and were also analyzed for a panel of other surface molecules (Table S1, see also below). The percentage of CD4+ CTLs corresponding to double positive (GZMA+ KSP37+ GZMK+) CD4+ T cells was significantly higher in patients compared to HD (p=0.0373, Figure 2b,c). As expected, CD8+ TEMs were nominally, while not significantly more frequent in GPP. Flow-cytometry of T cell subpopulations based on previously established sets of markers confirmed an uneven distribution of CD4+ T cells with a significantly higher proportion of activated CD4+ T cells (CD4+ Eff; p=9.5E-03) and of CD4+ TEMs (p=0.0332; Figure S2a), but a significantly reduced one of naïve CD4+ T cells (p=8.0E-04). In contrast, we did not detect similarly significant alterations of CD8+ effector and naïve T cells, although we detected nominal differences between GPP and HD, especially in activated CD8+ T cells (CD8+ Eff; Figure S2a).

CD56+ NK cells (GZMA+ KSP37+ GZMK+) were even less common in the group of the two GPP patients and of one additional MPO-deficient one compared to HD (p=0.0213; Figure
and \textit{FGFBP2} was solely expressed on two further cell types, CD4$^+$ CTLs and CD8$^+$ TEMs (Figure 2a,b). Therefore, we considered \textit{FGFBP2} as an appropriate marker for these two cell clusters. In order to confirm that CD4$^+$ CTLs and CD8$^+$ TEMs were more frequent in GPP patients in general, we performed targeted gene expression analyses on RNA from whole blood in an extended case control group. This group consisted of five HD and five GPP patients without disease-contributing variants in \textit{IL36RN} and \textit{MPO}, three patients with one (n=1) or two (n=2) \textit{IL36RN} variants, five patients with one (n=4) or two (n=1) variants in \textit{MPO} and two patients with variants in both genes. The expression of \textit{FGFBP2} was significantly higher in patients with at least one variant in either or both genes compared to the group of non-carriers (patients and HD) (p=0.0015; Figure 2d). Although \textit{FGFBP2} was also expressed in NK cells in the scRNAseq data, the numbers of NK cells were rather evenly distributed between patients and controls as shown in scRNAseq analysis (Figure 1c) and or even significantly lower observed upon flow cytometry of intracellular markers (Figure 2c). Therefore, we were confident that the proportion of CD4$^+$ CTLs and to a lesser extent CD8$^+$ TEMs were the source of this disease-related difference. The higher proportion of CD4$^+$ CTLs and higher expression of \textit{FGFBP2} in GPP2 compared to GPP1 (Figures 1c, 2d) supported this aspect.

As many of the analyzed GPP patients obtained immune-modulating therapies, we assessed whether the expression level of \textit{FGFBP2} in groups of patients with different therapeutics was comparable. Although the range of expression levels varied widely within groups, the median values were comparable, indicating no considerable effects due to therapy (Figure S2b). In each treatment group with more than a single patient, the gene expression of \textit{FGFBP2} was lowest in patients without variants, and we observed evidence for marginal significance for a gene-dose effect (p=0.02; Figure S2c).
Considering the differences in innate lymphoid cells (ILCs) between four patients and five HD in scRNAseq data, we determined frequencies of ILCs by flow cytometry. Two patients were part of the scRNAseq screening group, and all patients carried different combinations of variants in MPO and/or IL36RN: GPP1: 2 MPO and 1 IL36RN variant, completely MPO-deficient; GPP2: 2 MPO variants, partially MPO-deficient; GPP3: 1 MPO variant, partially MPO-deficient; GPP4: 2 IL36RN variants (Table S1). Frequencies of total ILCs in lymphoid cells were nominally, though not significantly higher in four GPP patients compared to five HD (Figure S3). The same was true for ILC3s in a comparison of three GPP patients to the same group of HD (p=0.08).

In addition, we measured a broad spectrum of other cell types - including neutrophils, T helper cells, Tregs, different subsets of monocytes and NK cells - in the two GPP patients of the scRNAseq experiment and one additional patient and four HD (patients carrying different genetic variants as indicated for intracellular markers above, Table S1) by flow cytometry. We did not observe major differences in most cell counts, while the frequency of Th02 subpopulations showed largest nominal differences with a delta mean of >30% between GPP and HD (Figure S2a; data not shown). Although the frequencies of Th01 and Th17 cells varied widely in patients and/or HD, we did not observe evidence for statistical differences.

Pathway analyses in certain clusters of PBMCs

Differentially expressed genes in single cell clusters were used for pathway analysis to identify biologically relevant alterations. In CD4+ CTLs, this revealed negative regulation of leukocyte apoptotic process and positive regulation of cytokine production as the most enriched pathways (Figure 3). DEGs in CD8+ TEMs were most significantly enriched for
regulation of B cell activation. Analyses of DEGs in CD4+ TEM and CD16+ monocytes indicated an enrichment in response to interferon-γ (IFN-γ) and further immune responses e.g. cell killing and response to peptides. These results are in line with an activated state of CD4+ CTLs, CD8+ TEMs, CD4+ TEMs and CD16+ monocytes, even though both GPP patients received immunosuppressive treatment (GPP1: IL-17 inhibitors and methotrexate; GPP2: TNFα-blocker).

