The Immune Memory Response and Metabolic Requirements of Human Gamma Delta T Cells

Dissertation

zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Bonn 2023

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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List for publications

<u>Suen, T. K.</u>*, Al, B.*, Placek, K., & Netea, M. G. (2023). Innate (learned) memory. Journal of Allergy and Clinical Immunology. DOI: 10.1016/j.jaci.2023.06.014
*Contribute equally
The published version is reprinted in the Appendix of the thesis

<u>Suen, T. K.</u>, Moorlag, S. JCFM., Li, W., LJ de Bree, C., Koeken, V. ACM., Mourits, V.P., Dijkstra, H., Lemmers, H., Bhat, J., Xu, C.J., Joosten, L.A.B., Schultze, J.L., Li, Y., Placek, K. and Netea, M.G. (2023). BCG vaccination induces innate immune memory in gamma delta T cells in humans. Journal of Leukocyte Biology. DOI: 10.1093/jleuko/qiad103
The published version is reprinted in the Appendix of the thesis

Roring, R.J., Debisarun, P.A., Botey-Bataller, J., **<u>Suen, T. K.</u>**, Bulut, O., Kilic, G., Koeken, V.A., Sarlea, A., Bahrar, H., Dijkstra, H., Lemmers, H., Gössling, K. L., Rüchel, N., Ostermann, P. N., Müller, L., Schaal H., Adams O., Borkhardt A., Ariyurek Y., de Meijer, E. J., Kloet S., Oever, J. ten, Placek, K., Li, Y., Netea, M. G. (2022). MMR vaccination induces a trained immunity program characterized by functional and metabolic reprogramming of γδ T cells. The Journal of Clinical Investigation. bioRxiv, 2022-11. Under revision.
Preprint: DOI: https://doi.org/10.1101/2022.11.24.516894
The preprint version is reprinted in the Appendix of the thesis

<u>Suen, T. K.</u>, Al, B., Ulas, T., Reusch, N., Bahrar, H., Bekkering, S., Bhat, J., Kabelitz, D., Schultze, J.L., van de Veerdonk, F.L., Roeters van Lennep, J., Riksen, N.P., Joosten, L.A.B., Netea, M.G., Placek, K. (2023). Mevalonate Metabolism fuels pro-inflammatory function of Vδ2 T cells. Submitted.

Al, B., Bruno, M., Roring, R. J., Moorlag, S. JCFM., **<u>Suen, T. K.</u>**, Klück, V., Liu, R., Debisarun, P.A., Gaal, O., Bhat, J., Kabelitz, D., van de Veerdonk, F. L., Joosten, L.A.B., Netea, M.G., Placek, K. (2023). Peripheral T Cell Populations are Differentially Affected in Familial Mediterranean Fever, Chronic Granulomatous Disease, and Gout.
DOI: 10.1007/s10875-023-01576-7
The published version is reprinted in the Appendix of the thesis

<u>Suen, T. K.</u>, Al, B., & Placek, K. (2021). Cell-to-cell proteome variability: life in a cycle. Signal Transduction and Targeted Therapy, 6(1), 229. DOI: 10.1038/s41392-021-00655-8

Dolfi, L., **<u>Suen, T. K.</u>**, Ripa, R., & Antebi, A. (2021). Sperm cryopreservation and in vitro fertilization techniques for the African turquoise killifish Nothobranchius furzeri. Scientific Reports, 11(1), 17145. DOI: 10.1038/s41598-021-96383-8

Acknowledgements

Abstract

γδ T cells are unconventional T cells that can mediate pro-inflammatory, cytotoxic, phagocytotic and professional antigen presenting properties. Sharing the features of both adaptive and innate immunity, γδ T cells also showed signs of induction of both adaptive immune memory and innate immune memory responses. These multi-functional cells therefore play indispensable roles in acquired immunity, host defense against infection and immune surveillance against cancer. In the first part of this thesis, I reviewed the concept and recent advances of innate immune memory and showed the discovery of the trained immunity phenotype in γδ T cells by BCG and MMR vaccination, demonstrating for the first time that γδ T cells can mount both adaptive and innate memory. In the second part of the thesis, I focused on investigating the effect of manipulating mevalonate metabolism on the immune function of γδ T cells as well as the metabolic and immune functional changes of γδ T cells in autoinflammatory and immunodeficient disorders. The result of these studies encouraged us to take into account the adverse effects of mevalonate pathway inhibitors on γδ T cells and provided possible reasons for the limited effectiveness and efficiency of the current γδ T cell-based therapy. Altogether, my thesis offered additional knowledge to facilitate the development of next-generation vaccines and a better γδ T cell-based therapy for cancer and infectious diseases in the future.

List of abbreviations

αβ	Alpha beta
AbTCR	antibody-TCR
ADCC	Antibody-dependent cellular cytotoxicity
ASK	Apoptosis signal-regulating kinase
ATACseq	Assay for transposase-accessible chromatin with sequencing
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
BTN	butyrophilin
BrHPP	bromohydrin pyrophosphate
C. albican	*Candida albicans*
CARD	caspase recruitment domain family member
CARs	chimeric antigen receptors
CCL	C-C Motif Chemokine Ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
CTLA4	Cytotoxic T-lymphocyte associated protein 4
2-DG	2-Deoxy-D-glucose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable
FOXP	Forkhead box protein
FPP	Farnesyl diphosphate
γδ	Gamma delta
GTP	Guanosine triphosphate
GVHD	Graft-versus-host disease
H3K27ac	Histone 3 lysine 27 acetylation
H3K4me1	Histone 3 lysine 4 mono-methylation
H3K4me3	Histone 3 lysine 4 tri-methylation
HIES	Hyper-IgE syndrome
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen – DR
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPP	Isopentenyl pyrophosphate
JNK	Jun N-terminal kinase
LAG3	Ligand lymphocyte activation gene-3
LCK	Lymphocyte-specific protein tyrosine kinase
L. monocytogenes	*Listeria monocytogenes*
LTK	Leukocyte Tyrosine Kinase
MAPK	Mitogen-Activated Protein Kinase
MDA5	Melanoma differentiation-associated protein 5

MET	Mesenchymal Epithelial Transition
MHC	Major histocompatibility complex
MMR	Measles, mumps, rubella
mTOR	Mammalian target of rapamycin
M. tuberculosis	*Mycobacteria tuberculosis*
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NF-kB	Nuclear factor kappa B
NKG2	Natural killer group 2 member
NLRs	NOD-like receptors
Oligo	Oligomycin
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed Cell Death Ligand 1
PRRs	Pattern-recognition receptors
RAC	Ras-related C3 botulinum toxin substrate
RAP	Ras-related protein
RAS	Rat sarcoma
RHO	Ras Homolog Family Member
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SARS-CoV	Severe acute respiratory syndrome coronavirus
S. aureus	*Staphylococcus aureus*
SCENITH	Single cell energetic metabolism by profiling translation inhibition
ScRNAseq	Single cell RNA sequencing
SEK	Stress-activated protein kinase (SAPK)/ERK kinase
STING	Stimulator of interferon genes
TAAs	Tumor-associated antigens
TCR	T cell receptor
TGF	Transforming growth factor
TIM3	T cell immunoglobulin domain and mucin domain 3
TLRs	Toll-like receptors
TNFa	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TYRO	Tyrosine-protein kinase receptor

Chapter 1

General Introduction

Discovery of γδ T cell

From γ chains to γδ T cells

T cells are known to be indispensable members of the vertebrate immune system in combating infectious diseases and cancer. The rearrangement of the T cell receptor (TCR) gene in T cells is critical to construct the heterodimer of TCRs that recognizes different antigens specifically. While the majority of T cells in humans are conventional T cells, expressing TCR with alpha (α) and beta (β) chains (1,2), Tonegawa and his research team discovered a third somatically rearranged gene in a clone of cytotoxic T cells which is termed the gamma (γ) chain in humans and mice (3,4). This surprising discovery encouraged further investigation of the organization, structure and gene recombinant diversity of the γ chain (5–7). Later, Brenner and his colleagues were able to isolate and sequence the delta chain from the T cell receptor (TCR) and further characterized the molecular structure of the chain from a subset of human T cells (8,9). Since then, the mystery of this T cell subset carrying a never-before-seen T cell receptor has been revealed. While γ locus is localized on chromosome 7 in humans and contains 14 variable gene segments, 2 constant regions and 5 joining segments, the delta (δ) chain is located on chromosome 14 within the TCRα locus (7,10,11). Due to the limited number of functional V, D and J segments, the combinatorial repertoire of γδTCR is smaller than that of αβTCR, resulting in a lower number of recombinants (12,13). However, the random insertion and removal of nucleotides at the coupling sites during the processing of rearranged gene segments lead to a junctional diversity of γδTCR genes, expanding the diversity of γδTCR.

γδ T cell subsets and where to find them

Based on chain recombination, γδ T cells can be separated into several subsets. In humans, γ2, γ3, γ4, γ5 and γ8 chains predominately pair with Vδ1 chain while γ9 chain predominately pairs with Vδ2 chain to form the T cell receptor (14–16). In 1988, two subsets of human T cells, called Vδ1 and Vδ2 T cells, were first identified due to the expression of the respective TCR chains using monoclonal antibodies (17). Further studies revealed more gamma delta T cell subsets such as Vδ3 and Vδ5 in humans, characterized by their distinct functional properties and distribution (18–20). While the Vδ2 subset is the main γδ T cell population found in the human and non-human primate peripheral blood consisting of up to 90% of γδ T cells, Vδ1, Vδ3 and Vδ5 T cells are mainly located in thymus, organs or epithelial tissues in humans (21).

The unique characteristics of γδ T cell

The specific expression of T cell receptors marks the major difference between γδ T cells and conventional αβ T cells, where the former express γ and δ chains and the latter express α and β chains. This also implies that the antigens recognized by γδ T cells are distinctive from their conventional counterparts. Indeed, γδ T cells not only recognize classical antigens presented by major histocompatibility complex (MHC)-like molecules, but they also recognize non-peptide antigens such as phosphoantigens in an MHC-unrestricted manner, as well as stress-induced molecules and lipids presented by CD1 molecules (22,23). Particularly, Vδ2 T cells

can respond rapidly to antigens without priming but require activation in the context of butyrophilin (BTN) (24–26). In 1995, Tanaka et al. revealed that isopentenyl pyrophosphate (IPP) and related prenylpyrophosphate derivatives produced by mycobacteria can activate the Vγ9Vδ2 T cells, leading to their proliferation in an MHC-unrestricted manner (27,28). Bürk et al. later confirmed the discovery and identified 5 other potent Vγ9Vδ2 T cell activators produced in eukaryotic cells (29). The startling discovery initiated the mining of potent γδ T cell activators, which resulted in the discovery of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a phosphoantigen that is 10^4 times more potent in activating Vγ9Vδ2 T cells than IPP (30). In fact, both IPP and HMBPP are intermediate molecules produced in mevalonate and non-mevalonate pathways, respectively, that sustain isoprenoid synthesis in organisms (31,32). The ability of γδ T cells to sense these metabolites highlighted their immune surveillance role in dysregulated metabolism. Furthermore, γδ T cells also play a role in stress surveillance. A previous study showed that stress-induced ligand annexin A2 can be recognized by human Vγ8Vδ3 T cells and stimulate proliferation of Vδ2-negative γδ T cells (33). Besides stress-induced molecules, self-lipid can also activate non-Vδ2 γδ T cells. Recent studies showed that Vδ1 T cells presented autoreactivity toward lipid antigens such as sulfatide and α-GalCer in the context of CD1 (34). Altogether, these studies described a distinctive nature of antigen recognition by the TCR of γδ T cells compared to αβ T cells.

Apart from TCR, γδ T cells also present higher expression of innate immune cell receptors such as natural killer group 2 member C (NKG2C) (35), NKG2D (36), Natural killer protein 30 (NKp30) (37,38), NKp44 (39) and NKp46 (40) compared to conventional T cells, which makes them a highly potential cytotoxic killer for infected and cancer cells. Upon activation by NKG2D, γδ T cells produce tumor necrosis factor alpha (TNF-α) and cytotoxic granules such as granzyme B and perforin, leading to the lysis of target cells (41,42). Interestingly, the cytotoxic properties of some γδ T cells require the orchestration of both NKG2D and TCR (41,43,44).

Similar to myeloid cells, γδ T cells also express a spectrum of pattern-recognition receptors (PRRs) that recognize various structurally conserved antigens derived from bacteria and viruses. One of these receptor families is the well-known toll-like receptors (TLRs). Studies have reported that TLRs can initiate anti-viral and bacterial responses in dendritic cells and monocytes by sensing pathogen-associated molecular patterns (PAMPs) such as lipoproteins, lipid-containing ligands and nucleic acids derived from different microbes and viruses (45). There are 12 functional TLRs of which only 10 have been identified in humans (46). According to the study by Pietschmann et al., TLR2 and TLR6 were detected in Vδ1 and Vδ2 T cells respectively, while TLR3 was detected in both subsets (47). Although TLR1 and TLR5 RNA expressions were marginal in both subsets, a TLR2 ligand cocktail containing Pam2Cys, Pam3Cys and FSL-1 can co-stimulate TCR-activated Vδ1 and Vδ2 T cells to produce interferon-gamma (IFN-γ), interleukin-8 (IL-8) and C-C Motif Chemokine Ligand 5 (CCL5) (47). Furthermore, a previous study also showed that the TLR3 ligand Poly l:C can co-stimulate TCR-activated Vδ2 T cells to up-regulate CD69 and secrete IFN-γ, IL-8 and chemokine(48). As a result, TLRs in γδ T cells also contribute to the adaptive immune responses during bacterial and viral infections.

Other receptors, including the stimulator of interferon genes (STING) receptor and the Notch receptor, were also found to modulate the immune response of γδ T cells (49,50). Ruben et al. showed that STING ligands diamidobenzimidazole and Merozoite Surface Antigen 2 can co-stimulate cytokine production and modulate tumor cell killing by Vδ2 γδ T cell lines (49). Another study showed that Notch receptor signaling is necessary to promote proliferation and IFN-γ production by γδ T cells (50). Inhibition of such signaling would inhibit the cytotoxic potential of γδ T cells. Moreover, the human atlas showed that RNA from other PRRs such as NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors and melanoma differentiation-associated protein 5 (MDA5) receptor also present on γδ T cells (51). However, their protein expression, function and contribution to the anti-bacterial and viral responses are yet to be elucidated.

The many faces of γδ T cells

Similar to CD4$^+$ αβ T cells, γδ T cells feature on both arms of pro-inflammatory and anti-inflammatory immunity on the basis of cytokine expression. This plasticity is found in the Vγ9Vδ2 T cell subset (52). For example, Vγ9Vδ2 T cells stimulated with phosphoantigen and IL-2 skew to produce Th1-type pro-inflammatory cytokines, while co-culture of IL-1β, transforming growth factor (TGF)-β, IL-6, IL-23 and phosphoantigen with Vγ9Vδ2 T cells will polarize them towards IL-17-producing Vγ9Vδ2 T cell (53). Whereas, upon stimulation by TGF-β1, IL-15 and phosphoantigen, Vγ9 T cells can develop into anti-inflammatory Forkhead box protein (FOXP)3$^+$ Treg Vδ2 T cells (54). A further study showed that the FOXP3$^+$ Treg Vδ2 T cell transformation was supported by the supplementation of vitamin C (55,56).

Besides the pro-inflammatory and anti-inflammatory roles of γδ T cells, they are also known to possess professional antigen-presenting properties. Brandes et al. were the first to report that γδ T cells are able to present MHC class II molecules: human leukocyte antigen-DR (HLA-DR) and costimulatory molecules: CD80, CD86, CD54 and CD40 upon activation (57). Additionally, γδ T cells can cross-present antigens to CD4$^+$ and CD8$^+$ αβ T cells, showing a strong supportive role of γδ T cells for their counterparts in modulating immune function (58).

In the same year, the professional phagocytotic property of γδ T cells was revealed. Wu and his colleagues observed the phagocytosis of *Escherichia coli* (*E. coli*) through antibody opsonization and CD16 by γδ T cells and confirmed their ability to present antigens (59). Another study also observed a similar phenomenon of trogocytosis by γδ T cells on cancer cells (60). Moreover, γδ T cells can also phagocytose *Plasmodium falciparum*-infected red blood cells and control parasite multiplication in malaria (61).

Although the majority of human γδ T cells are CD8$^-$CD4$^-$, small subsets of γδ T cells were identified to express CD8 or CD4 markers and are prevalent in certain locations and conditions (13,62). CD8$^+$ γδ T cells and CD8$^-$CD4$^-$ γδ T cells are minor subsets in the thymus but are found as the majority in the periphery (62). Whereas CD4$^+$ γδ T cells and CD8$^+$CD4$^+$ γδ T cells are a minority in the periphery but become dominant subsets in pathological conditions such as acute lymphoblastic leukemia (13).

Resembling the memory phenotype of αβ T cells, human γδ T cells can also be divided into naïve and 3 memory compartments based on the expression of the surface markers CD45RA and CD27: CD45RA⁺CD27⁺ (Naïve), CD45RA+CD27- (terminally differentiated), CD45RA-CD27+ (central memory) and CD45RA-CD27- (effector memory) (53,63). In this context, Vδ2 T cells have been the most studied γδ T cell subset. Dielie et al. demonstrated that naïve and central memory Vδ2 T cells were accommodated at the lymph node and did not show immediate effector functions (63). While terminally differentiated and effector memory Vδ2 T cells are recruited to sites of inflammation and readily present their effector functions. A further study showed that the capacity for proliferation and differentiation in response to stimulation differs in distinct memory subsets. For example, when stimulated with IL-2 and phosphoantigen, naïve and central memory Vδ2 T cells expand vigorously while terminally differentiated and effector memory Vδ2 T cells proliferate weakly (53). Incubation with IL-15 leads to the expansion of terminally differentiated and effector memory Vδ2 T cells, intermediate proliferation of central memory Vδ2 T cells and weakly proliferating naïve Vδ2 T cells (53). The terminally differentiated and effector memory subsets differentiated from purified IL-15-stimulated central memory Vδ2 T cells also show differences in robustness of IFN-γ production and cytotoxicity against tumor cells (53).

