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Peripheral T cell populations are differentially affected in monogenic autoinflammatory syndromes and gout

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Abstract

Purpose: Both monogenic autoinflammatory syndromes, such as Chronic Granulomatous Disease (CGD) and Familial Mediterranean Fever (FMF), and the common inflammatory disease gout are characterized by episodes of sterile inflammatory attacks in the absence of an infection. While these disorders encompass distinct pathologies due to differentially affected metabolic pathways and inflammasome activation mechanisms, their common features are the excessive production of interleukin (IL)-1β and innate immune cell hyperreactivity. On the other hand, the role of T cells and innate-like lymphocytes such as gamma delta (γδ) T cells in these pathologies is ill-defined. Methods: In order to widen our understanding of T cell involvement in FMF, CGD and gout pathology we developed multicolour immunophenotyping panels for flow cytometry and functional assays to characterise γδ T cells, as well as CD4 and CD8 T cell populations in terms of their cytokine production, activation status, memory or naive phenotypes, exhaustion status, homing receptor expression, and cytotoxic activity. Results: Our study is the first deep immunophenotyping analysis of T cell populations in FMF, CGD and gout patients. We found that CGD affects the frequencies and activation status of T cells, while gout impairs cytokine production capacity of Vδ2 T cells and reshapes homing receptor expression by T cells. FMF was characterized by only minor effects on T cell populations. Conclusion: Autoinflammatory syndromes differentially affect T cell compartments. Future studies are warranted to assess whether these phenotypical changes are relevant for disease pathology.

Keywords: autoinflammatory syndromes, gout, T cells, Familial Mediterranean Fever, Chronic Granulomatous Disease, γδ T cells
Introduction

Autoinflammatory syndromes are a recently described group of distinct heritable disorders, mostly affecting skin, joints, gut, and eyes, and are characterized by episodes of sterile fever and inflammation, mediated predominantly by cells and molecules of the innate immune system (1, 2). These autoinflammatory disorders are classically seen as interleukin (IL)-1β-mediated pathologies. IL-1β maturation and secretion are under control of inflammasomes, multiprotein complexes containing the cysteine protease caspase-1, that are activated both by infections as well as endogenous stimuli (e.g., metabolic stimuli, stress, etc). The same mechanisms regulate IL-18 secretion. Several distinct mechanisms lead to disturbances of this common pathway in autoinflammatory syndromes, mainly alterations in inflammasome activation (3) and in various metabolic pathways (4). While many of these syndromes have been originally characterised by the absence of auto-antibodies or auto-reactive T cells, CGD-causing mutations result in the presence of lupus-like autoantibodies and autoreactive T cells (5–7). Therefore, the lymphoid compartment might still be affected and contribute to the pathology of the diseases (8, 9). Yet, a systematic analysis of the T cell compartment, including innate-like gamma delta (γδ) T cells, has not been performed in these group of diseases.

The human T cell compartment is composed of conventional alpha beta (αβ) T cells and unconventional γδ T cells. The T cell receptor (TCR) of αβ T cells recognizes processed antigenic peptides presented by major histocompatibility complex (MHC). γδ T cells are distinct from conventional αβ T cells (CD4 T helper and CD8 cytotoxic T cells) in the rapidity of their initial response, the manner to recognize and respond to foreign or self-non-peptide molecules, as well as their tissue distribution within the body (10). In addition to the TCR composed of γ and δ chains, γδ T cells express innate receptors such as natural killer group 2 member D (NKG2D) and Toll-like receptors (TLRs) (11). Due to their unique characteristics, which encompass both adaptive and innate immune features, they are considered to be innate-like T cells.

IL-1β is known to regulate adaptive immune responses either by directly stimulating T cells or by acting on innate immune cells such as dendritic cells (12–18). The general effect of IL-1β on αβ T cells manifests in promoting their expansion, survival and cytokine production (19–22). While IL-1β potentiates responses of CD8 and various CD4 T cell lineages (19,21,23) it is mainly known to promote Th17 responses (24–38) as well as to induce IL-17-producing γδ T cells in mice (39). Interestingly, many autoinflammatory conditions, including FMF and gout, display upregulation of Th17-related cytokines (40–42). Not only IL-1β affects T cell responses, recent studies
have shown that CD8 and CD4 T cells can in turn modulate the production of IL-1β in myeloid cells (43–45). IL-18, a Th1 polarising factor, is also under the NLRP3 control (46). Moreover, a recent study has shown that NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) can assemble in CD4 T cells upon stimulation of the C5a receptor 1 (C5aR1) by the complement system, and this mediates Th1 immunity (47). Consistently, CD4 T cells from cryopyrin-associated periodic syndrome (CAPS) patients, which carry gain-of-function mutations in NLRP3 encoding gene, exhibit increased IL-1β and IFN-γ production (47). However, the contribution of CD4, CD8 and γδ T cells in the pathogenesis of many monogenic autoinflammatory syndromes and gout remains unknown and needs to be revealed.