Transcriptome analysis of neutrophils

The transcriptome of the three GPP patients, either partially or completely MPO deficient and with an additional deficiency of IL-36Ra in two patients (Table S1), and four HD clustered along PC2 (Figure 4a) in the principal component analysis. Gene ontology based pathway analysis of genes differentially expressed between the two groups (Figure 4b) revealed a highly significant enrichment of genes involved in complement activation alongside of SRP-dependent cotranslational protein targeting to membrane and negative regulation of viral genome replication (Figure 4c). In contrast, a comparison of neutrophil transcriptomes of GPP patients with IL36RN variants from previously published data (Catapano et al., 2020) indicated response to virus as the most significantly enriched pathway beside positive regulation of pattern recognition receptor signaling pathway. Although the numbers of GPP patients in our transcriptome analyses was similar to the previously published one and the bioinformatic methods were the same, the evidence for significance was about five orders of magnitudes higher for the classical pathway of complement activation in MPO-deficient patients compared to response to virus in IL36RN-deficient patients (Figure 4c, S4b).
**Discussion**

The present study contributes to the better understanding of GPP by an integrated analysis of circulating immune cells using scRNAseq. Cluster annotation using multimodal reference mapping (Hao et al., 2020) improved the resolution of clusters especially of CD4+ and CD8+ T cell subsets compared to annotation exclusively relying on expression of marker genes (Hao et al., 2020). By probing protein expression, we confirmed the expansion of CD4+ CTLs in MPO-deficient GPP patients and thereby multimodal reference mapping as a valid method. Similarly, though less precise, targeted gene expression of FGFBP2 reinforced the cluster annotation. Of note, this was even the case, although we did not perform scRNASeq, quantitative PCR (qPCR) and flow cytometry at the same time point. The selection of marker molecules followed by set-up of an individual flow cytometry panel and qPCR required time; therefore two time points were used for these procedures. Reassuringly, also an independent panel of immune cell phenotyping using flow cytometry confirmed an expansion of CD4+ effector cells and a reduction of CD4+ naïve cells.

Generally, clonal expansion of CD4+ T cells has been discovered in GPP patients before, but not differentiated further (Arakawa et al., 2018). Arakawa et al. interpreted the expansion of CD4+ T cells due to IL-17 producing Th17 cells. Our data does not support this interpretation due to similar frequencies of Th17 cells in GPP compared to HD. In chronic plaque psoriasis, CTL subsets of CD8+ T cells have been described as IL-17A producing cells (Hijnen et al., 2013, Ortega et al., 2009, Res et al., 2010), suggesting that CD4+ CTLs may produce IL-17 as well. Whether CD4+ CTLs in GPP were more abundant
due to clonal expansion could be determined in future studies via sequencing of T cell receptors at single cell level (single cell repertoire sequencing).

Considering a potential mechanism leading to abundant CD4+ CTLs in deficiency of IL-36Ra and MPO (Figure 5), previous murine studies indicated that those deficiencies lead to DC activation resulting in or enhancing T cell activation directly (Foster et al., 2014, Odobasic et al., 2013, Vigne et al., 2011). Activated CD4+ CTLs produce pro-inflammatory cytokines and mediate cytotoxicity against target cells in an MHC-II dependent manner (Takeuchi and Saito, 2017). Expression of MHC-II is typically limited to antigen-presenting cells, e.g. DCs; however, atypical expression has been observed in stimulated neutrophils and keratinocytes (Kambayashi and Laufer, 2014). Following CD4+ CTL mediated cytotoxicity, apoptotic cells need to be cleared by phagocytosis, a process previously shown to be impaired in MPO-deficiency for apoptotic neutrophils (Haskamp et al., 2020). Whether clearance of target cells of CD4+ CTLs might be impaired and whether other cells beside neutrophils might be affected, remains to be shown.

Previous ex vivo studies indicated a reduced proportion of CD4+ CTLs after incubation with TNFα blocker in patients with angina pectoris (Rizzello et al., 2006). Although we cannot exclude a general effect of therapies applied in our patients on CD4+ CTLs, our study did not reveal considerably differing expression of the CD4+ CTL marker gene FGFBP2 in five GPP patients treated with infliximab (Figure S2b) compared to other therapeutic groups. As patients without variants had the lowest expression of FGFBP2 within groups of the same treatment, we consider the influence of genetic variants in IL36RN and/or MPO as superior compared to the one by treatment.
Our findings provide a potential novel therapeutic approach in GPP, i.e., inactivating or depleting CD4+ CTLs. Besides infliximab, other monoclonal antibodies inhibiting cytotoxic T cells have been developed (Chemin et al., 2019). Patients with Crohn's disease have been successfully treated in a phase-II clinical trial with an antibody for NKG2D, a receptor on CD4+ CTLs (Allez et al., 2017). Currently in patients with rheumatoid arthritis, an inhibitor of the chemokine fractalkine is investigated; fractalkine can activate CD4+ CTLs via its receptor CX3CR1 (Tanaka et al., 2021).

CD4+ CTLs are also known to produce IFN-γ, which has been shown after their stimulation with PMA/ ionomycin as well as in mouse models infected with various viruses (Takeuchi and Saito, 2017). Recently, IL-36 signaling has been described to be correlated with type I IFN signaling in GPP (Catapano et al., 2020). In subsets of PBMCs of this study (CD4+ TEMs and CD16+ monocytes), DEGs were enriched for the pathway response to interferon, further supporting the relevance of IFN pathways in GPP.

Neutrophils play a key role in pustular psoriasis; and the detection of DEGs involved in the classical pathway of complement activation does not represent its first connection with GPP. In single elder studies, the authors demonstrated an involvement of the complement system in skin of pustular psoriasis patients and in MPO-deficient neutrophils (Stendahl et al., 1984, Tagami and Ofuji, 1977). The complement system is known to escalate inflammatory reactions, an aspect that might explain the multi-systemic and often episodic inflammatory nature of GPP. Of note, this pathway was not enriched in a dataset of previously published GPP patients carrying IL36RN variants (Catapano et al., 2020). Moreover, type I interferon signatures were not prominent in our analysis of these GPP patients with IL36RN variants, as it has been described in the larger group of GPP patients.
regardless from disease-relevant susceptibility alleles. We did not detect an expression of \textit{IL36RN} in neutrophils, while \textit{MPO} is primarily expressed in neutrophils and represents a major neutrophilic protein. Its deficient expression in the patients of this study might therefore influence the expression of other neutrophilic genes and lead to a rather specific enrichment of the \textit{classical pathway of complement activation} in comparison to enriched pathways in carriers of \textit{IL36RN} variants only.