Altogether, these studies and descriptions indicate that γδ T cells possess unique characteristics and can exert multiple functions compared to their counterparts (**Figure 1**).
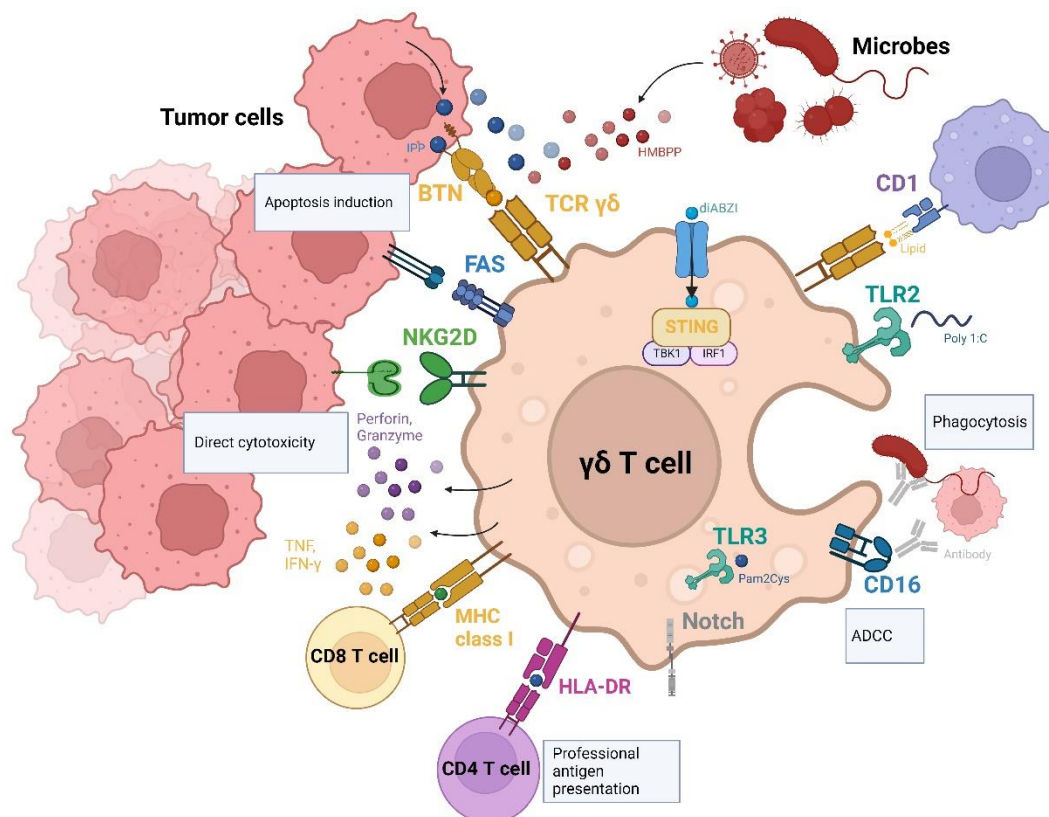
Figure 1 Unique characteristics of multifunctional γδ T cells (Created by Biorender)

The memory of γδ T cell

On one side, γδ T cells demonstrated typical features of innate immunity such as recognizing pathogen-unspecific and stress-related antigens, performing phagocytosis and mediating antibody-dependent cellular cytotoxicity (ADCC); on the other side, they showed common characteristics of adaptive immunity, such as somatic rearrangement of receptor genes. As γδ T cells possess immunologic features of both adaptive and innate immunity, it has been challenging to confine them to only one arm of the immune system (42,64,65). Can the memory character of γδ T cells reveal the answer? For decades, immune memory was considered a unique trait of the adaptive immune response. However, recent studies indicated that cells consisting of innate immunity can also "remember" a primary insult during vaccinations or infections and exert a more robust response upon a secondary challenge. This memory is called innate immune memory, also termed "trained immunity"(66,67). The difference between innate and adaptive memory might be marginal at the molecular level, but it is distinctive in speed, duration, and their ability to respond to secondary stimulation (68–70). Mechanistically, the former is dominantly triggered in myeloid cells and is more rapid, short-term, and pathogen-unspecific, while the latter is only mediated in conventional T and B cells and is slower, long-lasting and antigen-specific (71,72). Adaptive immunity resorts to gene recombination, while both adaptive immunity and trained immunity are mediated by a long-term functional modification, including epigenetic and metabolic rewiring after the primary stimulation. (A detailed review of trained immunity is underlined in **Chapter 2.**) Since γδ T cells bridge innate immunity and adaptive immunity, the question arises: which type of immune memory they are able to mount?

Adaptive immune memory of γδ T cells

In an early study, Hoft et al. stimulated peripheral blood mononuclear cells (PBMCs) from Bacille Calmette-Guerin (BCG)-vaccinated individuals with mycobacterial antigens *in vitro* and observed a drastic expansion of γδ T cells compared to the non-vaccinated donors (73). A similar result was observed in an *in vivo* study where γδ T cells displayed a rapid recall expansion when reinfected intravenously and bronchially with BCG in a non-human primate model (64). This enhanced proliferative response upon re-exposure suggests the capability of γδ T cells to develop a pathogen-specific memory during *Mycobacterium* infection. Furthermore, Suilman et al. showed that BCG-vaccinated individuals have a higher number of IFN-γ-producing γδ T cells upon *in vitro* restimulation, showing the development of pathogen-specific memory, which led to an increased effector response of γδ T cells (74). Moreover, the induction of adaptive memory in γδ T cells was also found in viral infections. Pitard et al. claimed that upon re-challenge with cytomegalovirus (CMV), CMV-positive patients show rapid secondary expansion of γδ T cells, indicating a memory-recall response of γδ T cells against viral infection (75).

Innate immune memory of γδ T cells

In the early studies, trained immunity was mainly addressed in myeloid cells such as monocytes and macrophages. Among these, pathogen-unspecific PRRs, such as the Dectin-1 receptor and TLRs, were involved in innate memory formation. Similarly, γδ T cells also possess pathogen-

unspecific receptors such as NKG2D and TLRs (42,45,76). Their TCR is also innate-like due to its ability to sense pathogen-unspecific and stress-related molecules. Based on these features, γδ T cells resemble their myeloid counterparts. But do they have the potential to develop innate immune memory upon activation? As mentioned, a key feature of innate immune memory is an enhanced immune response mediated by innate immune cells after secondary exposure to a heterologous stimulus. In an early study, Munk et al. reported that γδ T cells pre-expanded by *Mycobacteria tuberculosis* (*M. tuberculosis*) showed secondary expansion upon secondary challenge with an unrelated pathogen such as *Listeria monocytogenes* (*L. monocytogenes*), demonstrating the first pathogen-unspecific proliferating response of γδ T cells (77). Furthermore, Shen et al. showed that macaques vaccinated with an attenuated strain of *L. monocytogenes* present a higher number of IFN-γ-producing Vδ2 T cells upon rechallenge with *M. tuberculosis*, which may be contributing to the lower lung pathology and less weight loss of vaccinated macaques upon infection (78). Apart from that, such a pathogen-unspecific response has also been observed in viral infections in an earlier study. As such, Vδ2 T cells previously stimulated with phytohemagglutinin or mycobacteria showed an increased lysing ability of herpes simplex virus- and vaccinia-infected cells (79). Although these studies demonstrated the induction of heterologous responses by γδ T cells, further studies are required to determine the nature of this immunological memory. This thesis aims to provide further evidence of the induction of trained immunity characteristics in γδ T cells (**Chapter 3** and **4**).

Potential of γδ T cells in immune therapy

Due to the unique characteristics and cytotoxic potential of γδ T cells, they remain a strong candidate for the development of immune therapy against cancer and infections. Advantages of using γδ T cells in immune therapy include:

(1) γδ T cells, such as Vδ2 T cells, are easily expandable *in vitro*

The most common Vδ2 T cell expansion protocol used includes phosphoantigens or a family of drugs called bisphosphonates. Bisphosphonates, such as zoledronate, are clinically approved drugs used for osteoporosis treatment due to their high affinity for bone and suppressive effect on osteoclasts (80,81). Simultaneously, it can inhibit the farnesyl pyrophosphate synthases of the mevalonate pathway in cells, which leads to the accumulation of IPP, a potent activator for Vδ2 T cells (82,83). Upon zoledronate and IL-2 treatment of PBMC cultures, Vδ2 T cells expand and can reach a purity of 70-90% after 10-14 days. The efficiency of Vδ2 T cell expansion can be further optimized through additional cytokine treatment (84). With current expansion protocols, a tremendous number of effector cells can be obtained.

(2) γδ T cells exert cytotoxic function against various cancer cells

Microbes, like all eukaryotic cells, produce isoprenoid molecules to sustain their survival. For example, *E. coli* (85) and *M. tuberculosis* (86) produce HMBPP in the non-mevalonate pathway, while *Staphylococcus aureus (S. aureus)* produces IPP as isoprenoid (87). Both molecules serve as phosphoantigens which activate Vδ2 T cells to fight infected cells (88–90). Interestingly, many cancer cells have dysregulated mevalonate metabolism, in which they tend

to overproduce IPP to support their rapid growth rate (91). The recognition of these PAMPs allows γδ T cells to rapidly exert anti-cancer immune surveillance by killing the transformed cells.

(3) γδ T cells are activated in an MHC-unrestricted manner

As activation of γδ T cells is MHC-unrestricted, γδ T cells can still recognize cancer cells even though they downregulate MHC-I expression to escape immune surveillance. This allows γδ T cells to target tumors more effectively than conventional T cells (92,93). Due to the induction of side effects such as graft-versus-host disease (GVHD), using MHC-restricted αβ T cells as an allogeneic therapy product requires tremendous financial input, time and quality assurance (94). The adoptive transfer of γδ T cells with MHC-unrestricted recognition can avoid GVHD, favoring donors who are not eligible for autogenic transplantation (92).

(4) γδ T cells assist the immune response both directly and indirectly through antigen-presenting properties

In addition to pro-inflammatory and cytotoxic functions, γδ T cells can contain, process and present professional antigen-presenting properties like dendritic cells. This grants them a secondary approach to promote proliferation, cytokine production and cytotoxicity of conventional T cells and exert an effective immune response against infection and cancer (57,58).

γδ T cell role and immunotherapeutic potential in infectious diseases

The prevalence of different infectious diseases has threatened human health for centuries. Despite the advance of clinical treatment, infectious agents developed mechanisms such as mutation and antibiotic-resistant features to evade therapeutic intervention. With the aforementioned features, γδ T cells have high potential to be a key player in the upcoming combat with multi-drug-resistant infectious bacteria such as *M. tuberculosis*, *S. aureus*, *L. monocytogenes* etc (23,95). In fact, evidence has shown that γδ T cells are involved in the immune response toward different bacterial infections. For example, *M. tuberculosis* and BCG infections lead to the major expansion of Vδ2 T cells in macaques (64). A similar phenotype was also manifested in humans who suffered from the *Legionella micdadei* infection (96). It was found that the Pontiac fever developed during legionellosis was associated with persistent Vδ2 T cell expansion. Further studies claimed that γδ T cells also expand in different magnitudes during other bacterial infections such as salmonellosis, tularemia, brucellosis and ehrlichiosis (23). In addition to active expansion, γδ T cells can exert direct effector functions such as cytokine and cytotoxic granule production and indirectly promote recruitment of dendritic cells and macrophages through chemoattractants in listeriosis and mycobacterial infections (97–100). For instance, γδ T cells produce IFN-γ, TNF-α and IL-17, as well as trigger the maturation of dendritic cells upon mycobacterial infection (101). Apart from bacterial infections, recent studies demonstrated that γδ T cells expanded and produced TNF and IFN-γ in response to *Plasmodium falciparum* antigen stimulation (102–106). These studies indicated that γδ T cells may play an important role in fighting both bacterial and parasitic infections.

In addition, γδ T cells also show involvement in combating acute and chronic viral infections (107). In the last decade, newly emerged viruses such as the severe acute respiratory syndrome coronavirus (SARS-CoV1 and SAR-CoV2), Zika and influenza virus have rampantly spread around the world and caused serious damage to human health. Poccia et al. showed that SARS-CoV-infected healthcare workers presented a selective expansion of Vδ2 T cells and IFN-γ-dependent cytotoxicity against infected cells three months after disease onset (108). In the case of severe SARS-CoV2, γδ T cells were recruited from peripheral blood to the lesions of the lung in adult patients (109). Although the frequency of γδ T cells was overall lower in severe SAR-CoV2 patients than healthy donors, an upregulation of activation and exhaustion markers CD69, CD25 and programmed cell death protein 1(PD-1), respectively, was observed on γδ T cells of SARS-CoV2-infected adult patients, indicating the involvement of γδ T cells in the immune response to coronavirus infection (110–113). In acute Zika virus infection, Cimini et al. showed a substantial expansion of Vδ2 T cells with granzyme B and IFN-γ-producing ability upon phosphoantigen stimulation, showcasing the antiviral role of Vδ2 T cells against Zika virus (114). Regarding influenza virus infection, Qin et al. showed that respiratory epithelium actively recruited γδ T cells in a C-C chemokine receptor type 5 (CCR5) receptor-dependent manner (115). The activated γδ T cells featured increased IL-17A production, which further triggered the protective function of neutrophils and NK cells in the tracheal mucosa (116). A recent study showed that the activation of IL-17A producing-γδ T cells was mediated by sensing endogenous lipids released from H1N1 influenza virus-infected host cells (117).

In addition to acute viral infection, γδ T cells also play a role in host defense against chronic viral infection. For example, it has been reported that the decreased frequency of Vδ2 T cells in the peripheral blood of hepatitis B and hepatitis C patients may contribute to the pathology of liver infections (118,119). On the contrary, activating peripheral Vδ2 T cells can promote IFN-γ production and induce a non-cytolytic inhibition of hepatitis C virus replication, restoring the antiviral activity of the immune system (120). Chen et al. showed that IFN-α therapy, which enhances effector γδ T cells, may contribute to hepatitis B surface antigen clearance (121). In cases of human immunodeficiency virus (HIV) infection, the decrease of Vδ2 T cells may be involved in disease progression (122). Whereas, activated γδ T cells show the ability to induce ADCC and can block HIV replication by producing chemokines that block entry into CD4 T cells (122–124).

Altogether, these studies demonstrated that γδ T cells naturally take parts in the protection against bacterial, parasitic, acute and chronic viral infections, fuelling the idea of harnessing γδ T cells as a therapeutic tool to treat different infectious diseases. In the last decade, researchers have attempted to administer phosphoantigens, bisphosphonate, agonists or cytokines to facilitate the expansion and boost effector function of γδ T cells in fighting infectious invaders. Many of these applications have led to positive outcomes. For example, HMBPP-activated Vδ2 T cells expanded by cytokines: IL-12 and IL-15 can inhibit the growth of intracellular *M. tuberculosis* during mycobacterial infection *in vitro* (125,126). Also, application of the HMBPP and IL-2 can induce Vδ2 T cell expansion, which attenuates lung plague lesions caused by *Yersinia pestis* infection in macaques (127). Besides, Cimini et al. showed that Vδ2 T cells expanded by γδ T cell agonist (IPH1101) induce NKG2D-mediated cytotoxicity

towards ZIKA virus infected cells *in vitro* (128). In HIV positive individuals where Vδ2 T cell numbers are commonly reduced, expanding Vδ2 T cells using IPP or bisphosphonate in combination with IL-18 can boost the proliferation and cytotoxic function of Vδ2 T cells, restoring their anti-viral activity (129).

Recently, the development of new immunoantibiotics and agonists has allowed researchers to further optimize the efficiency of Vδ2 T cell activation. Conceptually, a potent inhibitor of the IspH enzyme in the non-mevalonate pathway in most gram-negative bacteria, can cause accumulation of HMBPP which then activate the proliferation and effector function of Vδ2 T cells. This method has a potential to synergize direct antibiotic action and provides an alternative approach in treating multi-drug resistant gram-negative bacterial infections. As mentioned, Vδ2 T cells is activated in the context of BTN. Targeting BTN3A with anti-BTN3A agonists may be a potential treatment strategy for infectious diseases. De Gassart et al. developed an anti-BTN3A agonist called ICT01 and showed that it can exert preclinical efficacy in inducing Vδ2 T cell activation (130,131). This agonist is currently in phase I/II clinical trials. Altogether these showcase that γδ T cells are popular immunotherapeutic candidate in treating infectious diseases.

γδ T cells-based immunotherapy in cancer

Cancer has remained one of the leading causes of death in human history, accounting for nearly 700 thousand deaths worldwide in 2022 (132). The immune system in the human body is an effective defense system against transformed cells. However, highly proliferating cancer cells developed various strategies to evade and deceive the immune system through their microenvironment, such as removing tumor-associated antigens (TAAs) or MHC class antigens to prevent them from being recognized by immune cells (133–136). Many immunotherapies using conventional αβ T cells cannot overcome this barrier and are therefore limited to ineffective anti-cancer activity. As mentioned, γδ T cells can exert the function of anti-tumor immunosurveillance in an MHC-unrestricted manner. Together with their cytotoxic properties against tumor cells, γδ T cells have been considered a highly potential tool for cancer treatment. In fact, the presence of γδ T cells is a positive prognostic factor in many tumor tissues (137,138). For example, a recent study showed that although lower γδ T cell abundance was found in the blood sample of bladder cancer patients compared to non-tumor patients, a high frequency of tumor-infiltrating γδ T cells, which are mostly composed of Vδ2 T cells, is associated with a better survival outcome (139). A high abundance of Vδ1 T cells also correlates with lower numbers of liver metastatic lesions in colorectal cancer patients (140). A bioinformatic analysis demonstrated that head and neck squamous cell carcinoma patients with a higher number of γδ T cells have a lower clinical stage and a greater survival advantage (141). The high frequency of γδ T cells is also positively associated with conventional T cell infiltration and the overall enrichment of genes associated with pathways related to immune effector function.

Mechanistically, γδ T cells show anti-tumor effector functions in several ways. Apart from the Th1 type response and cytotoxic granule release through activation of TCR and NKG2D, γδ T cells can also express death receptor ligands such as FAS ligand and tumor necrosis factor-

related apoptosis-inducing ligand (TRAIL) to cause apoptosis of tumor cells. Dokouhaki et al. illustrated that TRAIL produced by γδ T cells through NKG2D co-activation can induce apoptosis in lung cancer cells (142). Whereas FAS has been shown to be upregulated in some cancer cells upon chemical sensitization, FAS-mediated cytotoxicity is not the main pathway of γδ T cells inducing apoptosis in cancer cells (143–145). Besides, Vδ2 T cells can express CD16 which binds to the fragment crystallizable (Fc) region of antibodies and exert ADCC against cancer cells or infected cells (146,147). This was shown in the context of treatment using exogenous monoclonal antibodies(147). Apart from targeting cancer cells directly, γδ T cells can instruct their immune counterparts to coordinate an anti-tumor response (57,148,149). For instance, γδ T cells can co-stimulate NK cells using CD137 ligand to enhance the cytolytic activity of NK cells against hematopoietic and nonhematopoietic cancers (150). The professional antigen-presenting property and IFN-γ secretion of γδ T cells allow them to activate αβ T cells, therefore amplifying overall cytotoxicity and IFN-γ production (151). Caccamo et al. also showed that γδ T cells can assist antibody production and class switching of B cells through cytokine secretion and CCR5 expression (152).

All in all, this evidence confirms that γδ T cells are competent at fighting cancer and therefore could be a potent therapeutic tool. Indeed, many studies attempt to harness their functions to treat different tumors. One of the most recent advances in γδ T cell-based therapy includes the use of γδ T cell-specific engagers. This method adopts agonistic antibodies, which can induce conformational changes in BTN3A1 on tumor cells and increase their recognition by Vδ2 T cells (153). For instance, a humanized agonistic monoclonal antibody called ICT01 can engage all forms of BTN3A and selectively activate adoptively transferred Vδ2 T cells. This approach was proven to slow down the growth of hematologic and solid tumor xenografts in mice as well as extend their survival (131). Another class of γδ T cell engagers such as bispecific antibodies also facilitate the anti-tumor response of γδ T cells. As such, bispecific antibodies are designed to bind Vγ9 on γδ T cells and human epidermal growth factor receptor 2 on tumor cells (154). This allows activated γδ T cells to exert anti-tumor cytotoxicity in proximity to transformed cells and reduce the growth of pancreatic tumor xenografts in mice. Furthermore, a class of bispecific molecules called gamma delta TCR anti-CD3 utilizes the high affinity of Vδ2 TCR for phosphoantigens to re-direct conventional T cells against hematopoietic, solid tumor and acute myeloid leukemia (155).