Our study focuses on three diseases with known dysregulation of IL-1 cytokine production: FMF, the most prevalent monogenic autoinflammatory disease worldwide, Chronic Granulomatous Disease (CGD) which has both characteristics of autoinflammation and immunodeficiency, and gout, which is the most common form of inflammatory arthritis (48,49). FMF is a recessively inherited and caused by gain-of-function mutations in the MEFV (Mediterranean FeVer) gene encoding the pyrin protein (50). Pyrin is mainly expressed in innate immune cells (51) where the activated form of the protein promotes oligomerization of apoptosis-associated speck-like protein with a caspase-recruitment domain (ASC) and inflammasome formation resulting in IL-1β production (52). Although the expression of MEFV gene has not been detected in T cells by The Human Protein Atlas (53), it has been found in other source databases such as BloodSpot (https://servers.binf.ku.dk/bloodspot/).

Furthermore, numerous early studies have postulated the activation of T cell compartment during inflammatory attacks in FMF patients (8,54). Another monogenic autoinflammatory disorder, CGD, is caused by mutations in genes encoding components of the reduced nicotinamide dinucleotide phosphate (NADPH) oxidase complex: gp91phox, p22phox, p67phox, p40phox or p47phox, which generates reactive oxygen species (ROS) (55,56). As a result, phagocytes such as neutrophils, monocytes, and macrophages cannot properly clear phagocytized microorganisms leaving the body vulnerable to frequent infections and chronic inflammation (55). This leads to life-threatening bacterial and fungal infections and granuloma formation (57). In contrary, gout is not associated with monogenic mutations but is caused by increased concentrations of urate in the serum, which leads to the formation of monosodium urate (MSU) crystals in the joints (58). The pathogenesis of gout is known to be driven mainly by innate immune cell responses in which inflamasomes play a crucial role (59). As such, innate immune cells produce an excess amount of IL-1β upon engulfing MSU crystals (60). It has been shown that uric acid and MSU crystals have stimulatory effect also on T cells and can enhance T cell responses upon secondary stimuli (61,62).
Moreover infiltrated T cells were found in the tissues of gout patients (63). The involvement of T cells in the pathogenesis of gout is however poorly known.

Therefore, the aim of our study is to assess the lymphoid compartment with a focus on T cells in patients with FMF, CGD and gout.

Methods

Patient Recruitment

All gout, CGD and FMF patients gave informed consent to use leftover blood for research purposes. Blood draw from healthy volunteers were approved by the Ethical Committee of the Radboud University Medical Center (no. NL32357.091.0 and no. NL42561.091.12).

PBMCs staining for flow cytometry

PBMCs were isolated by density gradient centrifugation on Pancoll (Pan Biotech). PBMCs were washed with PBS and incubated with Fc block solution (BioLegend) for 10 minutes. The antibody mixes (Table S1) in staining buffer (BD Bioscience) were added and cells were incubated for 30 minutes at 4°C in the dark. Samples were washed and stored at 4°C in the dark until the reading.

Intracellular cytokine staining for flow cytometry

PBMCs were incubated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma-Aldrich) and ionomycin (1 µg/mL, Sigma-Aldrich) in the presence of Golgi Plug and Golgi Stop (BD Biosciences) in RPMI 1640 complete medium (10% fetal bovine serum, 1% of 100 mM sodium pyruvate (Gibco), 1% of 200mM glutamax (Gibco), 1% of 10,000 U/ml Penicillin and 1% of 10 mg/ml Streptomycin (Pan Biotech)) for 4 hours at 37°C. Then, cells were washed with cold PBS and Fc blocking followed by surface marker staining were performed as described above. Cells were washed with PBS and incubated in cytofix/cytoperm permeabilization solution (BD Bioscience) for 30 minutes at 4°C in the dark. Cells were washed with the washing solution (BD Bioscience) and antibody mix in the washing solution was added. After 30 minutes incubation at 4°C in the dark, the cells were washed and stored at 4°C in cell fixation solution (BD Bioscience) until analysis.