More generally, all patients analyzed in this study had severe disease and therefore received treatments with different immune-modulating therapeutics. We cannot exclude that one or several of those medications might have influenced (some of) our results, although an obvious effect was not observed when analyzing \textit{FGFBP2}'s expression. GPP accounts for a very rare psoriatic manifestation, at least in Europe. When selecting patients of such a rare entity for carrying disease-relevant variants (e.g. \textit{MPO}) and when considering additional availability of this selected group of patients for clinical visits, the numbers of participating patients decreased even further due to private/ disease-related reasons. This rather limited number of patients might explain the mostly marginally significant findings, although absolute values indicated rather large differences. Therefore, confirmation of our findings in independent study groups will be rather essential to elucidate the role of these cell types and pathways in GPP. Moreover, taking into account the heterogeneous combination of variants in up to five GPP genes so far (\textit{IL36RN, CARD14, AP1S3, SERPINA3, MPO}) indicates that the evaluation of gene-related effects can be rather challenging, and the selection of a genetically homogenous patient group might be desirable, but a theoretical construct. Previous publications suggested an oligogenic inheritance model of this rare psoriatic subtype (Haskamp et al., 2020, Mossner et al., 2018, Twelves et al., 2019). Therefore, an analysis including patients with
susceptibility variants in a single gene and without variants in any other known gene cannot take into account so far undetected susceptibility alleles that might differ within the selected group and also influence molecular signatures considerably.

Local interactions between keratinocytes and immune cells during an acute flare of GPP can alter the activation states of immune cells for example; therefore, the analysis of PBMCs and neutrophils from peripheral blood in more stable disease state might not reflect the same functional status as the one of immune cells present in diseased or symptom-free skin. Future studies using e.g. scRNAseq and Cellular Indexing of Transcriptomes and Epitopes by Sequencing from skin biopsies might elucidate the detection of different cell states and relevant molecules.

In sum, this study reveals CD4+ CTLs as a to our knowledge previously unreported cell type in the pathogenesis of GPP depending on variants in IL36RN and MPO. Additionally, we provide evidence for an involvement of the complement system in GPP.

**Material and methods**

**Patients and group of HD**

The study was approved by the ethical committee of the Friedrich-Alexander-Universität of Erlangen-Nürnberg. Written informed consent was obtained from each patient and control individual before enrolment, and investigations were conducted according to Declaration of Helsinki principles. The overall study group consisted of 13 patients with GPP, one with acrodermatitis continua suppurativa Hallopeau (ACH) and one with acute generalized exanthematous pustulosis (AGEP), while fresh peripheral blood for
scRNAseq and RNAseq of neutrophils was available in two and three patients, respectively. Previously described diagnostic criteria were applied (Haskamp et al., 2020). Clinical characteristics, variants in disease-genes and the participation in different parts of this study of all 15 female patients are presented in Table S1. Except for a heterozygous *AP1S3* variant in GPP7, the 14 further GPP patients did not carry coding variants in other disease-genes (*CARD14*, *SERPINA3* and *AP1S3*) apart from *IL36RN* and *MPO*. All ten HD were female, had a similar age (mean 48 years) compared to our patients (mean 53.9 years) and did not carry a disease-associated variant in *MPO* or *IL36RN*.

**Single cell RNA-sequencing of PBMCs**

We isolated PBMCs from whole blood of two patients and three HD using BD Vacutainers (BD, Franklin Lakes, NJ, USA). We performed scRNAseq in a total of 20,508 cells according to manufacturer’s instructions. Libraries were prepared using the Chromium controller (10X Genomics, Pleasanton, CA; USA) in conjunction with the single-cell 3' v2/v3 kit and sequenced on an Illumina HiSeq 2500 sequencer to a depth of 160 million reads/sample. Primary data analysis was performed as previously described (Haskamp et al., 2020); we used Seurat v3 (Stuart et al., 2019) for quality control, downstream data analysis and visualization. More details on filtering processes and data integration are given in the supplementary methods. Pathway analysis for DEGs in single cell clusters (Tables S2, S3) based on Gene Ontology was done using metascape (Zhou et al., 2019) (Table S4).

**Sequencing of RNA from neutrophils**

RNA of neutrophils of three patients and four HD was used for RNA sequencing. Libraries were generated using 200ng of RNA and the TruSeq Stranded mRNA Kit according to
manufacturer’s recommendations (Illumina, San Diego, USA). Libraries were sequenced on a HiSeq-2500 as 100bp single-end reads to a depth of at least 40 million reads. Details of the data analysis are given in the supplementary methods. Pathway analysis was performed as indicated for scRNAseq analysis using the thresholds indicated in Table S4. To compare our results to the previously published ones (Catapano et al., 2020) of RNA from neutrophils of GPP patients and control individuals, we applied the same procedures to the published raw data as described for our data. Targeted gene expression analysis was performed as previously described (Frey et al., 2020).

**Statistical analyses**

We performed statistical analyses using R v3.6.1 (R-Core-Team, 2013). Details on used statistical tests are given in supplementary methods.

**Data Availability Statement**

Datasets related to this article can be found at [https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/) hosted by Gene Expression Omnibus of NCBI (GSE182244 – transcriptomes of PBMCs; GSE181994 - neutrophil transcriptomes).

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Conflict of Interest Statement

M.S. was an investigator, speaker, consultant, or an advisory board member for Abbvie, Actelion, Amgen, BMS, Celgene, Galderma, GSK, Janssen Cilag, Leo, Lilly, MSD, Mundipharma, Novartis, Regeneron, Pfizer, Sanofi, UCB; clinical studies Abbvie, Actelion, Amgen, BMS, Celgene, Galderma, GSK, Janssen Cilag, Leo, Novartis, Pfizer, Regeneron, Sanofi, UCB. All other authors do not declare any competing interests.