Adoptive cell therapies are the most explored method for next-generation immunotherapeutics. Many initial studies applied phosphoantigens such as HMBPP, IPP and bromohydrin pyrophosphate (BrHPP) or aminobisphosphonates such as pamidronate and zoledronate to expand autologous Vδ2 T cells *ex vivo* and adoptively transfer them to patients (156,157). An optimized protocol was developed by using zoledronate, IL-2, IL-15 and vitamin C to improve the proliferative and immune effector functions of Vδ2 T cells (84). Xu et al. showed that adoptive transfer of these expanded cells can inhibit lung tumor growth and prolong the survival of humanized mice compared to the Vδ2 T cells expanded using the expansion protocol with zoledronate and IL-2 only (84). The abundance of Vδ1 T cells is limited in peripheral blood; however, in tumor infiltrates, they predominate and present great cytotoxic potency against cancer cells (158,159). Almeida et al. invented a two-step protocol using a

cytokine cocktail to expand Vδ1 T cells up to 2000-fold in cell culture bags (160). These *in vitro* expanded Vδ1 T cells can inhibit chronic lymphocytic leukemia tumor growth and prevent dissemination *in vivo*. Further approaches, such as γδ T cells transduced with chimeric antigen receptors (CARs) against TAAs or engineered αβ T cells retrovirally transduced with TCRγδ, were also developed to strengthen the potency of T cell-based immunotherapy against cancers (161,162). Capsomidis et al. showed that CAR γδ T cells presented increased cytotoxicity against GD2-expressing cancer cell lines compared to unmodified γδ T cells (163). Another CAR γδ T cell targeting Mucin 1 on various cancer cell lines also showed a higher effectivity in tumor cell lysis compared to CAR-αβ T cells (164). Recently, a novel antibody-TCR(AbTCR) construct by combining γ and δ chains with the antibody fragment antigen-binding domain was created by Xu et al. (165). Cells transduced by AbTCR showed similar anti-tumor activity but a less exhausted phenotype. Liu et al. showed that their engineered cell product can effectively decelerate hepatocellular carcinoma growth in xenograft models (166). These studies demonstrate that γδ T cells are a potential therapeutic tool in treating cancer.

Clinical trials of γδ T cells and their current limitations

Due to the safety and enormous potential of γδ T cells as immunotherapy against infectious diseases and cancers, many γδ T cell-based therapeutic approaches have been granted approval from the US Food and Drug Administration for clinical trials (167). Currently, 13 γδ T cell-based clinical trials are implemented for cancer therapy **(Table 1)** (138). Three of these clinical trials adopt γδ T cell engagers approaches, while 10 of these clinical trials are adoptive cell therapies. Among all adoptive cell therapy trials, two of them apply the CAR-transduced γδ T cell approach, while four of them employed γδTCR-engineered T cells.

Notwithstanding γδ T cells as a very prospective tool in treating infectious diseases and cancers, the past γδ T cell-based clinical trials faced many obstacles. Twenty-two γδ T cell-based clinical studies have been approved in the past decade (168,169). Although these therapies showed no significant adverse effects, most of these studies demonstrated incomplete or only partial responses. Only two studies, by Wilhelm et al. and Kobayashi et al., have achieved complete remission in haematological malignancies and renal cell carcinoma disseminated from lung metastases by using zoledronate and IL-2-activated γδ T cells (170,171). Taken together, this shows that current γδ T cell-based therapies are yet to be improved in order to be efficient and successful.

Several limitations to the therapeutic effectiveness of γδ T cells against cancer are speculated. For example, aminobisphosphonate and phosphoantigens lack target specificity in cancers *in vivo* (169). While phosphoantigens are easily degraded in the body, aminobisphosphonate tends to be absorbed by bones due to its high calcium affinity; therefore, it is not efficient in targeting cancer cells distributed outside of bones (172). Aminobisphosphonate also lacks the ability to cross the plasma membrane of cancer cells without an active transport mechanism. Although aminobisphosphonate on one side can sensitize cancer cells and inhibit their mevalonate pathway, leading to overproduction of IPP to activate Vδ2 T cells, this inhibition

is not specific to cancer cells and affects other cells in the system. In fact, it was reported that aminobisphosphonates such as zoledronate can induce the killing of human macrophages (173). Neutrophils that uptake zoledronate can lead to immune suppression of Vδ2 T cells (174). A proposal of increasing the concentration of bisphosphonate and applying drugs locally to induce higher γδ T cell expansion may also lead to overdose toxicities in cells (175–178). So far, the side effects of such inhibition on γδ T cells and their counterparts have not been widely addressed. Moreover, microenvironments of tumors, such as oxygen reserve and metabolite composition, can induce dysfunction and exhaustion in γδ T cells (179–182). As a result, the next generation γδ T cell-based therapies must overcome all these obstacles in order to successfully cure cancer.

Method	Cancer type	Clinical trial	Reference
γδ T cell engager method:			
BTN3A agonist antibody	Haematological tumors	NCT04243499	(183)
BTN3A agonist antibody	Solid tumors	NCT05307874	(183)
Bispecific antibody	Colon cancer	NCT04887259	(184)
Adoptive cell transfer method:			
Expanded allogeneic Vδ2 T cells	Lung cancer	NCT03183232 NCT03183219	(185)
Expanded allogeneic Vδ1 T cells	Chronic lymphocytic leukaemia; acute myeloid leukaemia	NCT03183219	(186)
Expanded chemotherapy-resistant γδ T cells	Glioblastoma	NCT04165941	(187)
NKG2D ligand-directed CAR Vδ2 T cells	Colon, ovarian cancer	NCT04107142	(188)
CD20-directed CAR Vδ2 T cells	B cell lymphoma	NCT04735471	(189)
αβ T cells engineered with Vδ2Vγ9 TCRs	B cell lymphoma	NCT04688853	(190)
αβ T cells engineered with CD19-directed antibody-TCRs	B cell lymphoma	NCT03415399	(191)
αβ T cells engineered with a-fetoprotein-directed antibody-TCRs	Hepatocellular carcinoma	NCT04502082	(166)
αβ T cells engineered with glypican-3-specific chimeric co-stimulatory receptors	Hepatocellular carcinoma	NCT04634357	(166)

Table 1 Current ongoing clinical trial associated with γδ T cells

Outline of the thesis

This thesis aims to decipher the potential of human γδ T cells to develop innate immune memory and to unravel the influence of cellular metabolism on the immune function of the cells. The thesis consists of 2 parts: the first part describes the concept and recent advances of innate immune memory (192), followed by the discovery of the trained immunity phenotype in γδ T cells induced by BCG and measles, mumps and rubella (MMR) vaccination (193). The second part focuses on the role of mevalonate metabolism in human γδ T cells. This part also includes the immune phenotyping and energy metabolism profiling of γδ T cells in two diseases.

Part I: Trained immunity of γδ T cells

As mentioned, immune memory can be mounted not only in adaptive immune cells but also in innate immune cells. The innate immune memory, also termed "trained immunity", allows cells to exert a more rapid and potent non-specific immune response to subsequent exposures and is accompanied by long-term functional modifications such as metabolic and epigenetic rewiring. In **Chapter 2**, the recent advances in understanding trained immunity formation are discussed. It provides an overview of the intracellular signaling, metabolic and epigenetic mechanisms underlying the formation of the innate immune memory, as well as the cell types that have the potential to mount it. Furthermore, the effects of disbalanced trained immunity on human health are discussed along with the potential of harnessing trained immunity as a therapeutic tool. In **Chapter 3** and **Chapter 4**, I demonstrate the novel discovery of trained immunity in γδ T cells. In **Chapter 3**, I show that trained immunity can be induced in γδ T cells upon BCG vaccination. The BCG vaccine is well-known for inducing trained immunity in different innate immune cells and inducing adaptive immune memory responses in γδ T cells. Whether BCG-induced immune memory in γδ T cells can have an innate character and therefore contribute to the cross-protective effect of the vaccine against heterologous infections has not been addressed. We combined *in vitro* immune functional assays with single-cell analysis of peripheral immune cells from BCG-vaccinated individuals to investigate whether human γδ T cells can develop trained immunity. In **Chapter 4**, the potential to induce trained immunity in human γδ T cells by another vaccine against MMR is investigated. MMR, like BCG, has been associated with vaccine-induced heterologous immunity (194,195), yet the mechanism of it is not known. A similar integrative approach as in the previous chapter was employed, including immune functional assays and single-cell analysis, to examine healthy adults who received either the MMR vaccine or a placebo and explore whether MMR caused a novel trained immunity phenotype in γδ T cells.

Part II: Cellular metabolism and the immune function of γδ T cells

As previously mentioned, intermediate metabolites of the isoprenid biosynthesis, such as IPP, serve as a potent activator of Vδ2 T cells, a dominant γδ T cell subset in human peripheral blood. Aminobisphosphonates such as zoledronate have been used to expand Vδ2 T cells due

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to their ability to enhance IPP production by inhibiting farnesyl diphosphate (FPP) synthase in the mevalonate pathway. Zoledronate, together with IL-2, was widely adopted to expand Vδ2 T cells for therapeutic use in many clinical trials. Although the expansion protocol using bisphosphonate has been recently optimized for producing more potent Vδ2 T cells, the efficiency of these cells in a therapy setting remains limited. This gives rise to our speculation that although the application of zoledronate in PBMC can efficiently boost the effector Vδ2 T cell numbers, its inhibitory effect also exerts directly on the mevalonate metabolism of Vδ2 T cells, modulating their immune functions. Indeed, mevalonate metabolism is essential for many cellular activities. In **Chapter 5**, we emphasize the importance of mevalonate metabolism and unravel the molecular mechanisms by which various mevalonate pathway inhibitors, including frequently prescribed drugs in clinics such as zoledronate and statins, affect the immune function of Vδ2 T cells. Using *in vitro* immune assays as well as *ex vivo* analysis of the patient's material, we characterized the role of the isoprenoid synthesis pathway for the pro-inflammatory and cytotoxic activities of Vδ2 T cells. Transcriptomic, epigenetic and kinase activity analyses were performed to decipher the underlying molecular mechanisms of the observed phenotypes. This study reveals the importance of mevalonate metabolism for the proper functioning of Vδ2 T cells and provides essential considerations for using amino bisphosphonate to expand Vδ2 T cells for therapeutic use.

An effective immune response is formed on the basis of sufficient effector cell numbers and the robust release of effector molecules. However, both processes require massive expenditures of energy in cells. In **Chapter 5**, we revealed that the proliferation and effector molecule production by Vδ2 T cells are uncoupled. Therefore, a balance of energy usage on cell proliferation and effector responses is necessary for a proper immune response outcome by Vδ2 T cells. The mevalonate pathway also governs energy production in cells. A product of the mevalonate pathway called ubiquinone is an important element for electron transporters in the electron transport chain of the mitochondria. The electron transport enables the production of ATP in the process of oxidative phosphorylation, which sustains energy expenditure in cells. The mevalonate pathway of Vδ2 T cells is blocked during *in vitro* expansion by zoledronate. However, whether that also poses a disturbance to energy production by consequent ubiquinone deficiency is yet to be addressed. In **Chapter 6**, we explored whether the energy metabolic profile of Vδ2 T cells is affected by mevalonate pathway inhibition using a flow cytometry-based method called single cell energetic metabolism by profiling translation inhibition (SCENITH). We further applied SCENITH to decipher how autoinflammatory and immunodeficiency diseases such as chronic granulomatous disease (CGD) patients and hyper-immunoglobulin E (IgE) syndrome (HIES) patients affect the energy metabolism and immune profile of Vδ2 T cells.

Finally, **Chapter 7** summarizes all the results of the thesis and discusses the future direction of the investigation.

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Part I

Trained immunity of γδ T cells

Chapter 2

Innate (learned) memory

Innate (learned) Memory

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Key words

Trained immunity, Immune memory, Metabolism, Epigenetics, Cellular signaling

The published version is reprinted in the Appendix of the thesis.

Own contribution

I contributed as a lead author together with Burcu Al in writing this review.

Summary

Plants and invertebrates that lack an adaptive immune system can exert an immune memory response towards rechallenges, illustrating that an immune memory mechanism other than adaptive immune memory exists (1). Recent large cohort studies further demonstrated that an enhanced immune response can be induced in Bacillus Calmette–Guérin (BCG)-vaccinated individuals and mouse models upon rechallenge with heterologous pathogens, indicating that a pathogen non-specific immune memory can be mounted in immune cells (2–5). This rapid and enhanced non-specific immune response upon subsequent antigenic challenges is defined as trained immunity (4,6). This memory response is accompanied by long-term functional modifications such as metabolic and epigenetic rewiring. Trained immunity has been revealed in many myeloid cell types, including monocytes and macrophages (7–11) and recently it has also been discovered in NK cells (12–16) and innate lymphoid cells (17–20). Not limited to immune cells, innate immune memory was also discovered in non-immune cells such as fibroblasts and epithelial cells (21–24). Recent discoveries suggested that innate immune memory is already initiated at the level of bone marrow progenitors, where hematopoietic stem cells and multipotent progenitors are reprogrammed and myelopoiesis is enhanced (25–28). Such induction is termed central trained immunity. On the contrary, peripheral trained immunity is well studied in macrophages present in peripheral organs like the lung and intestine (29–33).

The development of trained immunity is initiated by subsequent antigenic encounters by PRRs. One of the most studied receptors is the Dectin-1 receptor in monocytes. Upon stimulation by b-glucan, dectin-1 receptors initiated the PI3k/AKT/mTOR/HIF-1α signaling cascade, which led to changes in metabolic and epigenetic status and therefore induced trained immunity (34–37). Also, stimulation of TLRs can trigger MyD88 signalling and mediate trained immunity formation in microglia and mast cells. This signaling cascade upon stimulation results in metabolic reprogramming of the cells. Glycolysis is one of the most important metabolic pathways for the trained immunity. The typical metabolic change of β-glucan-, BCG- and oxLDL- induced trained immunity in monocytes, macrophages and progenitor cells is increased aerobic glycolysis (28,34,38,39). The upregulation of glycolysis initiates the PI3k/AKT/mTOR/HIF-1α pathway and inhibits conversion of pyruvate to acetyl-CoA (38,40). This leads to an enhancement of glutaminolysis to support the TCA cycle in mitochondria (34). The accumulation of different intermediate metabolites at the TCA cycle also plays a role in trained immunity induction (34) , and immune tolerance (41). On the other hand, training with different dosages of β-glucan, BCG or oxLDL also modulates the oxidative phosphorylation rate of the cells (42,43). Apart from that, cholesterol (28), fatty acid (44,45) and methionine (34) were recently found to have a pronounced impact on trained immunity. Furthermore, the metabolic changes in cells further lead to epigenetic rewiring, a hallmark of trained immunity. The modification of histone tails is one of the major inductions. Studies showed that specific histone marks such as histone 3 lysine 4 mono-methylation (H3K4me1), H3K4 tri-methylation (H3K4me3), and H3K27 acetylation (H3K27ac) were increased at the gene regulatory elements of proinflammatory genes in β-glucan-trained monocytes (8). Other layers of epigenetic modification, including DNA methylation (46) and interference of long non-coding RNAs (46,47) , are also known to associate with trained immunity.

Induction of trained immunity enables innate immune cells to react more rapidly and effectively during reinfection (48–52) and promotes tissue repairing (22,53,54). However, defects or excessive manifestation of trained immunity can also lead to detrimental consequences such as sepsis (55), tumor progression (34,56–58), autoimmune (59–61) and neurodegenerative diseases (62–64). Therefore, a balanced induction of trained immunity is

important to maintain individuals' health. By harnessing and suppressing trained immunity in an appropriate setting, it can be used as a therapeutic advantage to maximize the hosts' well-being.

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Chapter 3

BCG vaccination induces innate immune memory in gamma delta T cells in humans

BCG vaccination induces innate immune memory in gamma delta T cells in humans

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Summary: Characterization of memory responses of human gamma delta T cells to BCG vaccination.

Running title: gamma delta T cell immune memory

Keywords: γδ T cells; immune memory; trained immunity; BCG vaccine

> **The published version is reprinted in the Appendix of the thesis.**

Own contribution

I contributed by performing the preliminary experiments and *in vitro* immune functional assay of γδ T cell. I also contributed to partial writing of this manuscript.

Summary

Immunological memory allows the host to exert a more rapid and efficient immune response upon reinfection. Recent studies suggested that this immune memory is not only attributed to adaptive immune cells but also to innate immune cells. This property is termed "Trained immunity". Bacillus Calmette-Guérin (BCG) vaccine is well-known for mediating trained immunity in myeloid cells, which contributes to its cross-protective effect against heterologous infections. γδ T cells have been shown to mount immune memory upon exposure to mycobacteria and undergo secondary expansion with enhanced effector molecule release during reinfection (1–3). However, the γδ T cell immune memory was mainly addressed in the context of adaptive memory. We hypothesized that by sharing both properties of adaptive and innate immunity (1,4,5), γδ T cells are not only able to mount adaptive memory but are also eligible for the induction of innate immune memory.

In this part of the thesis, we combined single cell analysis by flow cytometry and RNA-sequencing to study whether the BCG vaccine, a well-known agent inducing trained immunity in myeloid cells, may also lead to the formation of trained immunity phenotypes in γδ T cells in humans. In order to better understand the functional responses of human peripheral blood γδ T cells, we first examined the immune response of γδ T cells toward different heat-killed pathogens, including *M. tuberculosis*, *E. coli*, *S. aureus* and *C. albicans*. We observed that γδ T cells upregulated activation markers CD25 and CD69 in response to all pathogens. Particularly, *S. aureus* and *C. albicans* were the most potent activators of γδ T cells. We found that the BCG vaccine did not change the frequency of γδ T cells and their activation status but boosted the perforin production. However, upon *in vitro* stimulation with *M. tuberculosis*, we observed a higher fold change in the number of IFN-γ-producing γδ T cells in BCG-vaccinated donors 2 weeks after vaccination than non-vaccinated controls, confirming the generation of specific adaptive immune memory in γδ T cells. BCG-vaccinated donors also showed a higher increase in TNF- and IFN-γ release when stimulated by *C. albicans*. This enhanced immune response to a BCG-unrelated agent indicated the induction of innate immune memory in γδ T cells. We also observed that approximately half of the BCG-vaccinated donors show an increase in cytokine production capacity by γδ T cells upon *E. coli* and *S. aureus* stimulation compared to non-vaccinated donors. This is consistent with the previous studies showing the heterogeneity in trained immunity induction by BCG, rendering some donors to be good responders while others to be weak responders.

To examine the transcriptional changes induced in γδ T cells by the BCG vaccine, we performed single-cell RNA-seq on PBMC collected before and three months after BCG vaccination upon stimulation with or without LPS. We identified γδ T cell clusters based on the high expression of TRDC, TRGC1, TRGC2 markers and five distinct subpopulations. Our analysis showed that the majority of γδ T cells did not upregulate the trained immunity response genes, indicating that less than half of the γδ T cells were trained. However, one of the cell clusters showed a high expression of *IFNG*. This subset was characterized by an enrichment of up-regulated genes in the GO pathways related to "response to interferon-gamma" and "interferon-gamma mediated signaling". Cell-cell communication analysis revealed that IL family ligands from monocytes were potential ligands to regulate *IFNG* receptor signaling in this γδ T cell cluster, while *IFNG* was also a highly expressed ligand in this cluster and was predicted to interact with trained immunity response genes in monocytes. Our analysis suggested that γδ T cells in BCG-vaccinated donors present enhanced responses upon heterologous rechallenge, indicating the induction of trained immunity in γδ T cells by the BCG vaccine.

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Chapter 4

MMR vaccination induces a trained immunity program characterized by functional and metabolic reprogramming of γδ T cells

MMR vaccination induces a trained immunity program characterized by functional and metabolic reprogramming of γδ T cells

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The preprint version is reprinted in the Appendix of the thesis.

Own contributions

I contributed by performing the immune functional assay and metabolic profiling of γδ T cell. I also contributed writing the method part of this manuscript.

Summary

BCG is an attenuated live vaccine with a cross-protective effect. Another well-known live vaccine, the MMR vaccine, has also been known to contribute to heterologous protection against nontarget infections (1,2). In this part of the thesis, we combined immune functional assays and next-generation transcriptome sequencing to investigate whether the MMR vaccine can induce trained immunity against SARS-CoV-2 and a range of other microbial stimuli in a randomized placebo-controlled trial of MMR-re-vaccinated individuals. We applied proximity extension assay technology (Olink) to assess targeted proteomics biomarkers before and one month after MMR vaccination. We did not observe a major change in plasma proteome composition in individuals between the baseline measurement and one month after the MMR vaccination. Among all the analyzed parameters, the most significant hit was a pancreas-associated protein involved in lipid metabolism. In the protein subcategories, the inflammation-related proteins had the highest number of changes, with an upregulated trend in individuals after vaccination. Most of the suggestive hits of the cardiometabolic-related proteins were related to immunological processes directly or indirectly, while immunology-related proteins showed an upregulation in oncology and neurology panels in individuals after vaccination. We found that the number of circulating leukocytes significantly increased in MMR-vaccinated individuals one month after the vaccination, mainly due to the increasing number of myeloid cells. Altogether, these indicated that the systemic inflammatory status after MMR vaccination increased.