Data Analysis
Flow cytometry data was analyzed using FlowJo (version 10.0) software. Graphs were generated using GraphPad Prism (version 8.4.3) software. Non-parametric Mann-Whitney t-test was applied to calculate statistical significance. Two-tailed P values were considered statistically significant if below 0.05. Significant P values were shown with asterisks as follows * < 0.05, ** < 0.01, *** < 0.001

Results
To characterize the T cell populations in patients with autoinflammatory syndromes and gout in steady state, we applied several flow cytometry multi-color immunophenotyping panels on peripheral blood mononuclear cells (PBMCs) isolated from patients in between febrile episodes and from healthy controls (Supplementary Table 1). We hypothesize that T cell populations can undergo phenotypical and functional changes caused by recurrent inflammation, deficiencies in metabolic pathways in monogenic autoinflammatory syndromes, or hyperuricemia in gout. T cell subpopulations were investigated in-depth for their 1) activation status and naïve/memory phenotype, 2) susceptibility to apoptosis, 3) exhaustion status, 4) cytokine production ability, 5) homing potential, 6) adhesion and 7) cytotoxic markers.

CGD affects the distribution of peripheral immune cells
First, we determined whether the autoinflammatory diseases affect immune cell distribution in peripheral blood. Our gating strategy enabled discrimination of γδ, CD4, and CD8 T cell subpopulations as well as B cells, CD56+ natural killer (NK) cells, regulatory T cells and NKT-like cells (Figure 1 and Figure S1). Two main subsets of γδ T cells have been described in humans: Vδ1 and Vδ2, which mainly reside within epithelial tissues or are found in peripheral blood, respectively (64). Because Vδ2 T cells are the most prevalent γδ T cell population in human peripheral blood consisting up to 90% of the total γδ T cells (65), they were the main focus among γδ T cells in this study. We found significantly decreased percentages of Vδ1, Vδ2, CD4 T cells, and CD56+ NK cells in CGD patients compared to healthy controls (Figure 1a, b, c, h). The distribution of cell populations within PBMCs remained largely similar in FMF and gout patients comparing to healthy controls (Figure 1).

Naïve and memory T cell compartments are not affected by autoinflammatory syndromes
To further scrutinize T cell populations in autoinflammatory syndromes and gout, we analyzed the distribution of naïve (T_{Naive}), effector memory (T_{EM}), central memory (T_{CM}) and terminally differentiated T cells (T_{EMRA}) based on CD45RA and CD27 expression (Figure S2) (66). These distinct T cell subsets differ in their effector function...
such as: T\textsubscript{Naive} cells (CD45RA\textsuperscript{+}CD27\textsuperscript{+}) do not mediate effector immune responses effectively, T\textsubscript{CM} (CD45RA\textsuperscript{+}CD27\textsuperscript{+}) cells have a higher sensitivity to antigenic stimulation and proliferative potential, T\textsubscript{EM} (CD45RA\textsuperscript{+}CD27\textsuperscript{−}) cells exhibit rapid effector function and lack of proliferative capacity, while T\textsubscript{EMRA} (CD45RA\textsuperscript{+}CD27\textsuperscript{−}) cells represent the most differentiated type of memory cells and express high levels of cytotoxic molecules (66). We observed a large variability in distribution of different T cell subpopulations between individuals, and no significant changes could be detected between patients and healthy controls (Figure S2b, c, d). Consistently, we did not observe significant changes in the expression of other naïve and memory markers: CD127 and CD45RO, respectively (data not shown). Thus, the distribution of different effector subpopulations among T cells is not affected in gout and autoinflammatory syndromes.