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Author Contributions Statement
Conception and design: SH, UH; acquisition of data: SH, CB, DP, ABE, JB, RM, DW, MS, UH; formal analysis: SH, BF, IB, AS, IA, SU, PK; analysis and interpretation of data: SH, UH; original draft preparation: SH, UH; review and editing: SH, BF, IB, AS, IA, CB, DP, ABE, JB, RM, DW, MS, SU, PK, UH.
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Figure Legends

**Figure 1:** Single cell transcriptome of PBMCs in GPP versus HDs. **a-b** Reference uniform manifold approximation and projection (RefUMAP) with 31 annotated clusters colored by cell type (a, as in Table S3) and by sample (b). c Normalized proportions of cell type per proband based on refUMAP annotation and sorted by descending proportions of GPP cells. Numbers at the top correspond to raw cell counts per cluster; shaded colors indicated less common clusters with ≤30 cells counts in the whole study group.

**Figure 2:** Selection of marker genes for CD4+ and CD8+ T cells and verification of proportions of T cell subpopulations at protein and RNA level. **a** RefUMAP colored by gene expression of four markers for CD4+ CTLs and CD8+ TEMs. **b** Cell clusters of effector T cells and their defining markers used in flow cytometry. c Percentage of cell subpopulations analyzed by flow cytometry using combinations as given in (b) corresponding to single/ double/ triple positive protein expression of GZMA, GZMK and KSP37 (FGFPB2) in CD4+ T cells, CD8+ T cells and NK cells; *CD8+ T cells not negative for any of the three markers were summed up. **d** Targeted gene expression of RNA from whole blood of 20 individuals grouped as following: HD and GPP without (w/o) variant; gene names – GPP patients with variant(s) in indicated gene(s). GPP1, GPP2 and GPP3 labeled by colored circles.

**Figure 3:** Enrichment of pathways in effector T cells and CD16+ monocytes. Pathway analysis based on enrichment of gene ontology (GO) terms for differentially expressed genes (DEGs) in CD4+ CTLs, CD8+ TEMs, CD4+ TEMs and CD16+ monocytes.

**Figure 4:** Transcriptome analysis of neutrophils. **a** Principal component analysis of transcriptomes in neutrophils of three MPO-deficient GPP patients and four healthy donors (HD).
Volcano plot illustrating the logarithm of the adjusted p-value versus the logarithmic fold change of every gene. Green dots correspond to genes with a fold change >1.5, blue dots to those with an adjusted p-value <0.05 and dots colored in red fulfil both criteria. Pathways analysis based on enrichment of GO terms for DEGs in neutrophils.

Figure 5: Model of CD4+ cytotoxic T cells (CTLs) in GPP patients with deficiency of the interleukin-36 receptor-antagonist (IL-36Ra) and/or myeloperoxidase (MPO). Unopposed IL-36 signaling due to IL-36Ra deficiency and/or absent MPO promotes activation of dendritic cells (DCs). DCs activate CD4+ T cells via antigen presentation in an MHC-II dependent manner. T cells expressing the IL-36 receptor (IL-36R) can also be directly activated by IL-36 cytokines. After activation, CD4+ T cells proliferate and differentiate to CD4+ CTLs. The effector functions of CD4+ CTLs are the production of pro-inflammatory cytokines and cytotoxicity against MHC-II expressing target cells, which present TCR-specific antigens. Cytotoxicity is mediated by the secretion of e.g. perforin and granzymes.
- **CD4⁺ CTL**
  - GO:00200107: negative regulation of leukocyte apoptotic process
  - GO:0001819: positive regulation of cytokine production
  - GO:0008630: intrinsic apoptotic signaling pathway in response to DNA damage
  - GO:0043550: regulation of lipid kinase activity
  - GO:0006413: translational initiation
  - GO:0043600: regulation of multi-organism process
  - GO:0193322: positive regulation of protein modification by small protein conjugation or removal
  - GO:0046677: response to antibiotic
  - GO:0043588: skin development

- **CD8⁺ TEM**
  - GO:0050864: regulation of B cell activation
  - GO:0000055: nuclear-transcribed mRNA catabolic process
  - GO:0046677: response to antibiotic
  - GO:0046718: viral entry into host cell
  - GO:0031331: positive regulation of cellular catabolic process

- **CD4⁺ TEM**
  - GO:0002764: immune response regulating signaling pathway
  - GO:0001906: cell killing
  - GO:0034341: response to interferon-gamma
  - GO:0031349: positive regulation of defense response
  - GO:0002694: regulation of leukocyte activation
  - GO:0010712: regulation of collagen metabolic process
  - GO:0009617: response to bacterium
  - GO:0002521: leukocyte differentiation
  - GO:003618: regulation of glycoprotein metabolic process
  - GO:0043491: protein kinase B signaling
  - GO:0090303: positive regulation of wound healing
  - GO:0006568: cellular defense response
  - GO:0032675: regulation of interleukin-6 production
  - GO:0019083: viral transcription
  - GO:0052548: regulation of endopeptidase activity

- **CD16⁺ Mono**
  - GO:0034341: response to interferon-gamma
  - GO:01901652: response to peptide
  - GO:0045669: positive regulation of osteoblast differentiation
  - GO:0010035: response to inorganic substance
  - GO:0002761: regulation of myeloid leukocyte differentiation
  - GO:0002521: leukocyte differentiation
  - GO:0001944: vasculature development
  - GO:0002262: myeloid cell homeostasis
  - GO:0001667: ameboidal-type cell migration
  - GO:0050727: regulation of inflammatory response
  - GO:0007568: aging
  - GO:0045936: negative regulation of phosphate metabolic process
  - GO:0006991: generation of precursor metabolites and energy
  - GO:0048589: developmental growth
  - GO:0002366: leukocyte activation involved in immune response
  - GO:0007229: integrin-mediated signaling pathway
  - GO:0009636: response to toxic substance
  - GO:0048469: cell maturation
  - GO:0002576: platelet degranulation
  - GO:0071356: cellular response to tumor necrosis factor
a