Immune functional assays on PBMC cultures indicated that cytokine production upon stimulation with LPS, *C. albicans* and viral antigens (poly(I:C), R848, Influenza A (H1N1) and SARS-CoV-2) was not affected by MMR vaccination. An integrated analysis of single-cell RNA-seq and ATAC-seq on the PBMCs of MMR-vaccinated donors showed that MMR vaccination did not cause major changes in PBMC composition. However, every cell type except CD4$^+$ T cells showed a pronounced transcriptional change. Particularly, γδ T cells present the most changes in their transcriptome, while chromatin accessibility was only impacted by MMR vaccination in CD14$^+$ monocytes. Further investigation into the γδ T cells showed that genes involved in cellular respiration and ATP synthesis were downregulated in γδ T cells, indicating a metabolic shift in these cells. Employing immune functional assays, we found that there was a significant decrease in Vδ2 T cells in the PBMC of MMR-vaccinated donors, while the expression of CD27 and CD45RA, which determine the memory phenotype of T cells, was not altered. Strikingly, the TNF and IFN-γ production by these cells significantly increased when compared to the non-vaccinated donors, indicating that Vδ2 T cells present an enhanced response against secondary stimulation similar to the phenotype of trained immunity in classical monocytes.

Using SCENITH, we found that Vδ2 T cells in MMR-vaccinated donors display higher protein synthesis upon stimulation compared to Vδ2 T cells in non-vaccinated donors. A decrease in glycolytic capacity and an increase in mitochondrial dependency were observed in stimulated Vδ2 T cells, consistent with the observation of metabolic shift in γδ T cells revealed by transcriptomic analysis. Altogether, our study showed that γδ T cells display trained immunity characteristics and may play an important role in the mechanisms underlying MMR-induced trained immunity.

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Part II

Metabolism and the immune functions of γδ T cells

Chapter 5

Destabilized intracellular signaling impairs Vδ2 T cell effector function upon mevalonate pathway inhibition

Destabilized intracellular signaling impairs Vδ2 T cell effector function upon mevalonate pathway inhibition

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Own contribution
I performed the majority of experiments including planning, performing and analysis and I drafted this manuscript.

Abstract

Mevalonate metabolism is essential for proper functioning of eukaryotic cells. Widely prescribed drugs, like statins and bisphosphonates, inhibit specific enzymes in the mevalonate pathway and modulate immune responses. Intermediate metabolites of the pathway activate innate-like Vδ2 T cells, while bisphosphonates effectively expand these cells for potential therapeutic use. Yet, the role of the mevalonate metabolism in Vδ2 T cells is poorly defined. We show that *in vitro* and *in vivo* inhibition of the mevalonate metabolism results in compromised cytokine production and cytotoxic activity of Vδ2 T cells. Impaired Vδ2 T cell function is accompanied by global transcriptome changes. Protein prenylation and kinome analysis unraveled dysregulated signaling pathways as the leading cause of the reduced effector function of Vδ2 T cells upon mevalonate pathway inhibition. Our findings reveal the importance of mevalonate metabolism for the proper functioning of Vδ2 T cells and provides important considerations for improving their therapeutic use.

Introduction

Cellular metabolism plays an integrative role in supporting immune cell functions. The mevalonate pathway is an important metabolic process which leads to the synthesis of isoprenoids and sterols in eukaryotes, archaea and some bacteria and is the initial step for many biological processes (**Fig. 1a**)(1,2). The entry substrate for the pathway acetyl-CoA is transformed into several intermediate metabolites such as β-hydroxy β-methylglutaryl-Coenyzme A (HMG-CoA), mevalonate, isopentenyl pyrophosphate (IPP) and farnesyl pyrophosphate (FPP) (**Fig. 1a**). FPP can then be used for cholesterol synthesis, the production of dolichol or protein prenylation: geranylgeranylation and farnesylation (3). Cholesterol is an important lipid component of cellular membranes where it modulates their fluidity and permeability (4). Being a component of lipid rafts, it is also involved in receptor signaling (5). Dolichol is a lipid carrier for the protein N-glycosylation, a post-translational modification that changes the nature of target proteins(6) which can affect protein localization, stability, interaction with other proteins and function (7–9). While protein prenylation increases protein anchoring to cellular membranes and therefore affects intracellular trafficking between cellular membrane compartments (10). All these processes are important for immune cell functions.

A deficiency of mevalonate kinase, which phosphorylates mevalonate in the isoprenoid biosynthesis pathway, causes hyper-IgD syndrome (HIDS). The syndrome is characterized by recurring attacks of fever and other inflammatory symptoms such as joint pain, swollen lymph nodes, skin rash, headaches and abdominal pain (**Fig. 1a**)(11). An accumulation of non-processed mevalonate in HIDS has been shown to cause an inflammatory phenotype in innate immune cells (12). The mevalonate pathway is also one of the most manipulated biological pathways in therapeutic interventions. Statins, for example, are used to lower cholesterol levels and reduce the risk of cardiovascular disease by inhibiting the rate limiting enzyme HMG-CoA reductase in the mevalonate pathway (13,14). Nitrogen bisphosphonates, such as zoledronate, which are potent inhibitors of farnesyl pyrophosphate synthase, are the most used drugs to treat osteoporosis (15,16). Recent studies have shown that these drugs not only alleviate the severity of targeted diseases, but also exert effects on the immune system (17,18). Statins can modulate immune cell function in various ways depending on the cell type and condition, but generally they show immunosuppressive effects(19–21). Intravenous administration of zoledronate induces transient fever in most patients (22). The inflammatory reaction might be caused by the activated V delta 2 (Vδ2) T cells, a unique group of human γδ T cells which recognize the intermediate metabolite of the mevalonate pathway, IPP (23–25). Zoledronate treatment leads to the accumulation of IPP (**Fig. 1a**) which serves as a phosphoantigen for Vδ2 T cells and causes their activation and expansion (26,27).

The T cell receptor (TCR) of Vδ2 T cells is composed of Vδ2 and Vγ9 chains and recognizes non-peptide molecules such as aforementioned IPP(23,24) and microbe-derived (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) in MHC molecule unrestricted manner (28,29). Upon activation, Vδ2 T cells produce pro-inflammatory cytokines, mainly TNF and IFN-γ (30). They also exert cytotoxic or phagocytotic properties against infected and tumor cells (31,32). Due to their effective anti-cancer activity, easy *in vitro* expansion with

phosphoantigens and bisphosphonates and suitability for allogenic transfer, they sparked interest in cancer immunotherapy (33). Yet, the effectiveness of zoledronate-expanded Vδ2 T cells in cancer patients remains limited (34,35). While zoledronate has been proven to be an efficient Vδ2 T cell expanding agent, its effect on mevalonate metabolism and Vδ2 T cell function is ill defined.

In this study, we aim to assess the role of the mevalonate pathway in Vδ2 T cell function. We showed that the mevalonate metabolism fuels cytokine and cytotoxic granule production by these cells. Especially protein prenylation and signal transduction, downstream mevalonate pathway, are important for the proinflammatory and cytotoxic functions of Vδ2 T cells. This study provides an insight into the off-target effects caused by drugs such as statin and nitrogen bisphosphonate on the proper functioning of Vδ2 T cells, as well as into the challenges of the common zoledronate-based Vδ2 T cell expansion protocol.

Results

In vitro inhibition of the mevalonate pathway compromises cytokine production by Vδ2 T cells.

To determine the role of mevalonate metabolism for Vδ2 T cell function we incubated PBMCs from healthy donors with pharmacological inhibitors targeting different enzymes in the mevalonate pathway: fluvastatin (Statin, the HMG-CoA reductase inhibitor), zoledronate (Zol, the farnesyl transferase inhibitor) and 6-fluoromevalonate (6FM, the mevalonate-PP decarboxylase inhibitor) as well as IPP (the downstream substrate of the mevalonate pathway which activates and expands Vδ2 T cells) and non-treated cultures (RPMI alone) as controls in the presence of IL-2 (**Fig. 1b**). After 12 days of culture, we assessed the proliferation and cytokine production capacity of Vδ2 T cells by flow cytometry (**Fig. 1c-f** and **Extended Data Fig. 1a**). As expected, the numbers of Vδ2 T cells in IPP- and zoledronate-treated cultures increased significantly (**Fig. 1c,d**)(26,27). The number of Vδ2 T cells in fluvastatin-treated culture was reduced at the higher dose (5μM) (**Fig. 1c, d**), while the number of Vδ2 T cells in 6-fluoromevalonate-treated cultures was comparable to that in non-treated cultures (**Fig. 1c, d**). This could be due to impaired proliferative capacity of Vδ2 T cells or increased cytotoxicity upon treatment with higher doses of statin (**Fig. 1d**, **Extended Data Fig. 1b,** and **Extended Data Fig. 1c**). Cytokine production assessment revealed that Vδ2 T cells incubated in RPMI medium alone or treated with IPP were potent producers of TNF and IFN-γ (**Fig. 1c, e, f**), while in the presence of higher doses of fluvastatin (**Fig. 1c, e, f**) and atorvastatin (**Extended Data Fig. 1d**) the percentages of live TNF- and IFN-γ-producing Vδ2 T cells decreased. The percentages of TNF- and IFN-γ-producing Vδ2 T cells were also decreased in the presence of other mevalonate pathway inhibitors: zoledronate and 6-fluoromevalonate (**Fig. 1c, e, f**). Similarly, fluvastatin- and zoledronate-treatment reduced numbers of IFN-γ-producing conventional CD4 T cells but only fluvastatin treatment affected cytokine-production by CD8 T cells (**Extended Data Fig. 1e, f**). These data indicate that *in vitro* inhibition of mevalonate metabolism leads to impaired cytokine production by T cells.
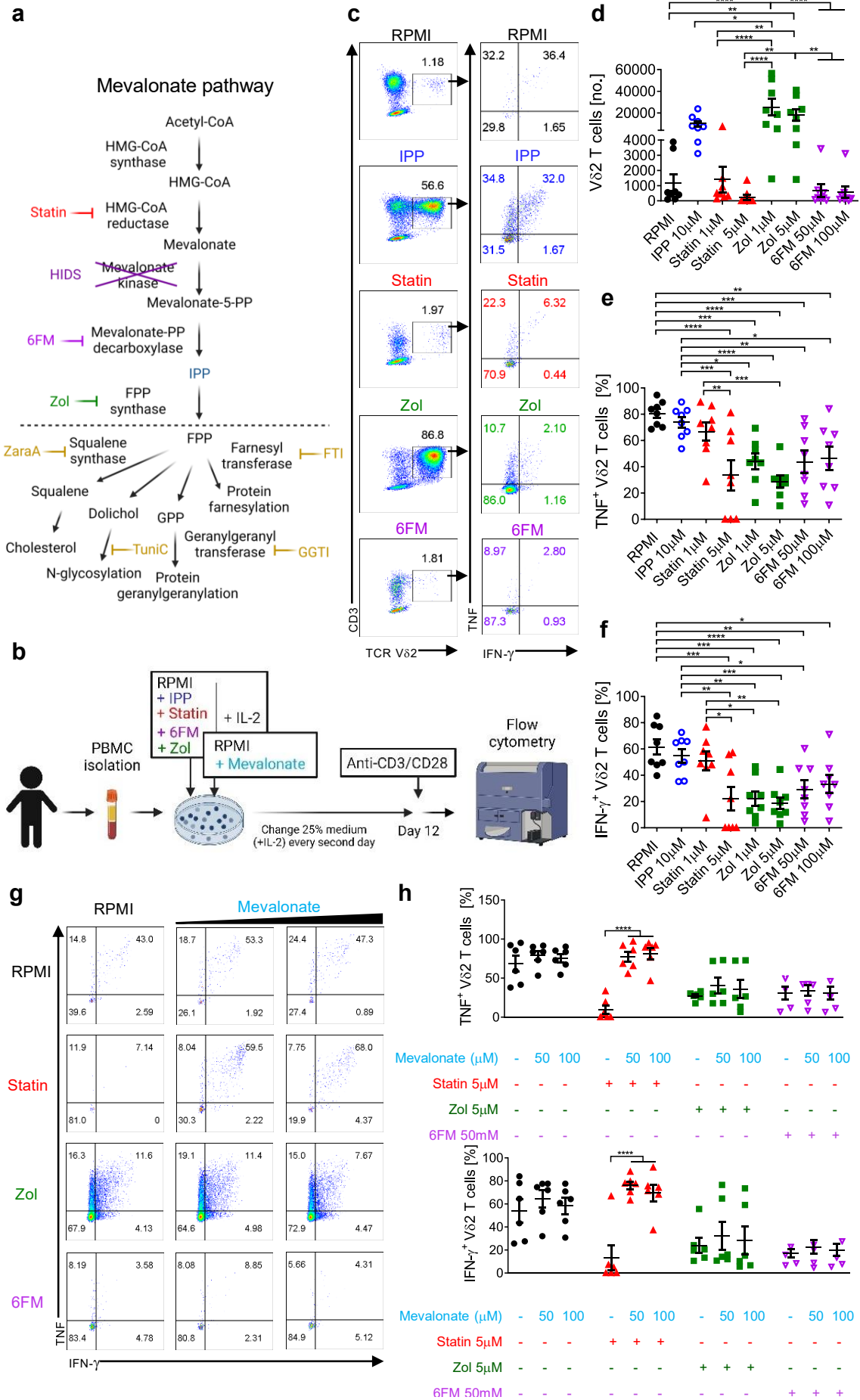
Fig. 1: *In vitro* **inhibition of mevalonate pathway results in compromised cytokine production by Vδ2 T cells. a,** Schematic representation of the mevalonate pathway, the inhibitors used in the study and downstream biological processes: fluvastatin (Statin) inhibits HMG-CoA reductase; Hyper-IgD syndrome (HIDS) is caused by deficiency in mevalonate kinases; 6-fluoromevalonate (6FM) inhibits mevalonate-5-PP decarboxylase; zoledronate (Zol) inhibits FPP synthase; zaragozic acid (ZaraA) inhibits squalene synthase; Tunicamycin (TuniC) alters N-linked glycosylation of proteins; GGTI 2133 inhibits geranylgeranyl transferase; and FTI 277 (FTI) inhibits farnesyl transferase; **b,** Experimental setup for *in vitro* inhibition of mevalonate pathway and rescue experiment with mevalonic acid. **c-f,** Flow cytometry analysis of Vδ2 T cells in 12-days PBMC cultures treated with indicated inhibitors (Mean ± SEM, n=8): **c,** representative dot plots showing % of Vδ2 T cells in PBMC cultures (left column) and % of TNF$^+$ and IFN-γ$^+$ Vδ2 T cells (right column); **d,** cumulative numbers of Vδ2 T cells; **e,** cumulative percentages of TNF$^+$ and **f,** IFN-γ$^+$ Vδ2 T cells; **g,** Representative FACS plots showing % of cytokine-producing Vδ2 T cells in PBMC cultures treated with indicated inhibitors in the presence or absence of mevalonic acid (50μM or 100μM); **h,** Cumulative % of TNF$^+$ and IFN-γ$^+$ Vδ2 T cells in PBMC cultures treated with indicated inhibitors in the presence or absence of mevalonic acid (Mean ± SEM, n=6). each dot represents one donor in **d-f** and **h.**, repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05. HMG-CoA: β-hydroxy β-methylglutaryl-Coenyzme A; HIDS: hyper-IgD syndrome; 6FM: 6-fluoromevalonate; mevalonate-5-PP decarboxylase:mevalonate diphosphate decarboxylase; GPP:geranyl pyrophosphate; FPP: farnesyl pyrophosphate.

To validate that the effect of the drugs on cytokine production is due to the inhibition of mevalonate metabolism, we supplemented PBMC cultures with mevalonic acid (**Fig. 1g, h**). Mevalonic acid alone had no effect on Vδ2 T cells while the supplementation of mevalonic acid to fluvastatin-treated cultures restored the viability and cytokine production by Vδ2 T cells (**Fig. 1g, h** and **Extended Data Fig. 1c**) as well as conventional CD4 and CD8 T cells (data not shown). This indicates that the decreased cytokine production and increased cells death in fluvastatin-treated cultures are the effects of the mevalonate deficiency upon the blockage of the pathway and not directly caused by the drug cytotoxicity. As expected, mevalonate addition to the PBMC cultures treated with downstream inhibitors: zoledronate or 6-fluoromevalonate had no effect on Vδ2 T cells (**Fig. 1g,h**). Altogether these data indicate that the effect of the inhibitors on Vδ2 T cell function is mediated by the suppression of mevalonate metabolism.

In vivo inhibition of the mevalonate pathway compromises cytokine production by Vδ2 T cells.

To verify whether our *in vitro* observations translate to the *in vivo* settings we performed a longitudinal cohort study on patients with hypercholesterolemia who were prescribed statin-therapy. We assessed the numbers and cytokine production capacity of Vδ2 T cells from these patients before and after 3-months of statin treatment (**Fig. 2a**). The numbers of Vδ2 T cells in patients were comparable to those in healthy donors (**Fig. 2b**). In accordance with our *in vitro* findings, the percentage of TNF- and IFN-γ-producing Vδ2 T cells markedly decreased after 3-months of statin-treatment (**Fig. 2c**). Statin-treatment also affected the cytokine production capacity of CD4 and CD8 T cells (**Extended Data Fig. 2a**, **b**) but to a lesser extent than those of Vδ2 T cells (**Fig. 2c**).