**V\textsubscript{d}2 and CD8 T cells patients exhibit increased activation status in CGD**

CD38 and CD69 are induced on T cells upon activation and therefore are commonly used as markers of activated or differentiated cells (67,68). We observed elevated proportions of CD38- and CD69- expressing V\textsubscript{d}2 T cells and CD69-expressing CD8 T cells in CGD patients (Figure 2a, d, f), while the reduced number of CD38-expressing CD4 T cells was observed in gout (Figure 2b). Overall, this data suggests that the activation status of T cells is increased in CGD. We further assessed the expression profile of the death receptor CD95 (Figure S3a), which is known to induce apoptosis (69). The numbers of CD95-expressing V\textsubscript{d}2 T cells and CD4 T cells were elevated in CGD and in gout patients, respectively (Figure 3a, b). The number of CD95-expressing CD8 T cells was also increased in gout patients, but did not reach statistical significance, possibly due to the small number of donors (Figure 3c). Increased activation of T cells can lead to exhaustion of these cells. Our results show that the expression of the exhaustion marker PD-1 is not affected in patients in comparison to healthy controls (Figure 3d-f and S3b), while numbers of CTLA-4-expressing CD8 T cells increases in FMF and gout patients (Figure 3g-I and S3c). It is however important to mention that the numbers of CTLA-4 expressing cells were very low, questioning a functional relevance of this observation. This data indicates that FMF and gout conditions may affect the exhaustion status of CD8 T cells.

**Cytokine production by V\textsubscript{d}2 T cells, but not conventional αβ T cells, is impaired in gout patients**

The effector function of T cells is largely driven by their cytokine production potential. Previous clinical studies demonstrated that cytokine production patterns are disrupted in patients with autoinflammatory syndromes and these changes might help to distinguish different autoinflammatory syndromes and their severity (70). We
assessed whether capacity to produce cytokines by T cells is also affected in patients with FMF, CGD and gout. Our analysis revealed a significant reduction of IFN-γ- and TNF-α-producing Vδ2 T cells in gout (Figure 4a, d and S5). There were no significant changes in cytokine production among CD4 and CD8 T cells in all patients. We also analyzed production of other cytokines including: IL-4, IL-9, and IL-17α, however we could not detect significant production of these cytokines (Figure S5c and data not shown). Thus, Vδ2 T cells are more susceptible to metabolic alterations in cytokine production than conventional T cells.

Gout reshapes the homing receptor expression pattern on T cells

Immune cell migration to inflamed tissues is an important component of the inflammatory processes. We therefore examined the expression profile of homing receptors to better understand the migratory status of Vδ2, CD4, and CD8 T cells in the autoinflammatory syndromes and gout (Figure 5, S6 and S7). The expression of the following chemokine receptors was assessed by flow cytometry: CCR2 (Figure 5a-c and S7a), which induces cell recruitment to sites of inflammation (71); CCR5 (Figure 5d-f and S7b), which regulates cell trafficking to the site of inflammation but also retention in tissues (72); CCR7 (Figure 5g-i and S7c), which mediates T cell migration from the blood to secondary lymphoid tissues (73); CCR8 (Figure 5j-i and S7d) and CCR4 (Figure S6a-c and S7e), which are skin homing receptors (74, 75) as well as CXCR3 (Figure S6d-f and S7f), which functions as a homing receptor to sites of infection and inflammation (76). We observed increased numbers of CCR5-expressing CD4 T cells and reduced numbers of CCR7-expressing CD4 and CD8 T cells in gout patients (Figure 5e, h, i). While the only significant change observed in FMF patients was the increased number of CCR8+ Vδ2 T cells, the same trend was observed for CD4 and CD8 T cells (Figure 5j). The homing properties of T cells seem not to be affected in CGD patients as no significant change in the receptor expression pattern was detected in comparison to healthy controls. Other homing receptors: CCR4 and CXCR3, did not show alterations in expression among T cell subpopulations in autoinflammatory patients and gout compared to healthy controls (Figure S6). Therefore, our results indicate that the expression patterns of the homing receptors are the most affected in gout. Whether these changes result in altered migratory potential of T cells in gout needs to be addressed experimentally.

The adhesion potential of T cells is not affected by autoinflammatory syndromes and gout

Adhesion potential of T cells is critical for cell migration, activation and cytotoxic function. LFA-1 and CD54 interaction determines the strength and duration of cell-to-cell contact and therefore cell function (77). This LFA-
1/CD54 interaction on T cells influences their cytokine production profile, efficiency of activation, migration through tissues and cytotoxic properties. Our assessment of CD54 and LFA-1 expression on T cells did not reveal any significant changes in all examined autoinflammatory syndromes (Figure 6a-c, S8a-c, S9a, d). Among T cells, CD8 and Vδ2 T cell can directly kill target cells (78, 79). We examined whether this cytotoxic property is also affected in T cells by analyzing expression of CD56, NKG2D and CD16 (80) (Figure 6d-g, S8d, e, and S9b, c, e). The expression of the three molecules correlates well with each other as well as with a high content of perforin and granzyme B and cytotoxic CD8 T cell function (81, 82). They have been therefore suggested to mark cells with cytotoxic properties. We did not observe significant differences in cell frequencies expressing the three markers between patient groups and controls (Figure 6d-g and S8d, e).