PC2: 19% variance

PC1: 29% variance

b

Log_2 fold change

-Log_10 P

GPP1, GPP2, GPP5, HD1, HD2, HD5, HD11

GO:0006958: complement activation, classical pathway
GO:0006614: SRP-dependent cotranslational protein targeting to membrane
GO:0045071: negative regulation of viral genome replication
GO:0015671: oxygen transport
GO:007597: blood coagulation, intrinsic pathway
GO:002337: modified amino acid transport
GO:0200047: regulation of cell-cell adhesion mediated by cadherin
GO:0002831: regulation of response to biotic stimulus
GO:0200106: regulation of leukocyte apoptotic process
GO:00015012: heparan sulfate proteoglycan biosynthetic process
GO:000243: positive regulation of reproductive process
GO:1902624: positive regulation of neutrophil migration
GO:0045576: mast cell activation
GO:0051701: interaction with host
GO:0097061: dendritic spine organization
GO:0042982: amyloid precursor protein metabolic process
GO:0045861: negative regulation of proteolysis
GO:0014068: positive regulation of phosphatidylinositol 3-kinase signaling
GO:0032465: regulation of cytokinesis
MPO: myeloperoxidase
IL-36: interleukin-36
IL-36Ra: interleukin-36 receptor antagonist
IL-36R: interleukin-36 receptor

unrestrained IL-36 signaling & absent MPO activate DC

T cell activation via antigen presentation and IL-36

differentiation & proliferation of CD4+ CTLs

effector functions of CD4+ CTLs promoting inflammation
Supplementary Table 1: Genetic and clinical characteristics of 15 patients with generalized pustular psoriasis analyzed in this study.

<table>
<thead>
<tr>
<th>ID</th>
<th>IL36RN variant(s)</th>
<th>MPO variant(s)</th>
<th>Medication scRNAseq samples (PBMC)</th>
<th>Medication RNA samples (whole blood)</th>
<th>Medication RNA samples (neutrophils)</th>
<th>age of onset of GPP</th>
<th>course of pustular psoriasis</th>
<th>concomitant disease</th>
<th>trigger</th>
<th>sample included for</th>
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<tbody>
<tr>
<td>GPP1_1 &amp; GPP1_2</td>
<td>het. c.115+6C&gt;T</td>
<td>hom. c.2031-2A&gt;C</td>
<td>GPP1_1: Secukinumab (IL-17A inhib.), Methotrexate; GPP1_2 Ixekizumab (IL-17A inhib.), Methotrexate</td>
<td>Secukinumab (IL-17A inhib.)</td>
<td></td>
<td>29</td>
<td>continuous</td>
<td>geographic tongue, PsA, Hypertension, polyarthriti</td>
<td>Surgery, contrast agent used for CT scans</td>
<td>scRNAseq, RNAseq, RT-qPCR, FACS, ILCs</td>
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<tr>
<td>GPP2_1 &amp; GPP2_2</td>
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<td>c.het. c.995C&gt;T/(p. Ala332Val), c.2031-2A&gt;C</td>
<td>Infliximab (TNFα inhib.)</td>
<td>for both samples: Infliximab (TNFα inhib.)</td>
<td>Infliximab (TNFα inhib.)</td>
<td>2</td>
<td>episodic</td>
<td>PPP, nail dystrophy</td>
<td>infection, reduce or stop of medication, stress, alcohol</td>
<td>scRNAseq, RNAseq, RT-qPCR, FACS, ILCs</td>
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<td>GPP3</td>
<td>none</td>
<td>het. c.2031-2A&gt;C</td>
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<td>Prednisolone (steroid medication), Azathioprine (inhibitor of purine synthesis)</td>
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<td>RT-qPCR, FACS, ILCs</td>
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<td>ID</td>
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<td>MPO variant(s)</td>
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<td>course of pustular psoriasis</td>
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<td>trigger</td>
<td>sample included for</td>
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<td>het. c.1705C&gt;T/(p. Arg569Trp)</td>
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<td>RT-qPCR</td>
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<td>GPP7* (AGEP)</td>
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<td>het. c.1555_1568del/(p.Met519Profs*21)</td>
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<td>no immunosuppressive therapy</td>
<td>-</td>
<td>48</td>
<td>episodic irregular</td>
<td>PPP, geographic tongue, stress, medication</td>
<td>RT-qPCR</td>
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<td>GPP8</td>
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<td>het. c.T752C/(p.Met251Thr)</td>
<td>-</td>
<td>Ustekinumab (IL-12/IL-23 blocker)</td>
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<td>9</td>
<td>continuous</td>
<td>PsV, geographic tongue, PsA, polyarthriti s</td>
<td>reduction or stop of medication</td>
<td>RT-qPCR</td>
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<td>GPP9 (ACH)</td>
<td>het. c.142C&gt;T/(p. R48W)</td>
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<td>-</td>
<td>Adalimumab (TNFα inhib.), Methotrexate</td>
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<td>PsA</td>
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<td>-</td>
<td>Mycophenolate Mofetil (inhibits activated lymphocytes)</td>
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<td>61</td>
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<td>none</td>
<td>medicati on</td>
<td>RT-qPCR</td>
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<td>ID</td>
<td>IL36RN variant(s)</td>
<td>MPO variant(s)</td>
<td>Medication scRNAseq samples (PBMC)</td>
<td>Medication RNA samples (whole blood)</td>
<td>Medication RNA samples (neutrophils)</td>
<td>age of onset of GPP</td>
<td>course of pustular psoriasis</td>
<td>concomitant disease</td>
<td>trigger</td>
<td>sample included for</td>
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<td>stress</td>
<td>RT-qPCR</td>
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<td>stress</td>
<td>RT-qPCR</td>
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<td>Secukinumab (IL-17A inhib.)</td>
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<td>40 episodic irregular</td>
<td>PsV, PsA, polyarthritis flue, infection, stress</td>
<td>RT-qPCR</td>
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<td>GPP14</td>
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<td>U</td>
<td>U</td>
<td>U</td>
<td>RT-qPCR</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** c.het: compound heterozygous, FACS: fluorescence-activated cell sorting, het.: heterozygous, hom.: homozygous, ILCs: innate lymphoid cells, inhib. = inhibitor, PsA= psoriatic arthritis, PsV= psoriasis vulgaris, RNASeq: RNA sequencing of RNA from neutrophils, RT-qPCR: reverse transcriptase quantitative PCR on RNA from peripheral blood cells, scRNAseq: single cell RNA sequencing from PBMCs, U= unknown, # indicate two samples of the same patient.