Furthermore, we recruited patients with hyper-IgD syndrome who have a deficiency in mevalonate kinase (**Fig. 2d, e**). Consistently, we observed no significant change in Vδ2 T cell numbers but a reduced proportion of cytokine-producing Vδ2 T cells in patients compared to healthy controls. Yet, this condition had no effect on conventional T cells (**Extended Data Fig.**

2c,d). Altogether our *in vitro* and *in vivo* data show that mevalonate metabolism plays an important role in cytokine production by Vδ2 T cells.
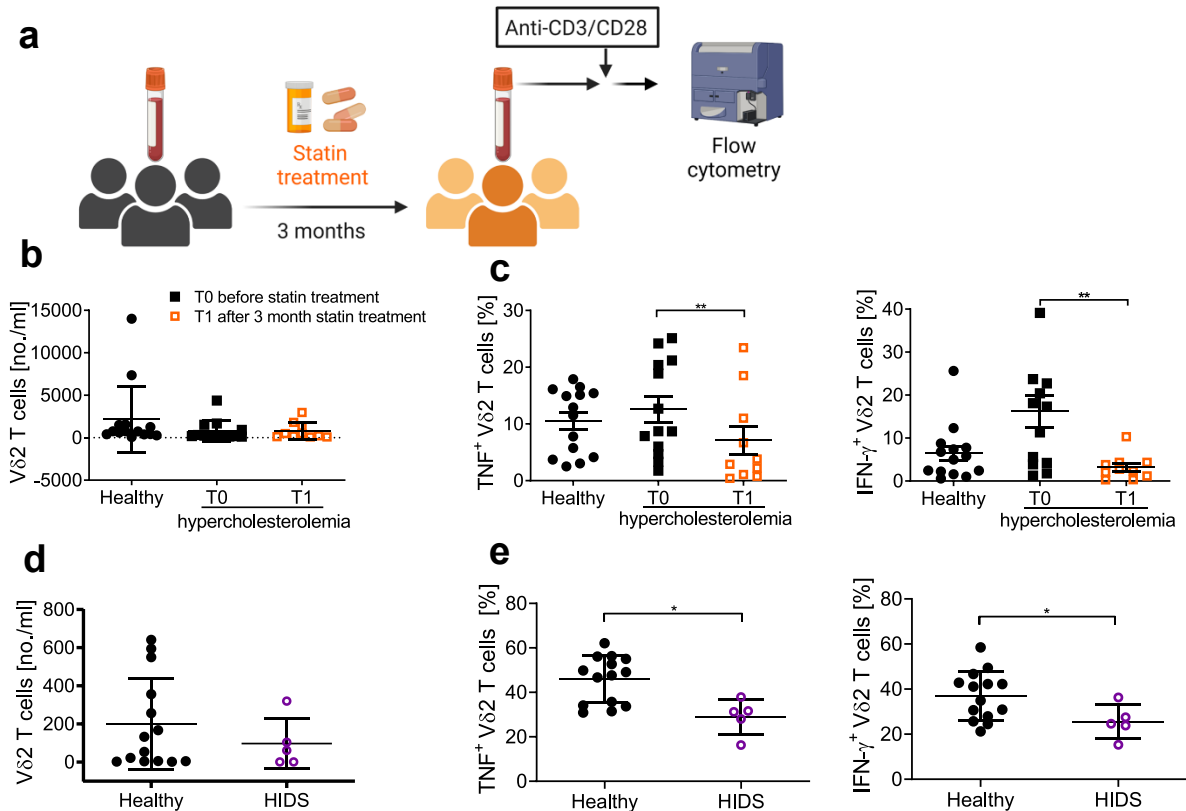
Fig. 2: *In vivo* **inhibition of mevalonate pathway results in compromised cytokine production by Vδ2 T cells.** **a,** Schematic representation of flow cytometry analysis of peripheral blood from patients with hypercholesterolemia before and after 3-months of statin treatment; **b,** Number of Vδ2 T cells and **c,** % of TNF$^+$ and IFN-γ$^+$ Vδ2 T cells in healthy individuals and patients with hypercholesterolemia before and after 3-months of statin treatment (Mean ± SEM, n=13: patients T0; n=10 patients T1; n=14: healthy donors; Mann-Whitney test: patients and healthy donors; Wilcoxon test: before and after treatment, P value * < 0.05). **d,** Number of Vδ2 T cells and **e,** % of TNF$^+$ and IFN-γ$^+$ Vδ2 T cells in healthy individuals and patients with Hyper-IgD syndrome, (Mean ± SEM, n=5: patients; n=14: healthy donors); Mann-Whitney test, P value * < 0.05.

Mevalonate pathway inhibition induces transcriptome changes in Vδ2 T cells.

To further determine the effect of mevalonate pathway inhibition on transcriptome and chromatin landscapes, we sorted the Vδ2 T cells from fluvastatin- and zoledronate-treated as well as IPP- and non-treated PBMC cultures and performed RNA-sequencing (RNA-seq) along with the Assay for Transposase-Accessible Chromatin using Sequencing (ATAC-seq) (**Fig. 3** and **Extended Data Fig. 3**). Principal component analysis of RNA-seq data revealed clustering of samples based on culturing conditions where IPP- and zoledronate-expanded Vδ2 T cells showed the most similarity in the transcriptional programs (**Fig. 3a** and **3b**). The highest number of transcripts was differentially up- and down-regulated in IPP- and zoledronate-treated Vδ2 T cells when compared to the Vδ2 T cells from RPMI-treated cultures (**Fig. 3c, d** and **Extended Data Fig. 3a**). Among mutually upregulated transcripts in IPP- and zoledronate-treated Vδ2 T cells vs. RMPI alone we found genes related to the regulation of cell cycle (e.g. *CDK6*, *CDCA7L*), proliferation (e.g. *PDGFRB*, *MYB*) and replication (e.g. *PIR*) (**Fig. 3b, d**). Furthermore, some transcripts related to immune functions were both mutually

a

PC 2 : 14.4% variance

PC 1: 39.5% variance

condition   ● RPMI   ● IPP   ● Zol   ● Statin

b All differentially expressed genes

donor
condition

GIPR
GPI
SLC2A3
PDGFRB
PIR
CCR2
MYB
CDCA7L
CDK6

TNF
IRF4

TLR3
CD160

XCL2
XCL1

IL7R

donor
a297
a148
a268

condition
RPMI
IPP
Zol
Statin

c

■ up-regulated genes
■ down-regulated genes

Number of genes

IPP vs RPMI   Zol vs RPMI   Statin vs RPMI

d

IPP vs. RPMI

−log10(padj)

log2(FoldChange)

XCL1

XCL2

FOSB   PLXDC1

ANKRD35
NFKBIZ
PLXNA4   TSHZ3
CD160

HLA-DRA   MSC

NCF4   MAST4   FABP5
IRF4   EGFL6
CCR2   BCAT1
CDK6   PIR   PDGFRB
MYB
CDCA7L

Zoledronate vs. RPMI

−log10(padj)

log2(FoldChange)

XCL2
XCL1

PLXDC1   DHRS3
IFIT2   NCAM1
CNR2
C1orf162
PLXNA4   CD160   DENND2D
ANKRD35
IGFBP2

NCF4
CTSH
CCR2   MSC
IRF4
CD70   TNFRSF11A
CDK6   PIR   MAST4
TNF   ITGA2
CDCA7L
GPAT3
PDGFRB
MYB

Fluvastatin vs. RPMI

−log10(padj)

log2(FoldChange)

GIPR
GPI   ENPEP

SLC2A3
HILPDA   COTL1
SUSD3
DUSP2   PGK1
WNT10B   HSPA1B

MYO1E

USP9Y

UTY

IL7R

KIF1B
AHR   TNFRSF9
RCBTB2   CX3CR1
SYK   RHOB
TLR3

55

Fig. 3: Mevalonate pathway inhibition causes transcriptome changes in Vδ2 T cells. a-g, RNA-seq analysis of Vδ2 T cells isolated from 12-days-PBMC cultures treated with IPP, zoledronate (Zol), fluvastatin (Statin) or RPMI alone (n=3); **a,** Principal component analysis colored by experimental conditions: RPMI (black), IPP (blue), Zol (green) and Statin (red). **b,** Clustered heatmap showing 479 differentially regulated genes between RPMI, IPP, Zol and Statin conditions. Selected genes are annotated. **c,** Number of differentially expressed genes in IPP, Zol and Statin conditions vs. RPMI. **d,** Volcano plot demonstrating differentially upregulated and downregulated transcripts. Grey points indicate genes with p-values > 0.05 and $\log_2$fold change > sigFC=-2 and < sigFC=2. Blue points indicate genes with p-values < 0.05 and $\log_2$fold change < sigFC=-2. Red points indicate genes with p-values < 0.05 and $\log_2$fold change > sigFC=2. **e,** Enrichment dot plots for IPP over RPMI, Zol over RPMI and Statin over RPMI treatments, showing most significantly enriched GO terms. The topmost enriched terms with adjusted P-values ≤ 0.1 are demonstrated. **f,** hCoCena Integrated group fold change (GFC) heat map showing hierarchical clustering and gene modules identified by hCoCena analysis for the RPMI, IPP, Zol and Statin-treated groups. Numbers and bar-plots on the right side reflect the sizes of the modules. **g,** Functional enrichment of hCoCena-derived modules using the GO gene set database. Selected top terms were visualized.

up- (e.g. CCR2, IRF4), while some were mutually down-regulated (e.g. XCL, CD160) in IPP- and zoledronate-treated Vδ2 T cells (**Fig. 3b, d**). Among all conditions fluvastatin-treated Vδ2 T cells presented the lowest number of up-regulated transcripts vs. RPMI, which mainly related to immune function (e.g. *IL-7R*, *TLR3*) (**Fig. 3b, c, d** and **Extended Data Fig. 3a**). On the other hand, significantly down-regulated transcripts in fluvastatin-treated Vδ2 T cells were in large proportion related to metabolic processes (e.g. *GIPR*, *GPI*, *SLC2A3*) (**Fig. 3b, d**). While we did not detect significant differences in *IFNG* expression between all conditions based on differential expression analysis, the *TNF* transcript was upregulated in zoledronate-treated cells (**Fig. 3b, d**). Gene Ontology (GO) enrichment analysis further revealed the up-regulation of immune effector processes, cell adhesion and adaptive immune response in IPP- and zoledronate-treated compared to RPMI-treated Vδ2 T cells (**Fig. 3e**). Genes that were upregulated in zoledronate-treated Vδ2 T cells also showed enrichment in positive regulation of cell activation involved in immune response, leukocyte mediated immunity and cell-cell adhesion (**Fig. 3e**). The genes upregulated in zoledronate- and fluvastatin-treated Vδ2 T cells showed enrichment in GO pathways related to leukocyte mediated immunity, regulation of immune effector processes and adaptive immune response (**Fig. 3e**). Conversely, genes upregulated in fluvastatin-treated Vδ2 T cells were enriched in GO terms related to negative regulation of immune effector processes including leukocyte mediated immunity and cytotoxicity, while the downregulated genes showed enrichment in many metabolic processes such as glycolysis and phosphorylation (**Fig. 3d**). To further characterize differentially mobilized functional gene modules in Vδ2 T cells between conditions, horizontal construction of co-expression networks and analysis (hCoCena) was applied (**Fig. 3f, g** and **Extended Data Fig. 3b**)(36). hCoCena allows the analysis of all conditions at the same time, identifying common and condition-specific co-expressed genes. The resulting network consisted of 5786 nodes (genes), connected by 63226 edges (co-expression relationships) (**Extended Data Fig. 3b**). Ten modules were identified using unsupervised Leiden clustering, highlighted by specific colors, representing groups of genes with similar expression patterns (**Fig. 3f,** left), while the heatmap visualizes the modules' mean group fold changes (GFCs) across all conditions (**Fig. 3f,** right). The genes grouped in each module were used for GO enrichment analysis. Consistent with the phenotype observed this analysis revealed that gene modules up-regulated in IPP and zoledronate-treated Vδ2 T cells (gold) were enriched in GO terms related to leukocyte activation and adhesion and amino acid transport as well as mitotic nuclear division and

organelle fission (lightgreen) (**Fig. 3g**). In contrast, the down-regulated modules in these conditions showed enrichment in GO terms associated with mitochondrial function (maroon) and response to viruses (turquoise) (**Fig. 3g**). The down-regulated modules in fluavastatin-treated Vδ2 T cells showed enrichment in processes related to cell division (lightgreen) (**Fig. 3g**). Furthermore, the close analysis of the transcription factor transcripts are known to directly regulate *IFNG* and *TNF* expression, such as *TBX21*, *STAT*, *NFAT* etc. could not explain the effect of inhibitors on the cytokine levels in Vδ2 T cells (**Extended Data Fig. 3b**). Altogether,
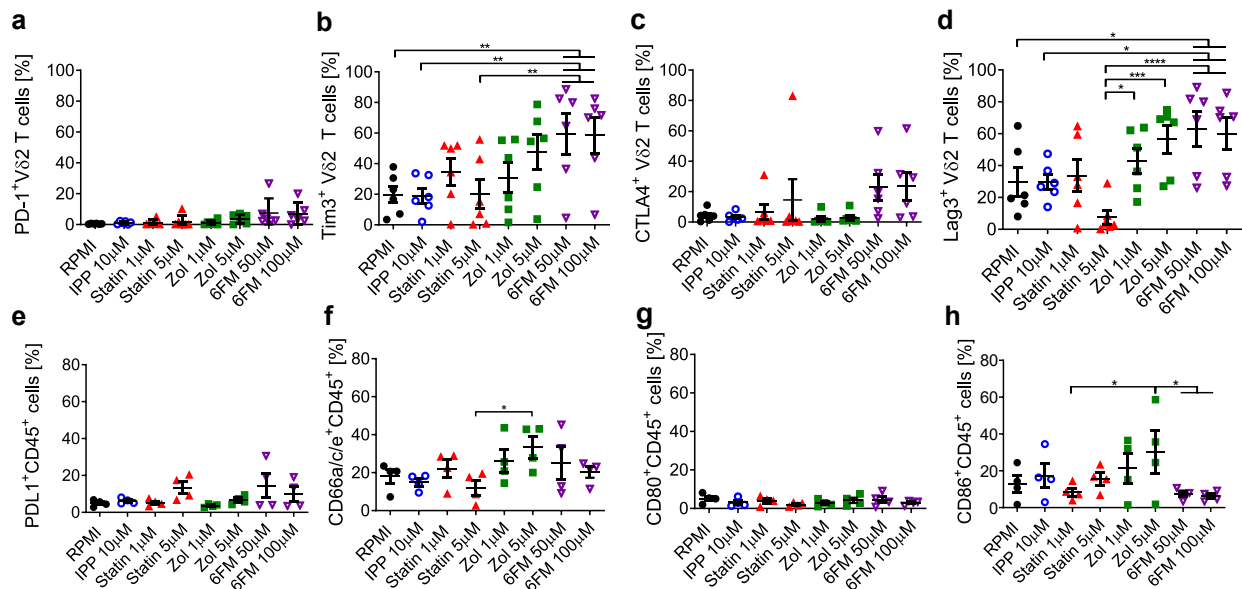
Fig. 4: Zoledronate- and 6-fluoromevalonate upregulate the expression of exhaustion markers on Vδ2 T cells. a-h, Flow cytometry analysis of Vδ2 T cells in 12-days PBMC cultures treated with indicated inhibitors: Cumulative % of **a,** PD-1$^+$, **b,** Tim3$^+$, **c,** Lag3$^+$ and **d,** CTL4$^+$ Vδ2 T cells (Mean ± SEM, n=6); Cumulative % of **e,** PDL1$^+$, **f,** CD66a/c/e$^+$, **g,** CD80$^+$ and **h,** CD86$^+$ expression on CD45$^+$ cells (Mean ± SEM, n=4). Each dot represents one donor. (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value $* < 0.05$).

the RNA-seq data unraveled the modulatory effect of mevalonate metabolism on the transcriptome of Vδ2 T cells. We further assessed whether mevalonate pathway inhibition affects the transcriptome by modulating chromatin accessibility. The ATAC-seq analysis demonstrated that the chromatin accessibility of differentially expressed transcripts, including *TNF* and *IFNG* loci, was not significantly affected in IPP-, zoledronate- and fluvastatin- vs. RPMI alone-treated Vδ2 T cells (**Extended Data Fig. 3c**). We further investigated other mechanisms fueled by mevalonate metabolism that are involved in the regulation of cytokine production in Vδ2 T cells upon restimulation.

Zoledronate- and 6-fluoromevalonate upregulate the expression of exhaustion markers on Vδ2 T cells.

An underlying mechanism leading to compromised cytokine production may be in large part the exhaustion of Vδ2 T cells in long-term cultures. Indeed, the RNA-seq analysis revealed the upregulation of *TIM3* exhaustion marker in Vδ2 T cells from zoledronate-treated cultures (**Extended Data Fig. 4**). To determine whether inhibition of mevalonate metabolism causes exhaustion, we assessed the protein expression levels of immune-suppressive checkpoint

receptors such as PD-1, TIM3, CTLA-4 and LAG3 on the Vδ2 T cells by flow cytometry (37–40) (**Fig. 4a-d**). IPP, zoledronate and fluvastatin treatment did not significantly increase the numbers of Vδ2 T cells expressing these receptors compared to RPMI alone condition, whereas 6-fluoromevalonate significantly upregulated the numbers of TIM3- and LAG3- expressing cells (**Fig. 4a-d**). Although the enhanced expression of checkpoint receptors is a strong indicator of immune cell exhaustion, their inhibitory effect is only exerted in the presence of their ligands. Therefore, we assessed the expression of the checkpoint receptor ligands: PDL1,
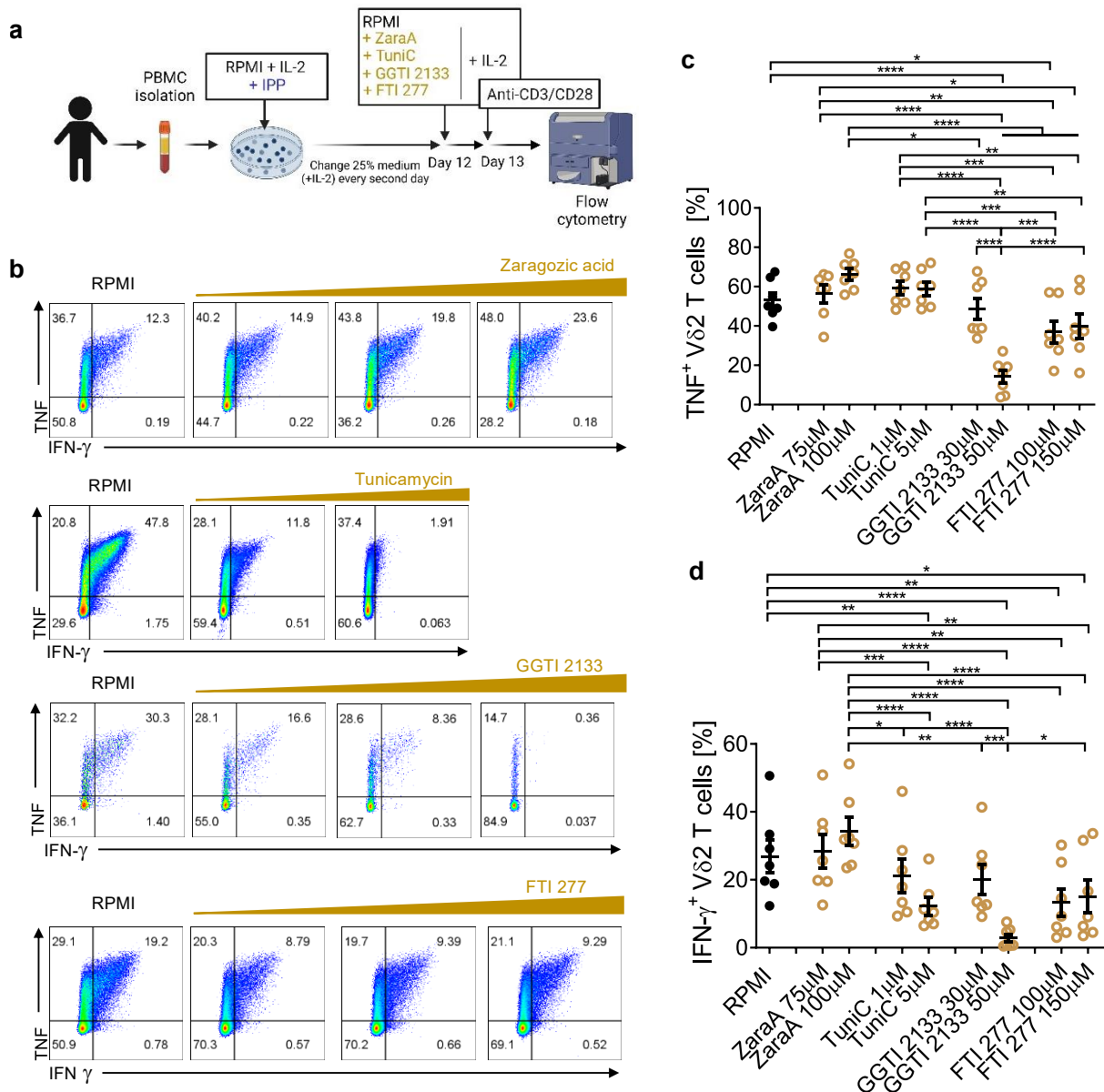
Fig. 5: Inhibition of protein prenylation impairs TNF and IFN-γ production by Vδ2 T cells. a, Experimental setup for the *in vitro* inhibition of downstream mevalonate pathways. PBMCs were first expanded with IPP in the presence of IL-2 for 12 days. On day 12, the indicated inhibitors: zaragozic acid (75μM, 100μM), tunicamycin (1μM, 5μM), geranylgeranyl transferase inhibitor (GGTI2133; 30μM, 50μM) and farnesyl transferase inhibitor (FTI277; 100μM, 150μM) were added for overnight incubation. **b-d,** Flow cytometry analysis of Vδ2 T cells in PBMC cultures treated as described above (Mean ± SEM, n=7): **b,** Representative dot plots showing % of TNF+ and IFN-γ+ Vδ2 T cells in PBMC cultures; **c,** cumulative % of TNF+ and **d,** IFN-γ+ Vδ2 T cells in PBMC cultures. Each dot represents one donor. (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05).