Discussion

In this study, we characterized three populations of blood lymphocytes: innate-like Vδ2, CD4, and CD8 T cells in CGD, FMF and gout patients. Our comprehensive immunophenotyping revealed the differential alterations in lymphoid compartment in terms of i; distribution of circulating immune cell types, ii; expression of cells surface markers and cytokines in the examined autoinflammatory disorders.

We found that the distribution of T cell populations was the most affected in CGD patients, in whom Vδ1, Vδ2, CD56+ NK and CD4 T cell percentages were diminished (Fig. 1). Previous studies reported contradictory observations, where increased, reduced or not affected T cell numbers were shown (83–86). The discrepancy between findings might be due to the differential treatment of patients at the time of analysis, different distribution of patients’ age or causative mutations in p91phox or p47phox coding genes within cohorts. Indeed, age-related differences in the distribution of T cells in CGD patients were reported where individuals older than 3 years displayed reduced numbers of CD4 and CD8 T cells (83). This is consistent with our observation that focuses on adult subjects. The cause of abrogated T cell numbers might result from the reduced proliferative capacity (85); but whether intrinsic, due to deficiency of the NADPH oxidase complex, or extrinsic, as a result of disturbed immune homeostasis; remains to be determined.

T cells have been shown to express the functional phagocyte-type NADPH oxidase that is activated upon TCR stimulation (87). Deficiency in its activity in mouse results in enhanced activation of MEK-Erk pathway and augmented Th1 and Th2 responses (88) as well as reduced differentiation and suppressive activity of regulatory T cells (88,89). Although we did not observe significant alterations in IFN-γ production by T cells in CGD, our
data revealed a trend towards increased numbers of TNF-α- and IFN-γ- producing CD4 T cells (Fig. 4), in line with mouse data (87) and with human CGD biopsies from inflamed tissues (90). Contradictory results were found in a study of human cohort where reduced IFN-γ production by CD4 T cells, but enhanced Th17 differentiation were reported (91). We did not find elevated numbers of IL-17α- producing T cells in CGD (Fig. 3 and data not shown). This discrepancy might originate from the different mutations causing the disease in our cohort. While Horvath et al. investigated X-linked form of CGD, caused by p91phox mutations, our cohort is mainly composed of patients with p47phox mutations (Table S1). These two forms of disease might have distinct pathophysiology (92,93). A study with increased number of inclusions will reveal if there is indeed a difference in T cell phenotype between these subgroups of patients.

Our analysis revealed increased number of CD69-expressing CD8 T and γδ T cells in CGD patients (Fig. 2). CD69 is considered as an early activation and a tissue retention marker (94). Increased expression on circulating T cells in CDG patients is less likely to be due to recent activation of these cells since the patients did not display any signs of acute attacks at the time of examination but could be rather due to the retention of these cells in the inflamed tissues. Yet, the numbers of CD69+ T cell are, as expected, very low within circulating lymphocytes. The significance of CD69 overexpression in CGD needs to be determined, especially at the sites of inflammatory attacks.

While most of studies of CGD focus on conventional αβ T cells, this is the first report to our knowledge characterizing unconventional γδ T cell populations using human material. Apart from reduced numbers of these cells, the expression of activation markers CD38 and CD69 (Fig. 2, S3) as well as death receptor CD95 (Fig. 3) was significantly elevated on Vδ2 T cells. This suggests an increased activation status and susceptibility to undergo apoptosis, the latest possibly explaining reduced numbers of Vδ2 T cells in CGD (Fig. 1). Our analysis has also revealed reduced number of CD56+ NK cells (Fig. 1). Early studies show normal cytotoxic function of NK cells from CGD patients (95). This suggest that development, rather than the functions of NK cells, is affected by NADPH complex deficiency. The analysis of a bigger cohort is necessary to confirm these speculations.