*GPP7 with AGEP carried an additional heterozygous AP1S3 variant c.97C>T/ p.Arg33Trp.*
Supplementary Table 2: Selected cell types and their frequency in PBMCs of healthy donors (HD) and of GPP patients. Abbreviations of cell names as in Table S3.

<table>
<thead>
<tr>
<th>cell types</th>
<th>Frequency of PBMC in percent (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HD</td>
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<tr>
<td>CD14⁺ monocyte</td>
<td>15.99</td>
</tr>
<tr>
<td>CD16⁺ monocyte</td>
<td>5.55</td>
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<tr>
<td>CD4⁺ naïve T cell</td>
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<tr>
<td>CD4⁺ proliferating T cell</td>
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<tr>
<td>CD4⁺ CTL</td>
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<tr>
<td>CD4⁺ TCM</td>
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<tr>
<td>CD4⁺ TEM</td>
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<td>CD8⁺ naïve T cell</td>
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<tr>
<td>naïve B cell</td>
<td>2.37</td>
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<tr>
<td>intermediate B cell</td>
<td>1.81</td>
</tr>
<tr>
<td>memory B cell</td>
<td>2.62</td>
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Supplementary Table 3: Definition of abbreviations used for cell types in multimodal reference mapping as indicated by Hao et al. (2020).

<table>
<thead>
<tr>
<th>cell types</th>
<th>description</th>
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<tbody>
<tr>
<td>ASDC</td>
<td>dendritic cells defined by the expression of AXL and SIGLEC6</td>
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<tr>
<td>B intermediate</td>
<td>intermediate B cells</td>
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<tr>
<td>B memory</td>
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<td>B naive</td>
<td>naive B cells</td>
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<td>CD14 Mono</td>
<td>CD14$^+$ monocytes</td>
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<td>CD16 Mono</td>
<td>CD16$^+$ monocytes</td>
</tr>
<tr>
<td>CD4 CTL</td>
<td>CD4$^+$ cytotoxic T lymphocyte</td>
</tr>
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<td>CD4 Naive</td>
<td>CD4$^+$ naïve T cell</td>
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<tr>
<td>CD4 Proliferating</td>
<td>CD4$^+$ proliferating T cell</td>
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<tr>
<td>CD4 TCM</td>
<td>CD4$^+$ central memory T cell</td>
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<tr>
<td>CD4 TEM</td>
<td>CD4$^+$ effector memory T cell</td>
</tr>
<tr>
<td>CD8 Naive</td>
<td>CD8$^+$ naïve T cell</td>
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<tr>
<td>CD8 Proliferating</td>
<td>CD8$^+$ proliferating T cell</td>
</tr>
<tr>
<td>CD8 TCM</td>
<td>CD8$^+$ central memory T cell</td>
</tr>
<tr>
<td>CD8 TEM</td>
<td>CD8$^+$ effector memory T cell</td>
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<td>cDC1</td>
<td>CD141$^+$ conventional dendritic cell</td>
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<td>cDC2</td>
<td>CD1C$^+$ conventional dendritic cell</td>
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<td>dnT</td>
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<td>Doublet</td>
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<td>gdT</td>
<td>gamma/delta T cell</td>
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<td>NK_CD56bright</td>
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<td>Treg</td>
<td>regulatory T cell</td>
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Supplementary Table 4: Different parameters for analyses of differentially expressed genes (DEG) and enrichment analyses. Abbreviations of cell names as given in Table S3.

<table>
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<tr>
<th>Cell type</th>
<th>No. of DEG</th>
<th>Threshold of adjusted p-value</th>
<th>Threshold of log2 fold change</th>
<th>No. of enriched pathways</th>
<th>Thresholds of adjusted p-value</th>
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<tbody>
<tr>
<td>CD4+ CTL</td>
<td>40</td>
<td>0.01</td>
<td>0.59</td>
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<td>Neutrophils</td>
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<td>0.05</td>
<td>0.59</td>
<td>19</td>
<td>9.143E-03</td>
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</table>
Figure S1: Further analyses on single cell transcriptomes of PBMCs from GPP and HD.  

a RefUMAP colored by gene expression level of known markers of cell types. 
b UMAP with 31 annotated cell types (left panel) and colored by sample (right panel; description of cell types as in Table S3). 
c UMAP colored by 17 clusters resulted from unsupervised clustering. 
d Normalized proportions of clusters per proband based on unsupervised clustering.
Figure S2: Extended analyses on T cell subpopulations and expression of marker gene *FGFBP2* in whole blood dependent on therapeutics and number of susceptibility variants. 

a Percentage of cell subpopulations in CD4+ T cells, CD8+ T cells, T helper cells and Tregs analyzed by flow cytometry using a published panel with significant p-values shown.