CD66a/c/e$^+$, CD80 and CD86 on PBMCs in our cultures (41–43) (**Fig. 4e-h**). A higher proportion, yet statistically not significant, of zoledronate-treated PBMCs expressed TIM-3 and CTLA-4 ligands: CD66a/c/e and CD86, respectively (**Fig. 4f, h**). Therefore, exhaustion mechanisms may only partially explain the reduced cytokine production capacity of Vδ2 T cells. We therefore hypothesized that the post-translational modifications downstream of the mevalonate pathway, such as N-glycosylation or prenylation, play a rather more important role in the cytokine production by Vδ2 T cells.

Inhibition of protein prenylation impairs TNF and IFN-γ production by Vδ2 T cells.
To decipher which biological processes downstream of the mevalonate pathway (**Fig. 1a**) affect the cytokine production of Vδ2 T cells, we pre-treated PBMC cultures with IPP and IL-2, as this condition expands the Vδ2 T cells but does not impair mevalonate metabolism and therefore does not affect the cytokine production capacity of Vδ2 T cells (**Fig. 5a**). After 12 days of expansion, the cultures were incubated for an additional 24 hours in the presence of different downstream mevalonate pathway inhibitors: zaragozic acid (ZaraA), tunicamycin (TuniC), geranylgeranyltransferase inhibitors (GGTI) and farnesyltransferase inhibitors (FTI) and the cytokine production by Vδ2 T cells was assessed (**Fig. 5**). Zaragozic acid, which blocks cholesterol synthesis by inhibiting squalene synthesis (**Fig. 1a**), did not significantly affect the number of cytokine-producing Vδ2 T cells (**Fig. 5b – d**). This is the opposite phenotype to the one caused by statin, zoledronate and 6-fluoromevalonate which inhibit cytokine production (**Fig. 1c**, **e, f**). To evaluate whether cholesterol levels in Vδ2 T cells are indeed affected by the inhibitors, we performed filipin staining (**Extended Data Fig. 5a**, **b**). Interestingly, the surface and intracellular cholesterol levels were not affected in Vδ2 T cells, it is therefore unlikely that fluvastatin, zoledronate and 6-fluoromevalonate affect TCR signaling through cholesterol depletion.In the presence of tunicamycin, which blocks the N-glycosylation process (**Fig. 1a**), the percentage of IFN-γ-producing Vδ2 T cells significantly decreased while the TNF-producing cells slightly increased (**Fig. 5b-d**). This is consistent with the fact that IFN-γ, but not TNF, undergoes N-glycosylation(44,45). Since only IFN-γ production by Vδ2 T cells is affected upon disruption of N-glycosylation, there are additional regulatory mechanisms which contribute to the Vδ2 T cell phenotype caused by fluvastatin, zoledronate and 6-fluoromevalonate.

Finally, we tested protein prenylation, which is another posttranslational modification found on many signaling proteins (**Fig. 1a**). Inhibition of protein prenylation by farnesyltransferase inhibitor FTI277 and geranylgeranyltransferase inhibitors: GGTI2133, GGTI298 and GGTI286 (**Fig. 1a**) decreased both TNF and IFN-γ production by Vδ2 T cells (**Fig. 5b-d** and **Extended Data Fig. 5c**). However, this trend was not observed when using another FTase inhibitor II (FTIII) (**Extended Data Fig. 5d**). This data indicates that protein prenylation, especially protein geranylgeranylation, downstream mevalonate pathway is important for TNF and IFN-γ production by Vδ2 T cells.
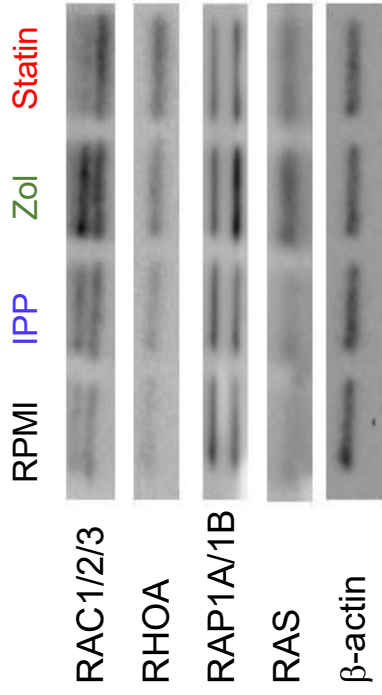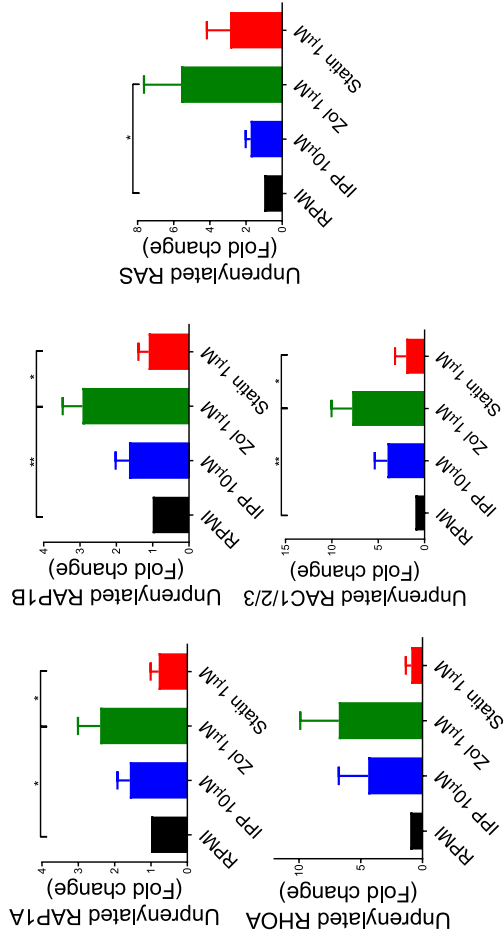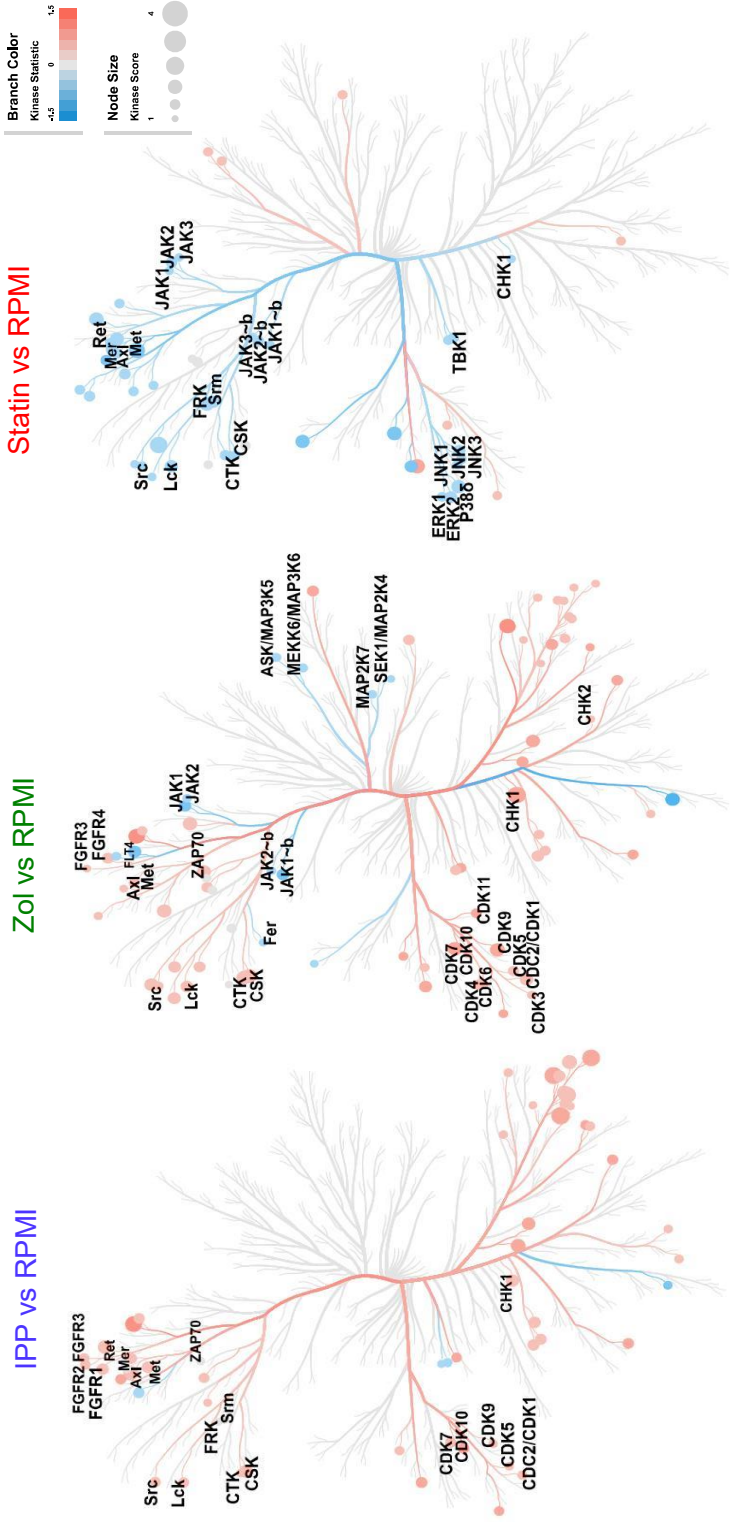
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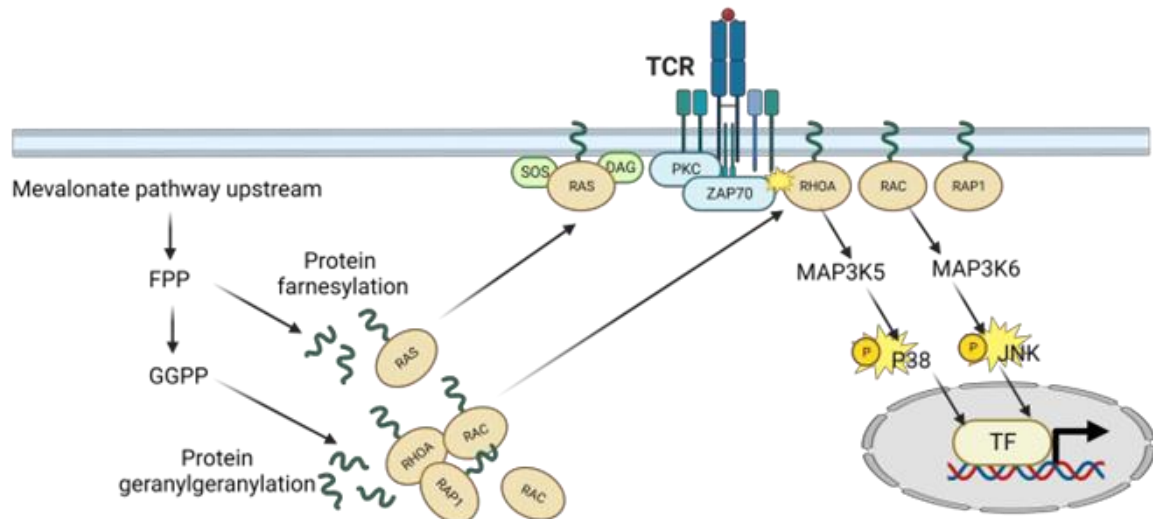
Fig. 6: Mevalonate pathway inhibition leads to accumulation of unprenylated small G proteins in the cytosol of Vδ2 T cells and perturbation of downstream signaling pathways. a, Representative Western Blot image showing enrichment of unprenylated small G proteins: RAC, RHOA, RAP1 and RAS in the cytosol of Vδ2 T cells isolated from 12-days-PBMC cultures treated with the indicated inhibitors. **b,** Quantification of cytosolic small G proteins in Western Blot using imageJ. Fold change was calculated over RPMI condition (Mean ± SEM, n=5) repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05); **c,** PamGene analysis of kinase activity in Vδ2 T cells isolated from PBMCs cultured 12 days with IPP, zoledronate, fluvastatin or RPMI alone and restimulated for 10 mins with anti-CD3/CD28 (n=7): Kinome tree showing kinases with up- and down-regulated activity in Vδ2 T cells. **d,** Schematic representation of the molecular mechanisms by which mevalonate metabolism fuels Vδ2 T cell function. Briefly, FPP generated in the mevalonate pathway serves for protein prenylation. Among prenylated proteins are small GTPases which when prenylated anchor to the cell membrane where they interact with various receptors including T cell receptor (TCR). Upon TCR stimulation small GTPases are activated and transduce the signal to the nucleus by activating downstream signaling pathways such as MAPK signaling. This results in transcription factors recruitment to the nucleus and induction of effector gene expression. Mevalonate pathway inhibition results in impaired protein prenylation and consequently compromised signal transduction upon TCR activation resulting in impaired Vδ2 T cell function. FPP, farnesyl pyrophosphate; GGPP, Geranylgeranyl pyrophosphate; Ras, Rat sarcoma; Rac, Ras-related C3 botulinum toxin substrate; RAP1, Ras-related protein 1; RHOA, Ras homolog family member A; MAP3K, Mitogen-Activated Protein Kinase Kinase Kinase; JNK, c-Jun N-terminal kinases; TF, transcription factor.

***In vitro* inhibition of mevalonate pathway leads to dysregulation of signal transduction pathways in Vδ2 T cells.**

Protein prenylation has been shown to play a role in signaling events important for T cell activation (46). For example, small GTPases are signal transducing molecules activated in response to TCR stimulation(47,48). When prenylated, they anchor to the cellular membranes where they can interact with various receptors(49)**.** To verify whether protein prenylation of small GTPases such as: RAC, RHOA, RAP1 and RAS is indeed compromised in Vδ2 T cells upon statin- and zoledronate-treatment, we extracted the cytosolic proteins from purified Vδ2 T cells derived from statin- and zoledronate-treated PBMC cultures and performed Western Blot analysis (**Fig. 6a, b**). A significant accumulation of unprenylated small G proteins such as RAC, RAP1A/B and RAS was observed in the cytosol fraction of zoledronate-expanded Vδ2 T cells compared to untreated (RPMI alone condition). Fluvastatin treatment resulted in a slight increase of unprenylated RAS while IPP-expanded Vδ2 T cells accumulated unprenylated RAC and RHOA, yet these changes were not significant (**Fig. 6a, b**). This data indicates that

protein prenylation in Vδ2 T cells is affected by mevalonate pathway modulation, yet each compound used has a specific effect.

To investigate whether signaling events downstream small GTPases such as MAP kinases: ERK, P38 and JNK signaling, important for many effector functions of T cells including cytokine production(50), are also affected due to the compromised protein prenylation upon mevalonate pathway inhibition, we performed the PamGene global kinase activity assay on TCR-activated Vδ2 T cells from our PBMC cultures (**Fig. 6c**, **d** and **Extended Data Fig. 6**). The PamGene technology allows the determination of numerous kinase activities based on the phosphorylation status of the target phosphosites. To that end, Vδ2 T cells were purified from the 12-days cultures and stimulated with anti-CD3/CD28 for 10 minutes prior to the kinome assessment. We found that among all treatment conditions IPP affected the smallest number of phosphosites compared to RPMI condition (**Extended Data Fig. 6a**). Zoledronate- and fluvastatin-treatment resulted in a higher number of phosphosites with decreased phosphorylation than IPP-treatment when compared to RPMI (**Extended Data Fig. 6a**). By performing the global kinase activity analysis, we observed the highest number of kinases with downregulated activity in statin condition and the highest number of kinases with upregulated activity in IPP and zoledronate condition (**Fig. 6c** and **Extended Data Fig. 6b**). In particular, IPP- and zoledronate increased the activity of the kinases related to proliferation, differentiation and cell cycle regulation such as AXL, a receptor kinase that binds to GAS6 and regulates cell proliferation and survival (**Fig. 6c**)(51); FGFRs, receptor kinases important for mitogenesis and differentiation(52); and CHKs, kinases which act as a key regulator of the cell cycle(53) (**Fig. 6c**). This is in line with the observed expansion of Vδ2 T cells in these cultures (**Fig. 1c, d**). Activities of serine threonine kinases downstream of small GTPases, such as ASK/MAP3K5 and SEK1/MAP2K4, were downregulated upon zoledronate treatment (**Fig. 6c**). Similarly, in statin-treated cells, the activities of kinases such as ERK, P38 and JNK were reduced in response to short TCR activation indicating that effector signaling cascades downstream of GTPases are impaired upon mevalonate pathway inhibition. Furthermore, most of the tyrosine receptor kinases, which are important for the initiation of receptor signaling such as LCK, LTK and MET, are affected in the statin-treated condition (**Fig. 6c**), indicating that the kinases upstream of GTPases are also highly affected. Contrary to IPP- and zoledronate-treated Vδ2 T cells, the kinases regulating proliferation and differentiation such as TBK1 and CHK1 were downregulated in statin-treated cultures in line with the decreased proliferation of Vδ2 T cells in this condition (**Fig. 1c, d**). Interestingly, our analysis did not reveal kinases commonly regulated by the two pharmacological inhibitors consistent with their distinct effect on the prenylation status of small G proteins (**Fig. 6a, b**). Overall manipulation of the mevalonate pathway results in dysregulation of the kinome in Vδ2 T cells, yet the effect of different inhibitors is specific (**Fig. 6d**).

Mevalonate metabolism is important for cytotoxic properties of Vδ2 T cells.