FMF is a widely studied monogenic autoinflammatory disease, in which T cell compartment has been best characterized so far. Early reports revealed increased number of CD4 and CD8 T cells (8) as well as increased number of IFN-γ-producing T cells in asymptomatic phase and during acute inflammatory attacks (96). The enhanced Th1 polarization in FMF patients was also suggested based on increased serum concentrations of IL-
18, IL-12 and IFN-γ irrespective of an attack-free or the acute inflammation phases (96–98). However, reduced, rather than increased, IFN-γ concentrations were found in PBMC cultures from FMF patients at different stages of the disease (99). We did not observe any significant differences in CD4 and CD8 T cell frequencies (Fig. 1) nor IFN-γ-producing cells (Fig. 4) in our cohort. This discrepancy might be due to differences in age: while our cohort included only adults (ages 35 to 55 years old) (Table S1), in some of the previous studies pediatric patients were examined (ages 2 to 17 years old) (8, 96). It is well known that the T cell compartment changes during a lifespan, therefore the differences in T cell status in children with FMF might disappear over time in adult patients due to the maturation of T cell compartment.

In our study the FMF condition showed only a very mild effect on T cells. We observed a significant increase in the expression of CTLA-4 and CCR8 homing receptor by CD8 and Vδ2 T cells (Fig. 3 and 5), respectively. CTLA-4 overexpression indicates the exhaustion of CD8 T cells, which might result from overactivation during the inflammatory attacks (54), while changes in CCR8 expression point to changes in migratory patterns of Vδ2 T cells. Yet, the frequencies of CCR8+ cell in peripheral blood are very low, in accordance with other studies (100, 101) but enriched in the skin (100). Examination of the immune cells in the skin of FMF patients will reveal whether γδ T cells are involved in inflammatory reaction at the site of rashes, for example.

Of note, one of our FMF patients has also Behçet disease (Table S1). As this patient exhibits huge increase in Vδ2 T cells, the observation also reported in previous studies (102) it did not cause any other outliers in our analysis. Yet, this data point needs to be interpreted with caution.

While inflammatory attacks in gout are mainly driven by cells of the innate immune system, T cells have been also found in the gouty tophi (63). Furthermore, a recent discovery of the NLRP3 assemblage in T cells (47), which is known to be activated by MSU crystals in innate immune cells and to drive inflammatory flares in gout (103) suggests that the adaptive immune cells can be also involved in gout pathology. Our analysis revealed significant differences in expression of homing receptors CCR5 and CCR7 as well as CD38 on CD4 and CD8 T cells in gout patients (Fig. 5 and Fig. 2) indicating that migratory pattern of T cells can be affected. While CCR7 regulates trafficking to lymph nodes and intestinal Peyer’s patches (104) CCR5 mediates migration and effector function of T cells to sites of inflammation (72). Increased frequency of CCR5+ CD4 T cells and reduced numbers of CCR7+ CD4 and CCR7+ CD8 T cells in gout (Fig. 5) suggests an enhanced recruitment of these cells to inflamed tissues. Consistently, gout patients have elevated concentrations of the CCR5 ligand: regulated upon activation
normal T cell expressed and presumably secreted (RANTES) (105, 106–108). CCR7 signaling has been shown to influence the Th1/Th2 balance by skewing CD4 T cell differentiation towards Th1 fate (109–112). The Th1 cells potentiate inflammatory responses to MSU crystals (42, 113). However, we did not find increased numbers of circulating IFN-γ+ T cells in gout condition (Fig. 4), consistent with recent reports (114, 115). Similarly, we did not find significant differences in IL-17-production by T cells (data not shown), despite previous studies reporting increased levels of Th17-related cytokines in gout (41, 42). However, our study shows significantly reduced numbers of cytokine-producing Vδ2 T cells (Fig. 4). Despite their innate-like character, γδ T cells are understudied in autoinflammatory disorders, but they are a major source of IL-17 production during the early onset of acute gout arthritis (41). The exact role of various T helper and especially unconventional γδ T cell subsets in gout remains to be determined. Especially further research is needed to evaluate whether the observed changes in Vδ2 T cells are due to hyperuricemia or MSU crystal deposition and how these cells act during gout flares.

We also found increased frequencies of CD95+CD4 and CTLA-4+CD8 T cells in gout patients (Fig. 3). Although, CD95 stimulation is known to induce cell death (116, 117), it has been also shown to enhance human T lymphocyte activation and proliferation (118) and therefore play a proinflammatory role. Whether elevated numbers of CD95-expressing T cells reflect their increased effector potential and therefore contribution to inflammation in gout remains to be assessed. CTLA-4 on the other hand transmits inhibitory signals and its expression points to the exhaustion phenotype (119). Increased numbers of CTLA-4+CD8 T cells in gout patients might indicate their overactivation during acute gout attacks and therefore exhausted phenotype in the steady state. Yet this hypothesis awaits verification.