b, c Targeted gene expression analysis of *FGFBP2* in 15 patients grouped by the corresponding systemic medication and colored by variants in *IL36RN* and *MPO* (b) or grouped by the number of variants in *IL36RN* and *MPO* (c).
Figure S3: FACS analysis of innate lymphoid cells (ILCs). a Flow cytometry analysis of ILCs of four GPP patients and four healthy donors (HD). HD2_2 and GPP4 were analyzed in a second experiment. Low number of total ILCs in GPP3 did not allow for reliable quantification of ILC subsets.
Figure S4: Transcriptome analysis of neutrophils as published by Catapano et al. (2020). a Principal component analysis including 19 transcriptomes of eight GPP patients and of eleven healthy donors (HD). Patients were either negative for IL36RN variants (n=5) or carried 1-2 IL36RN variants (n=3). b Pathway analysis based on enrichment of GO terms for DEGs between three carriers of IL36RN variants and healthy donors.
**Supplementary text contains details on materials and methods**

**Single cell RNA-sequencing of peripheral blood mononuclear cells**

In order to investigate the contribution of peripheral blood immune cells to GPP, we performed scRNAseq in total of 20,508 peripheral blood mononuclear cells (PBMCs) from two patients and three healthy donors (HD). In a first experiment, we could analyze patients GPP1 and GPP2 as well as HD1 and HD3. In the second experiment, we extended the analysis to HD2 and included cells/data of patient GPP1 in order to overcome the lower number of this individual’s cells sequenced in the first experiment.

We isolated PBMCs from whole blood using BD Vacutainers (BD, Franklin Lakes, NJ, USA) according to the manufacturers’ instructions. We aimed for 6,000 cells per sample. Libraries were prepared using the Chromium controller (10X Genomics, Pleasanton, CA; USA) in conjunction with the single-cell 3’ v2 kit (1st experiment) or single-cell 3’ v3 (2nd experiment) 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 (Haskamp et al., 2020) and used Seurat v3 (Stuart et al., 2019) for quality control, downstream data analysis and visualization.

We filtered cells with regard to the number of features (lower limit of 200; number of upper limit of 2,500 for single-cell 3’ v2 and of 4,000 for single-cell 3’ v3), percentage of mitochondrial RNA (<10: 1st experiment; <22: 2nd experiment) and the percentage of ribosomal proteins (>5%). Data sets were normalized and integrated using the functions sctransform (batch_var= sample) and “IntegrateData” with 30 dimensions, respectively. Multimodal reference mapping was performed according to the vignette online using Seurat v4 and a CITEseq reference dataset of 162,000 PBMCs combined with a panel of 228 antibodies previously described.
The quality of the mapping was assessed in several ways. After merging the reference data set and our data set, we obtained a UMAP (data not shown). All clusters were shared between the reference and our data (query). Therefore, we are confident that the reference data contains all cell types, present in our data. Along with the annotation per cell, the program also assigned a prediction score. The score varied substantially, especially in the clusters of effector T cells (data not shown). This prompted us to verify the proportions in patients and HD by flow cytometry.

We also determined the proportions of each sample normalized to the total cell number within each cluster (erythrocytes and platelets excluded) (Figure 1c) to ensure that differences in the composition of clusters are not due to a difference in total cell number per sample.

We determined differentially expressed genes (DEG) within a cluster with “FindMarkers” with default settings (Stuart et al., 2019). Cells other than erythrocytes containing more than 1% of hemoglobin transcripts were excluded from calculations. A threshold of a log fold change of 0.25 and a minimal expression in 10% of all cells per cluster was used, and we performed pathway analyses in clusters with at least 10% of genes significantly expressed (>0.4 absolute log fold change, <0.01 adjusted p-value). Pathway analysis for DEGs based on Gene Ontology was done using metascape (Zhou et al., 2019) with a threshold of an adjusted p-value 0.01 and of an absolute log2 fold change of 0.59 (Table S4) and a list of background genes defined as the top 90% of all expressed genes per cluster.

Flow cytometry-based assay of human PBMCs

Fresh whole blood was analyzed by immunophenotyping (IPT) by multicolor flow cytometry according to standard operating procedures as described earlier.
Supplementary material to Haskamp et al. (Donaubauer et al., 2020, Donaubauer et al., 2019, Ruhle et al., 2016). We gained whole blood samples from GPP1, GPP2, GPP4 and four HD at the same 2nd time point of experiments described above. The flow cytometric data acquisition was done on a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) with standard filter configuration. For data analysis, we used the Kaluza Flow Analysis Software (Beckman Coulter) according to the previously described gating strategy and defined the immune cell populations. The detection of intracellular targets (Granzyme A, Granzyme K and KSP37) was performed on PMBCs of patients GPP1, GPP2, GPP3 and four HD. We stained about 500,000 PBMCs with CD4-PerCP-CyC5.5, CD8-FITC, CD3-V450 and CD56-PerCP-CyC5.5 (all from Beckman Coulter) for 30 min in the dark. After intensive wash cycles, we consecutively stained for Granzyme A (Alexafluor 700), Granzyme K (PE-Cy7) and KSP37 (PE) (BioLegend, San Diego, CA, USA) using the Cyto-Fast™ Fix/Perm Buffer Set (BioLegend) according to the manufacturer’s instructions. Cells were also measured on a Gallios flow cytometer (Beckman Coulter) and analyzed with the Kaluza software package (Beckman Coulter). Intracellular markers were analyzed in separate batches to see expression on the following cell populations: CD3^pos^CD4^pos^CD8^neg^ or CD3^pos^CD4^neg^CD8^pos^ T cells and CD3^neg^CD56^pos^ NK cells similar to previous publications (Bade et al., 2005).

Identification of peripheral blood innate lymphoid cells (ILCs) by flow cytometry

To identify human ILCs by flow cytometry, single PBMC suspensions in 1x PBS supplemented with 1% FBS and 2mM EDTA were incubated with a FcR blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany) for 10 min at 4°C prior to staining cells with fluorochrome-conjugated antibodies directed against the following human antigens: lineage marker (including CD2 (RPA-2), CD3 (OKT3), CD14 (61D3), CD16 (CB16), CD19 (HIB19), CD56 (CB56), and CD235a (HIR2); eFlour450;
eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), CD11c (VioBlue; MJ4-27G12; Miltenyi Biotech), CD127 (APC/Vio770; REA614; Miltenyi Biotech), CD7 (FITC; CD7-6B7; BioLegend), CRTH2 (PE; BM16; Miltenyi Biotec), and CD117 (APC; 104D2; BioLegend). Total ILCs were identified as Lin⁻CD127⁺CD7⁺ single lymphoid cells as shown before (Schulz-Kuhnt et al., 2020). The three classical subsets of helper ILC were further differentiated based on their CD117 and CRTH2 expression as follows: ILC1s (CD117⁻CRTH2⁻), ILC2s (CRTH2⁺), and ILC3s (CD117⁺CRTH2⁻).