Apart from pro-inflammatory cytokine production, Vδ2 T cells possess cytotoxic activity(54,55). Therefore, we investigated whether mevalonate metabolism is also essential for the cytotoxic function of these cells. With the same *in vitro* experimental setup (**Fig. 1b**,
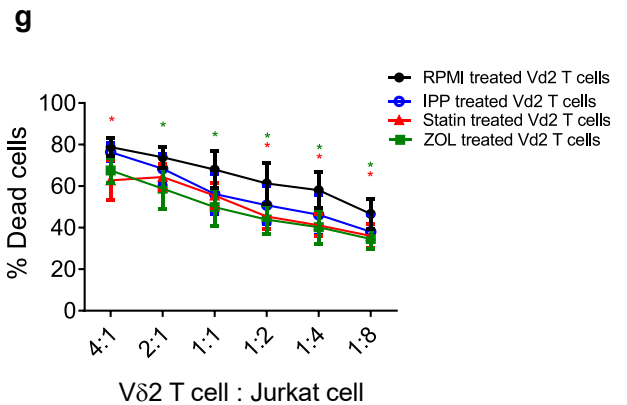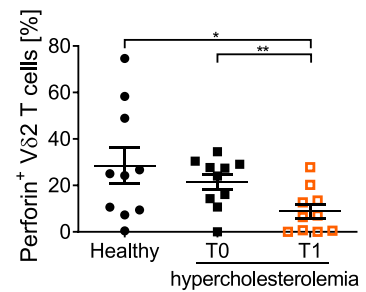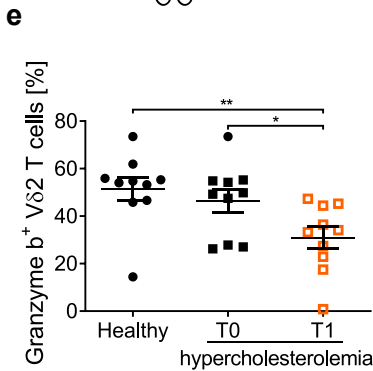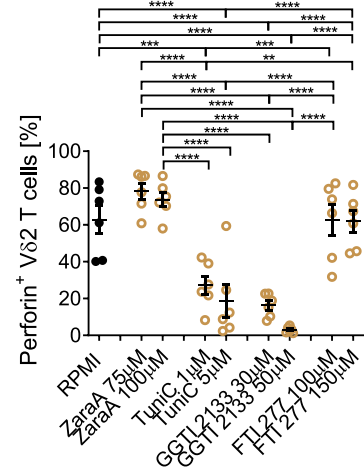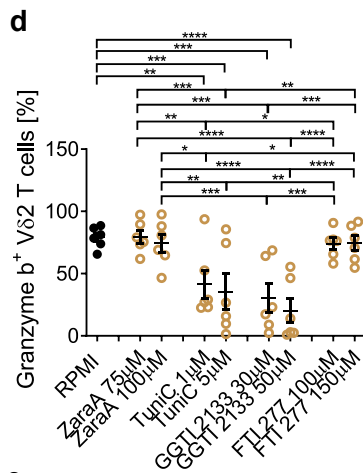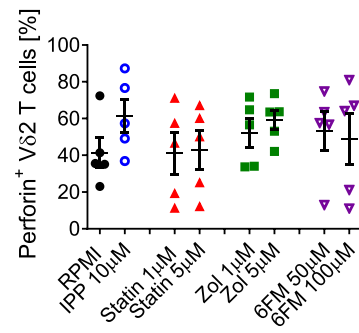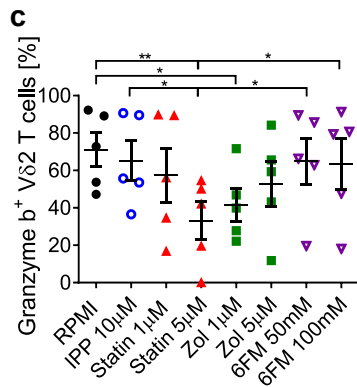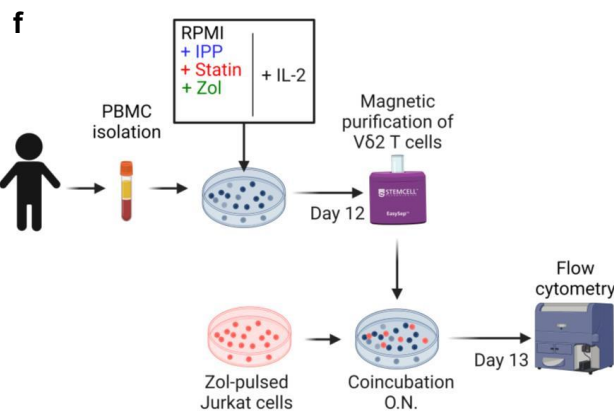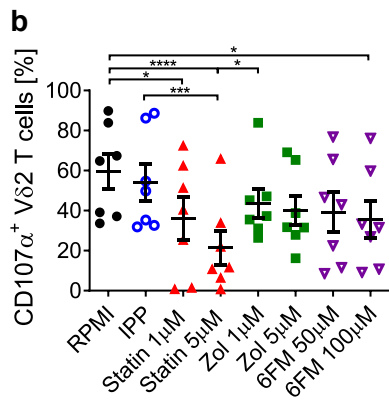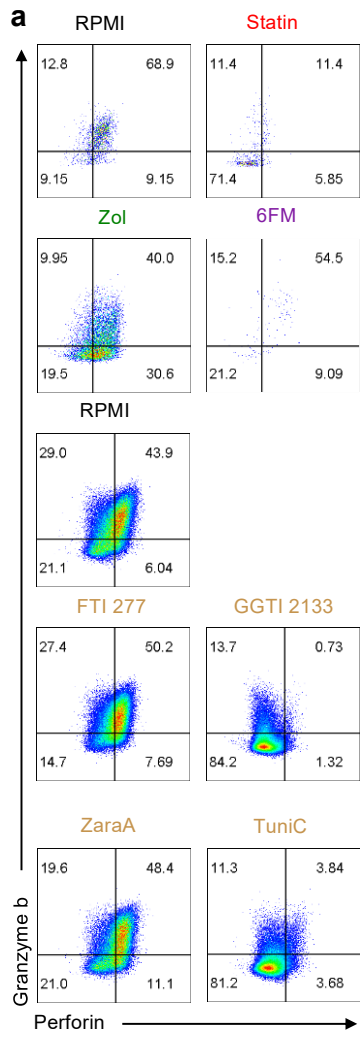
Fig. 7: Mevalonate metabolism is important for cytotoxic properties of Vδ2 T cells. a-d, Flow cytometry analysis of Vδ2 T cells in PBMC cultures treated as previously with indicated inhibitors (Mean ± SEM, A,C: n=5; B: n=7, D: n=6): **a,** representative dot plots showing % of granzyme B$^+$ and perforin$^+$ Vδ2 T cells; cumulative % of **b,** CD107a$^+$, **c-d,** granzyme B$^+$ (left panel) and perforin$^+$ (right panel) Vδ2 T cells in PBMC cultures after incubation with indicated inhibitors. (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05). **e,** Cumulative % of granzyme B$^+$ and perforin$^+$ Vδ2 T cells in patients with hypercholesterolemia before and after 3-months of statin treatment. (Mean ± SEM, n=10: patients T0, T1; n=10: healthy donors); (Mann-Whitney test: patients and healthy donors; Wilcoxon test: before and after treatment, P value * < 0.05). (B-E) Each dot represents one donor. **f-g,** *in vitro* Vδ2 T cell cytotoxic assay: **f,** Schematic representation of the experimental setup: PBMCs were incubated for 12 days with the indicated stimulus or inhibitors in the presence of IL-2. The Vδ2 T cells were isolated from PBMC cultures by magnetic purification and co-incubated with Jurkat cell at different cell to cell ratios. **g,** % of dead Jurkat cells after co-incubation with Vδ2 T cells assessed by flow cytometry (Mean ± SEM, n=5); (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05).

5a), we assessed the granzyme B and perforin production by Vδ2 T cells as well as the degranulation marker CD107a(56,57) (**Fig. 7**). Similar to cytokine production capacity, IPP did not affect the production of these cytotoxic molecules (**Fig. 7a**-**c**). 6-fluoromevalonate had only an effect on CD107a expression while zoledronate and fluvastatin at high doses reduced CD107a expression and granzyme B production by Vδ2 T cells (**Fig. 7a**-**c**). Furthermore, the percentage of granzyme B-producing Vδ2 T cells decreased in both tunicamycin- and GGTI2133-treated cultures (**Fig. 7d**), resembling the phenotypes of Vδ2 T cells in statin- and zoledronate-treated cultures. Tunicamycin and GGTI2133 treatment also significantly reduced the numbers of perforin-producing Vδ2 T cells (**Fig. 7d**). Furthermore, mevalonate pathway inhibition by a high dose of fluvastatin resulted in reduced cytotoxic molecule production by CD8 T cells (**Extended Data Fig. 7a**). We therefore postulate that the mevalonate pathway may support granzyme B and perforin production by T cells. We next assessed whether the cytotoxic properties of Vδ2 T cells are also affected upon statin treatment *in vivo*. Consistent with our *in vitro* findings, patients undergoing statin therapy displayed a reduced proportion of granzyme B- and perforin- producing Vδ2 T cells (**Fig. 7e**) but among conventional T cells only perforin-producing CD8 T cell numbers decreased (**Extended Data Fig. 7b**). This data shows that the mevalonate pathway is important for the production of cytotoxic molecules by Vδ2 T cells.

To determine whether these observations are of functional relevance, we established an *in vitro* cytotoxic assay (**Fig. 7f, g**). To this end, we purified Vδ2 T cells from PBMC cultures treated with mevalonate pathway inhibitors and co-incubated them with zoledronate-pulsed Jurkat cells (**Fig. 7f**). The percentages of dead target cells were assessed by flow cytometry. The addition of Vδ2 T cells purified from RPMI alone cultures increased the death rate of Jurkat cells confirming the cytotoxic properties of Vδ2 T cells (**Fig. 7g**) while the clearance of the target cells by the fluvastatin- and zoledronate- treated Vδ2 T cells was decreased, further validating that mevalonate metabolism plays a role in the cellular cytotoxic activity of Vδ2 T cells against cancer cells.

Discussion

Vδ2 T cells play an important role in host defense against infection and in anti-cancer immune surveillance. Thanks to their potent killing properties, easy expansion with bisphosphonate drugs and HLA-independent activity, they have been gaining increasing attention in the field of T cell therapies (58). Yet, the preliminary trials with zoledronate-expanded Vδ2 T cells in cancer patients showed limited effectiveness (34,35). Our study revealed that despite robust proliferation, zoledronate-treated Vδ2 T cells exhibit reduced pro-inflammatory activity. Furthermore, other inhibitors of the mevalonate pathway, such as statins and 6-fluoromevalonate, also compromised Vδ2 T cell function. These results point to the importance of mevalonate metabolism for proper Vδ2 T cell immune responses and urge consideration of the use of pharmacological inhibitors in the clinic.

Due to its ability to inhibit farnesyl pryrophosphate synthase (FPS), zoledronate is known to cause the accumulation of IPP and consequently expand Vδ2 T cells(59–62). Although neutrophils and monocytes are known to both take up zoledronate, only monocytes produce and present IPP in the context of butyrophilins to activate Vδ2 T cells (61,63). As butyrophilins 2A1 and 3A1 are crucial for the activation of Vδ2 T cells (64) , it would be of interest to assess the effect of inhibitors on the expression levels of these molecules in our PBMC cultures. Consistent with previous studies, Vδ2 T cells expanded to high numbers in our IPP- and zoledronate-treated PBMC cultures. On the contrary, inhibition of the mevalonate pathway using 6-fluoromevalonate does not result in any significant changes in Vδ2 T cell numbers, in line with the phenotype of hyper-IgD syndrome patients. This is likely due to the lack of IPP accumulation upon 6-fluoromevalonate treatment.

Drugs targeting the isoprenoid biosynthesis pathway, namely statins and bisphosphonates, have been used for treating cardiovascular disease and bone diseases, respectivey(65,66). Both groups of drugs exhibit pleiotropic effects affecting the immune system (16–18,67). While statins have been long known for their immunomodulatory properties(18), very few studies have examined zoledronate effect on immune cells (16,67,68). Here we show that different mevalonate pathway inhibitors distinctly modulate the proliferative capacity, cytokine production and cytotoxic activity of Vδ2 T cells in PBMC cultures. A high concentration of statins significantly decreases the number of Vδ2 T cells. This is consistent with previous studies showing impaired proliferation capacity of human T cells upon statin treatment (69,70). This could be due to the toxicity of the drug, which was previously reported on CD4$^+$ T cells (69). However, mevalonic acid restored the Vδ2 T cells viability and cytokine production in statin-treated PBMC cultures, indicating that mevalonate deficiency, but not other processes, are responsible for the reduced viability and function of statin-treated Vδ2 T cells. Our RNA-seq analysis revealed that many transcripts related to negative regulation of immune cell functions are upregulated by statin treatment, while numerous genes related to cell division as well as glucose metabolism are downregulated in fluvastatin-treated Vδ2 T cells. It is known that activation of T cells as well as IFN-γ production by mouse γδ T cells highly depend on energy derived from aerobic glycolysis (71–73).

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Our transcriptome data revealed significant changes in gene expression caused by IPP and inhibitor-treatment. For example, pathways related to cell cycle regulation and DNA replication are up-regulated in IPP- and zoledronate-treated Vδ2 T cells, which is in line with the observation that Vδ2 T cells rapidly expand upon IPP stimulation. Transcript expression of TNF is up-regulated in zoledronate-treated Vδ2 T cells. These observations are not coherent with the phenotype observed in Vδ2 T cells upon mevalonate pathway inhibition. One reason for this discrepancy might be due to the fact that RNA-seq analysis was performed on expanded but not restimulated cells in contrary to cytokine production assessment by flow cytometry where cells were restimulated for 4 hours. Our analysis suggests that the mevalonate metabolism affects cytokine production at the posttranslational level. Notwithstanding no further evidence, we also do not exclude that the up- and down-regulation of the transcripts in zoledronate- and fluvastatin-treated Vδ2 T cells may indirectly affect the proinflammatory responses of Vδ2 T cells such as cytokine protein translation, trafficking or secretion. In our study, we did not further investigate the role of specific genes suggested by the transcriptome analysis. We do not rule out other possibilities that contribute to the phenotype we observed. Although TNF transcript expression is differentially expressed upon mevalonate inhibition, our ATAC-seq analysis suggested no significant alterations in the chromatin accessibility at the *TNF* and *IFNG* loci upon fluvastatin and zoledronate treatment. Analysis of DNA methylation and histone modifications might provide better insights into epigenetic events taking place in Vδ2 T cells upon mevalonate pathway inhibition. For example, based on animal models and *in vitro* studies, it has been postulated that statins affect histone acetylation, while cohort studies suggest that statin therapy affects DNA methylation in blood cells (74–76). Furthermore, the use of histone deacetylase inhibitors has been shown to affect the effector functions of Vδ2 T cells (77), suggesting indeed the important role of epigenetic mechanisms in shaping Vδ2 T cell responses.

T cell exhaustion is a common cause of impaired T cell function. The expression of immune-suppressive check-point receptors and their ligands, such PD-1 receptor and PD-1 ligand (42), Tim3 and CD66a/c/e(78) or CTLA-4 and CD80 and CD86 (79) are classical indicators of immune cell exhaustion (80). We found a significant upregulation of some immune-suppressive check-point receptors by Vδ2 T cells upon mevalonate pathway inhibition. However, we did not observe significant changes in the expression of the ligands in our cultures. Furthermore, except of CD66a/c/e, the ligands were expressed by very few cells suggesting that cell exhaustion is not the main cause of attenuated Vδ2 T cell function in our cultures. This is in line with recent findings revealing that blocking PD-1 and CTLA4 receptors with antibodies does not improve the cytokine expression by zoledronate-expanded human γδ T cells (81). Apart from impaired cytokine production, T cells exhaustion is also defined as decrease in proliferation (37,82). Although zoledronate impaired the cytokine production by Vδ2 T cells, it induced high proliferation of Vδ2 T cells contradicting the hypothesis as cell exhaustion is a cause of impaired Vδ2 T cell function in our cultures.

Cholesterol is essential for stabilizing lipid raft structure and amending TCR signaling in T cells (83,84). Therefore, a decrease in cholesterol levels in T cells may affect effector signaling causing the arrest of proinflammatory effector action such as cytokine release. A previous study

showed that statins and zaragozic acid induce opposite effects on IFN-γ production by T cells(85). In our study, blocking cholesterol synthesis by zaragozic acid did not cause a decrease in cytokine production and fluvastatin treatment did not result in the depletion of cellular cholesterol. This observation is in line with previous studies on human T cells where authors postulate no effect of statins on cholesterol levels and lipid rafts (86,87). Indeed, T cells can maintain cellular cholesterol pools through extracellular uptake (88,89). Therefore, cellular cholesterol levels will only be affected if extracellular cholesterol is depleted, which is not the case in our PBMC cultures. Thus, it is unlikely that fluvastatin, zoledronate and 6-fluoromevalonate affect TCR signaling through cholesterol depletion. Although cholesterol synthesis is not responsible for the phenotype we observed, it most likely still plays a role in Vδ2 T cell function. Further investigation with potent cholesterol-depleting agents, such as methyl-β-cyclodextrin and in lipid-free medium, would unravel the function of cholesterol in Vδ2 T cell biology.

While having a distinct effect on proliferative capacity, all inhibitors: statins, zoledronate and 6-fluoromevalonate, comprised cytokine production by the Vδ2 T cells and to a lesser extent by CD4 and CD8 T cells. Similar results were observed in our *in vivo* studies involving statin-treated patients. Consistently, it has been reported that IFN-γ production by conventional T cells in mice and acute coronary syndrome patients is reduced upon statin treatment (69,90,91). Our data shows that inhibition of mevalonate metabolism affects T cell function and suggests that the two processes: effector molecule production and proliferation are uncoupled in Vδ2 T cells. In line with this hypothesis, an earlier study by Ryan et al. postulated a functional heterogeneity of the Vδ2 T cell compartment where the proliferative and effector capacities were mutually exclusive(92). Moreover, Vδ2 T cell function seems to be more sensitive to the modulation of the mevalonate pathway than the function of conventional T cells.

Our data revealed that protein prenylation downstream of the mevalonate pathway regulates Vδ2 T cell cytokine responses. We show that geranylgeranylation inhibitors cause a significant decrease in both TNF and IFN-γ production by Vδ2 T cells, while inhibition of farnesylation has a milder effect. In a normal condition, RAC, RHOA, and RAP1 are geranylgeranylated while RAS is farnesylated in order to initiate the signaling cascade from the cell membrane. Consistently, we found an accumulation of unprenylated small G proteins, namely RAC, RHOA, RAP1 and RAS in the cytosol of Vδ2 T cells isolated from zoledronate-treated PBMC cultures. Furthermore, prenylated RHOA and RAC play an important role in effector signaling in mouse CD4 T cells and the inhibition of prenylation leads to a decrease in IFN-γ production by these cells (85). We also observed an accumulation of unprenylated GTPases in fluvastatin- and IPP-treated cells, although this was not significant. While the depletion of RAS, RHO and RAB from the lipid rafts upon simvastatin treatment results in impaired intracellular signaling (86), the effect of IPP on protein prenylation requires further investigation. Of note, IPP stimulation might induce global increase of small GTPase levels including prenylated form. Our assessment did not allow us to look at the ratio of unprenylated vs prenylated forms due to technical limitations of Westernblot, which would be more relevant in this case.

Small GTPases regulate various signaling cascades such as Mitogen-Activated Protein Kinases (MAPK) signaling: c-Jun N-terminal kinase (JNK) and P38 that are involved in immune responses (93). The global kinase activity assay performed on Vδ2 T cells isolated from PBMC cultures revealed dysregulation of the kinome upon mevalonate pathway inhibition (94,95). While IPP and zoledronate had a mainly enhancing effect on kinase activity consistent with their Vδ2 T cell activating potential, statin caused a mainly reduction of phosphorylation activities. The activity of kinases related to proliferation, differentiation and cell cycle regulation was highly upregulated in IPP- and zoledronate-treated Vδ2 T cells which is consistent with the high proliferation rate of the cells in these conditions. Similarly, the activity of kinases downstream of TCR signaling was also upregulated, providing further evidence that IPP can potently activate the TCRVδ2. Among kinases whose activity was downregulated in Vδ2 T cells by zoledronate, we found MAPKs downstream of the GTPases such as MAP3K5 and its interaction partner MAP3K6, which activate the JNK and P38 kinase signaling pathway (96); MAP2K4, a key component of the P38 pathway that plays an essential role in T cell cytokine production and proliferation in mice (97); and MAP2K7, which is involved in the pro-inflammatory cytokine response in mouse macrophages (98). These show that JUN and P38 signaling may be hindered in Vδ2 T cells upon zoledronate treatment, which is in line with our Western Blot results showing an accumulation of the unprenylated form of their activating small GTPases: RAC and RHOA, respectively. On the other hand, the activities of many kinases related to TCR activation and signaling as well as many proliferation-related tyrosine receptor kinases such as TYRO3 and MER responsible for AKT and ERK signaling (99) were also significantly downregulated in fluvastatin-treated Vδ2 T cells, consistent with the decreased proliferative activity of the cells. Statin also compromised the activity of kinases downstream of GTPases such as JNK1,2,3, P38δ and ERK1,2. The reduced activity of these signal transduction pathways may be attributed to both decrease in receptor signaling and abrogation of small GTPase prenylation. Although no mutual kinases downstream of small GTPases and downregulated by zoledronate and fluvastatin were found, one commonly regulated phosphosite was found on the BTLN protein which regulates lymphocyte proliferation and cytokine production(100,101). It is therefore of interest to investigate the role of BTLN in Vδ2 T cells further. Therefore, we conclude that compromised intracellular signaling results in weakened effector function which manifests in decreased production of pro-inflammatory cytokines.