Overall, our findings indicate that autoinflammatory diseases are complex disease of immune dysregulation in which not only myeloid, but also lymphoid cell compartment is impacted. Despite the small size of our cohort, we were able to unravel significant changes in T cell populations. Performing broader examination of T cell compartment over the course of attack and resolution phase and at sites of inflammation, for example synovial fluid in gout, is necessary to reveal the involvement of these cells in the pathology of diseases. Furthermore, this is the first study to our knowledge characterizing unconventional γδ T cells in FMF and CGD patients. Our findings point to the involvement of the adaptive immune system in the pathology of certain autoinflammatory diseases and prompt the broader assessment of T cell involvement in gout, CGD and FMF.
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Conflicts of interest

L.A.B.J. and M.G.N. are scientific founders of TTxD. The authors declare that they have no competing interests.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

B.A. designed, performed and analyzed the experiments, wrote the manuscript; M.B., R.J.R., V.K., R.L., P.A.D., O.G. recruited patients; K.P., J.B. and D.K. designed the study; L.A.B.J. and M.G.N. conceptualized the study, K.P., conceptualized and supervised the study, wrote the manuscript. All authors commented on the manuscript, read and approved the final version.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. All patients gave informed consent to use leftover blood for research purposes. Blood draw from healthy volunteers were approved by the Ethical Committee of the Radboud University Medical Center (no. NL32357.091.0 and no. NL42561.091.12).

Consent to participate

The participants gave informed consent to participate in the study.

Consent for publication
The participants gave informed consent for publication.

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Figure legends

Figure 1. The distribution of circulating lymphocyte populations is significantly affected in CGD patients. a-h Percentages of different cell populations: CD3+ TCRVδ1+ cells (a), CD3+ TCRVδ2+ cells (b), CD3+ CD4+ cells (c), CD3+ CD8+ cells (d), CD3+ CD56+ NKT-like cells (e), CD3+ CD4+ CD25hi CD127lo regulatory T cells (f), CD3+ CD19+ B cells (g), CD3+ CD56+ NK cells (h) in freshly isolated PMBCs from healthy controls and patients determined by flow cytometry. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied. * p<0.05, ** p<0.01

Figure 2. CGD results in increased expression of activation markers on Vδ2 and CD8 T cells, while gout causes downregulation of CD38 on CD4+ T cells. a-f Percentages of Vδ2 (a, d), CD4 (b, e), and CD8 T cells (c, f) expressing CD38 (a, b, c) and CD69 (d, e, f) assessed by flow cytometry on freshly isolated PMBCs from healthy controls and patients. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied for statistical analysis. * p<0.05, ** p<0.01

Figure 3. Fas (death) receptor CD95 expression and CTLA-4 exhaustion marker expression on T cells is increased in some autoinflammatory syndromes. a-i Percentages of Vδ2 (a, d, g), CD4 (b, e, h), and CD8 T cells (c, f, i) expressing CD95 (a, b, c), PD-1 (d, e, f) and CTLA-4 (g, h, i) assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied for statistical analysis. * p<0.05

Figure 4. Gout patients show reduced numbers of IFN-γ- and TNF- producing Vδ2 T cells. a-f Percentages of IFN-γ- (a, b, c) and TNF- (d, e, f) producing Vδ2 (a, d), CD4 (b, e), and CD8 T cells (c, f) assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied for statistical analysis. * p<0.05, ** p<0.01
Figure 5. Homing receptors expression on peripheral T cells is differentially affected in autoinflammatory syndromes. a-l Percentages of Vδ2 (a, d, g, j), CD4 (b, e, h, k), and CD8 T cells (c, f, i, l) expressing CCR2 (a, b, e), CCR5 (d, e, f), CCR7 (g, h, i) and CCR8 (j, k, l) assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied. * p<0.05

Figure 6. T cells exhibit no change in expression of adhesion molecules and cytotoxicity markers in CGD, FMF, and gout. a-g Expression levels of CD54 (a, b, e), percentage of CD56-expressing cells (d, e) and expression levels of NKG2D (f, g) on peripheral Vδ2 (a, d, f), CD4 (b), and CD8 T (c, e, g) assessed by flow cytometry on freshly isolated PMBCs from healthy controls and patients. Dashed lines in y-axis show MFI of fluorescence minus one (FMO) control. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever. Grey triangle: FMF patient with Behcet disease. Non-parametric Mann-Whitney t-test was applied for statistical analysis. * p<0.05, ** p<0.01, *** p<0.001
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Supplementary Material

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Supplementary Table 1 Patients and healthy controls characteristics including sex, age, BMI, mutation and comorbidity if applicable. CGD: Chronic Granulomatous Disease, FMF: familial Mediterranean fever, NA: not available.
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**Supplementary Table 2** List of antibodies used for multi-color immunophenotyping flow cytometry analysis.