Respective isotype controls combined with fluorescence-minus-one stainings served as controls. Antibodies were incubated for 20 min at 4°C in the dark and subsequently fixed in 1x BD CellFix (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Measurements were performed using a MACSQuant 16 cell analyzer equipped with the MACSQuantify software (Miltenyi Biotec). Further data evaluation was performed with the FlowJo v10.06.01 software (BD Biosciences.). If less than 20 ILCs were gated in total, samples were excluded from further analyses of ILC subsets. GPP4 and HD2_2 were analyzed in a 2nd experiment.

Sequencing of RNA from neutrophils

RNA of neutrophils from GPP1, GPP2, GPP5 and four HD was used for next generation sequencing. Neutrophils were isolated using MACSxpress Whole Blood Neutrophil Isolation Kit in combination with MACSxpress Erythrocyte Depletion Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). For isolation of RNA, we used the RNAeasy Mini kit from Qiagen (Hilden, Germany). Beta-mercaptoethanol was added to RLT buffer as mentioned in the manufacturers’ protocol. The quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Sequencing libraries were generated using 200 ng of RNA and the TruSeq Stranded mRNA Kit according to manufacturer’s instructions (Illumina, San Diego, USA).
Libraries were sequenced on a HiSeq-2500 platform (Illumina) as 100 bp single-end reads to a depth of at least 40 million reads. Reads were converted to FASTQ format while masking Illumina adapter sequences (bcl2fastq v2.17.1.14, Illumina). Additionally, reads with more than 50% masked bases (fqtrim v0.9.5) were removed.

The filtered reads were mapped to the Homo sapiens reference transcriptome based on GRCh37 and the Ensembl gene annotation 99, using Salmon v1.1.0. Mapping to the GRCh37 reference genome, Ensembl gene annotation 99, was performed using STAR (version 2.6.1c). Mapped reads were quantified as reads per gene, while excluding exons shared between more than one gene (subread featureCounts v1.6.1). Based on this quantification, DEGs were determined using a negative binomial model as implemented in DESeq2 v1.30.1 under R v4.0.4. Results from significance tests were corrected for multiple testing (Benjamini-Hochberg).

The PCA plot for RNAseq samples is shown in Figure 4a. Pathway analysis was performed as indicated for scRNAseq analysis using the thresholds indicated in Table S4. To compare our results to the previously published ones (Catapano et al., 2020) of RNA from neutrophils of GPP patients and control individuals, we applied the same procedures to the published raw data as described for our data. The PCA plot of all RNAs including three carriers of one to two IL36RN variants, five non-carriers and eleven healthy donors and the enrichment pathways of DEGs in patients with IL36RN variants compared to healthy donors are depicted in Figure S3.

Targeted gene expression analysis was performed as previously described (Frey et al., 2020). We used 100 ng of RNA for cDNA synthesis. For analysis of FGFBP2, we performed quantitative PCR using the TaqMan probe Hs00230605_m1 (Thermo Fisher Scientific, Waltham, MA, USA). ACTB (order number 4352935E, Thermo
Fisher), B2M (4326319E, Thermo Fisher) and PGK1 (4326318E, Thermo Fisher) served as housekeeping genes.

**Statistical analyses**

We performed statistical analyses using R v3.6.1 (R-Core-Team, 2013). P-values for targeted gene expression was calculated by Wilcoxon test or Kruskal Wallis test (Figures 2d, S2c). To assess potential differences between patients and HD in flow cytometry of different cell subsets (Figure 2c, S2a), we performed a t-test as implemented in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).
Supplementary references


**Figure S1:** Further analyses on single cell transcriptomes of PBMCs from GPP and HD.  
- **a** RefUMAP colored by gene expression level of known markers of cell types.  
- **b** UMAP with 31 annotated cell types (left panel) and colored by sample (right panel; description of cell types as in Table S3).  
- **c** UMAP colored by 17 clusters resulted from unsupervised clustering.  
- **d** Normalized proportions of clusters per proband based on unsupervised clustering.

**Figure S2:** Extended analyses on T cell subpopulations and expression of marker gene *FGFBP2* in whole blood dependent on therapeutics and number of susceptibility variants.  
- **a** Percentage of cell subpopulations in CD4+ T cells, CD8+ T cells, T helper cells and Tregs analyzed by flow cytometry using a published panel with significant p-values shown.  
- **b, c** Targeted gene expression analysis of *FGFBP2* in 15 patients grouped by the corresponding systemic medication and colored by variants in *IL36RN* and *MPO* (b) or grouped by the number of variants in *IL36RN* and *MPO* (c).

**Figure S3:** FACS analysis of innate lymphoid cells (ILCs).  
- **a** Flow cytometry analysis of ILCs of four GPP patients and four healthy donors (HD). HD2_2 and GPP4 were analyzed in a second experiment. Low number of total ILCs in GPP3 did not allow for reliable quantification of ILC subsets.

**Figure S4:** Transcriptome analysis of neutrophils as published by Catapano et al. (2020).  
- **a** Principal component analysis including 19 transcriptomes of eight GPP patients and of eleven healthy donors (HD). Patients were either negative for *IL36RN* variants (n=5) or carried 1-2 *IL36RN* variants (n=3).  
- **b** Pathway analysis based on enrichment of GO terms for DEGs between three carriers of *IL36RN* variants and healthy donors.