Tagged fluorophores and corresponding vendors for each antibody are indicated.
Supplementary Figure 1 Representative core gating strategy of flow cytometry multi-color immunophenotyping panels in freshly isolated PBMCs. Duplets were excluded using FSC-H and FSC-W parameters, hematopoietic cells were gated based on CD45 expression. CD3 vs. TCR γδ gating was used to separate γδ T cells from conventional αβ T cells. TCR Vδ1 vs. TCR Vδ2 gating was applied on CD3⁺TCRγδ⁺ cells to further discriminate γδ T cell subpopulations. CD3⁺TCRγδ⁻ cells were further separated into CD4 T cells, CD8 T cells, and NKT-like...
cells based on CD4 vs. CD8 and CD56 expression. Regulatory T cells were identified among CD4 T cells by gating on CD25\textsuperscript{hi} and CD127\textsuperscript{-} cells. B cells and CD56\textsuperscript{+} NK cells were determined among CD3\textsuperscript{-} cells as CD19- and CD56-expressing cells, respectively.

**Supplementary Figure 2** Distribution of V\textsubscript{62}, CD4, and CD8 T cell subsets based on naïve-effector properties assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. (a) Representative CD45RA vs CD27 gating on V\textsubscript{62}, CD4, CD8 T cells indicating distinct T cell subsets. Terminally differentiated T cells (T\textsubscript{EMRA}): CD45RA\textsuperscript{-} CD27\textsuperscript{-}, naïve T cells: CD45RA\textsuperscript{+} CD27\textsuperscript{-}, central memory T cells (T\textsubscript{CM}): CD45RA\textsuperscript{-}
CD27⁺, and effector memory T cells (TEM): CD45RA⁻ CD27⁺. Distribution of the T cell subsets from healthy controls and patients were analyzed among Vδ2 T cells (b), CD4 T cells (c), and CD8 T cells (d) separately. FMF patient who also has Behcet disease is indicated in lighter color. Non-parametric Mann-Whitney t-test was applied for statistical analysis.

Supplementary Figure 3 Representative dot plots showing gating strategy for CD38 (a) and CD69 (b) on Vδ2, CD4 and CD8 T cells in freshly isolated PBMCs.
Supplementary Figure 4 Representative dot plots showing gating strategy for CD95 (a) and exhaustion markers PD1 (b) and CTLA-4 (c) on V62, CD4 and CD8 T cells in freshly isolated PBMCs.
Supplementary Figure 5 Representative dot plots showing gating strategy for IFN-γ- (a), TNF-α- (b) and IL17-α- (c) producing Vδ2, CD4 and CD8 T cells in freshly isolated PBMCs.
Supplementary Figure 6 Homing receptor expression on peripheral T cells. Percentages of CCR4- (a, b, c), and CXCR3- (d, e, f) expressing Vδ2, CD4, and CD8 T cells in freshly isolated PMBCs from healthy controls and patients. FMF patient who also has Behcet disease is indicated in lighter color.
Supplementary Figure 7 Representative dotplots showing gating strategy for CCR2 (a), CCR5 (b), CCR7 (c), CCR8 (d), CCR4 (e) and CXCR3 (f) on Vδ2, CD4 and CD8 T cells in freshly isolated PBMCs.
Supplementary Figure 8 LFA-1 and CD16 expression on peripheral T cells is not affected by autoinflammatory syndromes. Mean fluorescent intensity values MFI of LFA-1 (a, b, c), and % of cells expressing the cytotoxic marker CD16 (d, e) on Vδ2, CD4, and CD8 T cells assessed by flow cytometry on freshly isolated PBMCs if applicable. Dashed lines in y-axis show MFI of FMO controls. FMF patient who also has Behcet disease is indicated in lighter color. Non-parametric Mann-Whitney t-test was applied for statistical analysis.
Supplementary Figure 9 Representative dot plots showing gating strategy for CD54 (a), CD56 (b) and
NKG2D (c), LFA-1 (d) and CD16 (e) on Vδ2, CD4 and CD8 T cells in freshly isolated PBMC if applicable.