Apart from cytokine production, Vδ2 T cells execute their function by producing cytotoxic granules and killing infected and tumor cells (102). Here we show that mevalonate metabolism is also important for the cytotoxic activity of Vδ2 T cells. Our data revealed, the expression of cytotoxic molecules: granzyme B and perforin(31) is reduced in Vδ2 T cells in patients undergoing statin therapy. Granzyme B expression is also decreased by fluvastatin and zoledronate treatments of PBMC cultures. Furthermore, we found that N-glycosylation and protein geranylgeranylation are important processes for cytotoxic granule production by Vδ2 T cells. These findings are in line with reports revealing that granzyme B and perforin undergo N-glycosylation (103,104). Furthermore, the functional analysis of cytotoxic activity confirmed a lower clearance efficiency of fluvastatin- and zoledronate-treated Vδ2 T cells

towards cancer cells. Therefore, inhibition of the mevalonate pathway results in reduced cytotoxic function in Vδ2 T cells.

Taken together, our study revealed the importance of mevalonate metabolism for the proper effector function of Vδ2 T cells. Although all mevalonate pathway inhibitors used in the study resulted in compromised Vδ2 T cell function, the underlying molecular mechanisms of their action are different. While we focused our analysis on cholesterol synthesis, protein N-glycosylation and protein prenylation, other processes downstream of mevalonate metabolism such as ubiquinone synthesis might be affected by the inhibitors and contribute to the phenotype. Given the wide usage of mevalonate pathway inhibitors in clinics, our observations are vital for improving patient care and the effectiveness of treatment of the deadliest diseases: cardiovascular disease and cancer.

Methods

Cell lines and tissue culture

Jurkat cell line was a kind gift from Prof. Dr. Dietmar Schmucker at the University of Bonn. All cell lines were subjected to mycoplasma testing prior to experiments. Jurkat cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS; Pan Biotech) and 1% glutamax (Gibco).

Human study oversight

Buffycoats from healthy donors were obtained from Unikinikum Bonn following approval of the Ethics Committee for Clinical Trials on Humans and Epidemiological Research with Personal Data of the Medical Faculty of the Rheinische Friedrich-Wilhelms-Universität Bonn (approval numbers 148 /20 and 249/22). Human peripheral blood mononuclear cells (PBMCs) were isolated and reconstituted in RPMI medium (Pan Biotech) supplemented with 10% FBS (Pan Biotech), 1% sodium pyruvate (Gibco), 1% glutamax (Gibco) and 0.1% gentamicin (Gibco). All cells were maintained at 37°C, 5% CO_2 humidified incubator.

Clinical Study

Hypercholesterolemia patients were recruited as part of the clinical study approved by the Arnhem-Nijmegen Ethical Committee (no. NL72155.091.20, CMO 2020-6543) in Radboud University Medical Center, Nijmegen, the Netherland, according to the Declaration of Helsinki and Good Clinical Practice. All patients did not use cholesterol lowering drugs at inclusion in the study. They were subsequently treated with statins at the discretion of the treating physician (**Supplementary Table 1**).

Clinical Hyper Inflammatory Disease Study

Patients with Hyper-IgD syndrome were recruited as part of a clinical study approved by the Arnhem-Nijmegen Ethical Committee (no. NL32357.091.0 and NL42561.091.12) in Radboud University Medical Center, Nijmegen, the Netherland, according to the Declaration of Helsinki and Good Clinical Practice.

PBMCs isolation
Freshly collected buffy coats from healthy volunteers were transferred to a 200ml culture flask. The blood was diluted 1:4 with phosphate-buffered saline (PBS) buffer. The diluted blood was layered over Pancoll Human (Pan Biotech) and density gradient centrifuged (610 cf, 30 mins, $22^{\circ}C$) to separate human peripheral blood mononuclear cells (PBMCs) from red blood cells and granulocytes. The interphase rings containing the PBMCs were collected into new tubes and washed twice with cold PBS. The PBMCs were pelleted by centrifugation (1700 RPM, 15 mins, $4\ ^{\circ}C$), resolved and counted using trypan blue exclusion of dead cells on hemacytometer.

In vitro inhibition of mevalonate pathway
PBMCs reconstituted as above were incubated in RPMI alone or in the presence or absence of IPP (10 μM; Sigma), fluvastatin (0.5 μM, 1 μM, 5 μM, 10 μM; Sigma), atorvastatin (0.5 μM, 1 μM, 5 μM, 10 μM; Sigma), zoledronate (1 μM, 5 μM; Merck) or 6-fluoromevalonate (50 mM, 100 mM; Sigma) in 96-wells plate at $37^{\circ}C$ and 5% CO_2 for 12 days. All cultures were supplemented with IL-2 (100U; Peprotech). On day 12, the PBMC cultures were stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) at $37^{\circ}C$ and 5% CO_2 for 4 hours. The cultures were harvested for surface marker and intracellular staining and analyzed by flow cytometry.

In vitro rescue of mevalonate pathway inhibition
PBMCs reconstituted as above were treated with the stimuli and mevalonate pathway inhibitors as described above and supplemented with mevalonic acid (50 μM, 100 μM; Sigma) at $37^{\circ}C$ and 5% CO_2 for 12 days. On day 12, the PBMC cultures were stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) at $37^{\circ}C$ and 5% CO_2 for 4 hours. The cultures were harvested for surface markers and intracellular staining and analyzed by flow cytometry.

Clinical study of hypercholesterolemia patients
The peripheral blood from hypercholesterolemia patients was collected at baseline before statin treatment and after 3-months of statin treatment, as well as from healthy volunteers. PBMCs were isolated and reconstituted as described above and then stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) at $37^{\circ}C$ and 5% CO_2 for 4 hours. The cultures were harvested for surface markers and intracellular staining and analyzed by flow cytometry.

Clinical study of Hyper-IgD syndrome patients
The peripheral blood of Hyper-IgD syndrome patients and healthy volunteers was collected at baseline, PBMCs were isolated and reconstituted as described above. The PBMCs were then stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) at $37^{\circ}C$ and

5% CO_2 for 4 hours. The cultures were harvested for surface marker and intracellular staining and analyzed by flow cytometry.

In vitro inhibition of processes downstream mevalonate pathway

PBMCs ($5x10^6$ cells/well) were expanded by IPP (10 μM; Sigma-Aldrich) and IL-2 (100U; Peprotech) in 6-wells plate for 12 days. Then PBMCs were transferred to 96-wells plate ($0.5x10^6$ cells/well) and incubated with RPMI alone, zaragonzic acid (75 μM, 100 μM; Sigma-Aldrich), tunicamycin (1 μM, 5 μM; Sigma-Aldrich), geranylgeranyltransferase inhibitor 2133 (GGTI2133; 5 μM, 10 μM, 30 μM, 50 μM; Sigma-Aldrich), geranylgeranyltransferase inhibitor 286 (GGTI286; 30 μM, 50 μM, 75 μM, 100 μM; Sigma-Aldrich), geranylgeranyltransferase inhibitor 298 (GGTI298; 5 μM, 10 μM, 30 μM, 50 μM; Sigma-Aldrich), farnesyltransferase inhibitor 277 (FTI 277; 50μM, 100 μM, 150 μM; Sigma-Aldrich) or FTase inhibitor II (FTI II; 5 μM, 10 μM, 30 μM, 50 μM; Santa Cruz biotechnology) overnight. The PBMC cultures were stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) for 4 hours. The cultures were harvested for surface marker and intracellular staining and analyzed by flow cytometry.

Surface marker and intracellular staining by flow cytometry

PBMCs were incubated with the following antibodies against cell surface markers: anti-human CD3 Pacific blue (UCHT1; BioLegend), anti-human CD45 Brilliant Violet 605 (HI30; BioLegend), anti-human CD45 PE/Dazzle 594 (HI30; BioLegend), anti-human CD4 PE-Cy7 (OKT4; BioLegend), CD4 Brilliant Violet 605 (OKT4; BioLegend), anti-human PD-1 PerCP 5.5 (A17188B; BD Bioscience), anti-human CD8 APC-Cy7 (SK1; BioLegend), anti-human Lag3 Dazzle red (11C3C65; BioLegend), anti-human Tim3 PerCP-Cy5.5 (F38-2E2; BioLegend), anti-human CTLA4 Brilliant Violet 605 (BNI3; BioLegend), anti-human TCR Vδ2 FITC (REA711; Miltenyi Biotec), anti-human TCR Vδ2 APC (REA711; Miltenyi Biotec), anti-human CD80 PE (2D10; BioLegend), anti-human CD86 FITC (BU63; BioLegend), anti-human CD66a (CEACAM1) PE (ASL-32; BioLegend), anti-human CD274 (PDL1) FITC (MIH2; BioLegend), anti-human CD107a PE (H4A3; BioLegend), Live-or-Dye™ Fixable stain (Biotidium) and Live/dead™ Fixable Aqua Dead stain (Invitrogen) at 4 °C in dark for 30 mins. The cells were washed with PBS then permeabilized/fixed in Cytofix permeabilization/fixation reagent (BD biosciences) for 30 mins. The cells were washed with Cytofix permeabilization/washing buffer (BD biosciences) twice and incubated in Cytofix permeabilization/washing buffer with the following antibodies against intracellular markers: anti-human TNFα APC (MAb11; BioLegend), anti-human IFNγ PerCP-Cy5.5 (B27; BD Pharmingen), anti-human granzymeB Alexa Fluor 687 (BG11; BioLegend), anti-human Perforin PE-Cy7 (dG9; BioLegend) and anti-human CD3 Pacific blue (UCHT1; BioLegend) at 4 °C in dark for 30 mins. The cells were washed with PBS then fixed in CellFIX reagent (BD Bioscience) and stored at 4 °C in dark. Color compensation was done using OneComp eBeads (BD Bioscience). FACS analysis was performed on LSR II (BD Bioscience). Data analysis is performed using FlowJo vX.07 software.

RNA sequencing sample preparation

0.5 x10^6 Vδ2 T cells (CD45$^+$ CD3$^+$ TCR Vδ2$^+$) in each culturing condition from the corresponding donor were sorted from PBMC cultures on BD Aria3 flow cytometer and stored in QIAzol (Qiagen). Libraries were assembled by adapting the SMART-Seq2 protocol[105]. Qubit dsDNA HS Assay kit (Thermo Fisher Scientific) was applied for library quantification. Library size estimation was performed on the TapeStation 4200 High-sensitivity D500 assay system (Agilent Technologies). Pooled samples were then sequenced on a NextSeq500 using High Output v.2 chemistry (SR 75 bp). Raw data was collected as FASTQ files and demultiplexed with bcl2fastq2 v2.20.

RNA-seq data analysis

Pseudo-alignment was performed with Kallisto v.0.440 and mapped against the GRCh38p13 human reference genome (GENCODE v.27). Raw counts were imported, and low-count genes were excluded by pre-filtering (>10 reads in at least 3 samples and only protein coding transcripts) resulting in 13084 transcripts. DEseq2 pipeline was applied to normalize the count and rlog transform the data using default parameters[106]. Surrogate variable analysis (SVA) was used to identify latent variables (3 singificant SVs) that caused the batch effects and the variables were included in the DEseq2 model. Transformed counts were corrected for 3 SVAs using the function provided in the limma R package. All the present transcripts were submitted as input for principal component analysis (PCA). Standard differential expression analysis was performed for IPP-, zol- and statin-treated vs control comparisons using adjusted p-value threshold equal to 0.05 and a foldchange cut-off of 1.5. IHW was used as multiple testing parameter and log2 fold change shrinkage was applied. Hierarchical clustering of variable transcripts within the dataset were presented in the heatmap and the selected differentially expressed (DE) genes were annotated. The results from the differential genes expression analysis were visualizedas volcano plots. Gene ontology (GO) enrichment analysis was performed for DE transcripts in the respective comparison using gene ontology set of biological processes using the R tool clusterprofiler. The count and p-value (p<0.05) associated with each GO term were depicted in enrichment dotplot.

For co-expression network analysis all present and batch corrected transcripts were used as input. The inflection point was calculated, and 7184 top variable genes were included as input for hCoCena (horizontal construction of co-expression networks and analysis). Pairwise Pearson's correlation coefficients were calculated using the R package Hmisc (v4.1-1) to identify gene pairs whose expression patterns are positively correlated across all tested samples. A Pearson correlation coefficient cutoff of 0.912 (6023 nodes and 63226 edges) based on the balance of scale-free topology, number of graph components and edges was selected to construct an undirected co-expression network. Then, the "leiden" algorithm in igraph (v1.2.1) [the igraph software package for complex network research] was applied to perform unbiased clustering and this step was repeated 10 times. Modules with less than 40 genes were discarded. For each gene, the group fold changes (GFCs) for RPMI-, IPP-, zol- and statin-treated conditions were computed by calculating the average expression of that gene across all samples and determining the fold change of the gene's mean expression within each condition relative to the overall mean expression. Then, the hclust function (cluster package, version 2.1.0) was

used to perform agglomerative hierarchical clustering. The clinical parameters and conditions clustered by their GFCs were presented in heatmap. All clusters individually were used for GO and Hallmark enrichment analyses. The results were filtered for the 5 most significant terms with adjusted P-values ≤0.05 per cluster. If clusters are missing in the plot, then no significantly enriched terms were found.

ATAC-Sequencing sample preparation

0.1×10^6 Vδ2 T cells sorted on BD Aria3 flow cytometer as for the RNA-seq analysis. The cells were lysed in lysis buffer (10mM Tris-HCl, pH 7.4, 10mM NaCl, 3mM MgCl$_2$, 0.1% IGEPAL CA-630) and processed for nuclei isolation. Transposition reaction following the protocol of Buenrostro et al.(107) was performed using in-house Tn5 and DMF-Tn5 buffer. The transposed DNA was purified by MinElute Purification Kit (Qiagen) and amplified by PCR. Library quantification was performed using a Qubit dsDNA HS Assay Kit (ThermoFisher Scientific). Library size estimation was performed on the TapeStation 4200 High-sensitivity D500 assay system (Agilent Technologies). Paired-end Assay for Transposase-Accessible Chromatin using Sequencing (ATAC-Seq) was performed on pooled samples at NovaSeq SP flow cell with v.1 chemistry (PE 75 bp).

ATAC-Sequencing analysis

ATAC-Seq data preprocessing was based on the guideline on Galaxy training network(108,109).First, the FASTQ files were demultiplexed with bcl2fastq2 v2. The adapters were trimmed using Cutadapt (Martin, 2011). Then the sequencing reads were mapped using bowtie2 v 2.3.5 against the GRCh38p13 human reference genome (Langmead and Salzberg, 2012). Then duplicated reads were removed using Picard MarkDuplicates function. Bam files was exported and visualized by Integrative Genomic Viewer (IGV)(110).

Western blot sample preparation and image analysis

PBMCs (5×10^6 cells/well) were incubated with RPMI alone, IPP (10 μM; Sigma-Aldrich), fluvastatin (1 μM; Sigma-Aldrich) or zoledronate (1 μM; Sigma-Aldrich) in 6-wells plate for 12 days in the presence of IL-2 (100U; Peprotech). 0.5×10^6 Vδ2 T cells were purified from the PBMC cultures by Stemcell EasySep Release APC positive magnetic selection kit according to manufacturer's instructions and stored as pellets. The pellets were resuspended and lysed by RIPA buffer containing proteases and phosphatases inhibitors (Thermo Scientific) for 30 mins at 4°C. The lysates were then centrifuged at 14000g at 4°C for 10mins. The supernatant containing cytosolic fraction of protein lysate was collected. Pierce™ BCA protein quantification assay was performed to determine the concentration of protein in the lysate (Thermo Scientific). Lysate was then boiled together with Laemmli sample buffer (Bio-rad) for 5 mins at 95°C and proceed with SDS-pages. Equal amount of protein lysate (10μg) was separated on 10% Mini-PROTEAN® TBE-Urea Gel (Bio-rad) and transferred to Trans-Blot Turbo Mini 0.2 μm Nitrocellulose membrane (Bio-rad) using Trans-Blot Turbo™ Transfer System (Bio-rad). The blot was blocked in 5% non-fat dried milk (Roth) for 1 hour prior to overnight incubation with the following primary antibodies: anti-RAC 1/2/3 (Cell signaling), anti-RHOA (Cell signaling), anti-Pan RAS (Santa Cruz Biotechnology), anti-RAP1A/RAP1B

(Cell signaling), anti-b-actin (Biolegend), anti-phospho p44/42 MAPK (Erk1/2; Cell signaling), anti-P44/42 MAPK (Erk1/2; Cell signaling). Blot was washed 3 times with Tris Buffered Saline-Tween (TBS-T) and incubated with anti-mouse IgG (Cell signaling) or anti-rabbit IgG HRP-conjugated secondary antibodies (Cell signaling). The blot was incubated with Pierce™ ECL Western Blotting Substrate (Thermo Scientific) after 3 times washing with TBS-T and anaylzed by ChemiDoc imager (BioRad).

PamGene sample preparation and analysis
PBMCs were isolated from buffy coats of healthy human donors. The PBMC cultures ($5x10^6$ cells/well) were incubated with RPMI alone, IPP (10 μM; Sigma-Aldrich), fluvastatin (1 μM; Sigma-Aldrich) or zoledronate (1 μM; Sigma-Aldrich) in 6-wells plate for 12 days in the presence of IL-2 (100U; Peprotech). Vδ2 T cells were purified from the PBMC cultures by Stemcell EasySep Release APC positive magnetic selection kit. The purified Vδ2 T cells were stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience) and anti-CD28 (1μg/ml; CD28.2 BD Bioscience) for 10 min and immediately lysed by M-PER™Mammalian Extraction Buffer (Thermo Scientific) containing Halt™Phosphatase and Protease Inhibitor cocktail (Thermo Scientific). The extracted protein was quantified by Pierce™ BCA protein quantification assay (Thermo Scientific). The kinase activity of the protein lysate was determined on PTK PamChip® and STK PamChip® arrays (Pamgene International BV) and performed on a PamStation® according to the manufacturer instructions. Phosphorylation signals captured from hundreds of phosphosites on the array by the PamStation® were quantified using BioNavigator® software software 6.1 (PamGene International BV). Phosphosites that show significant difference compared to control (p<0.05) were further analyzed. Differentially active kinases were identified by Upstream Kinase Analysis (UKA) algorithm specify kinase-phosphosite relationship (Median Final score > 1.2). The result is depicted using Upstream Kinase Tool such as CORAL kinome tree base on the kinase functional class scoring(111,112).

In vitro cytotoxic assay
Jurkat cell line cultures (~$3x10^6$ cells/ml) were pulsed with zoledronate (10 μM; Sigma-Aldrich) in 10ml flask overnight prior to the co-culture with Vδ2 T cells. The cells were centrifuged at 1500 RPM at 4°C for 5mins and washed with their corresponding growth media two times. The pellets were then resuspended in RPMI containing 0.02% of gentamycin. Vδ2 T cells were purified from the PBMC cultures by positive magnetic selection (Stemcell EasySep Release APC positive magnetic selection kit) according to manufacturer's instruction. Effector Vδ2 T cells were co-incubated with the target cells at the effector cell:target cell ratio of 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8 overnight. The cultures were then analyzed by flow cytometry for dead cells and surface markers expression as described above.

Statistics
Acquired data from *in vitro* study was analyzed statistically in case of paired non-parametric comparison: Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test and Wilcoxon test, and unpaired non-parametric comparison: Mann-Whitney test were

applied. All statistical analysis was performed on graphic analysis software GraphPad Prism 8.4 (GraphPad Software Inc.). result considered as significant with P value < 0.05 (*), < 0.01 (**), < 0.001 (***) or < 0.0001 (****). Westernblot protein quantification analysis was done using imageJ.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
RNA-seq and ATAC-seq data have been deposited at EGA and are publicly available as of the date of publication. The accession number is EGAD00001011322.

Extended data
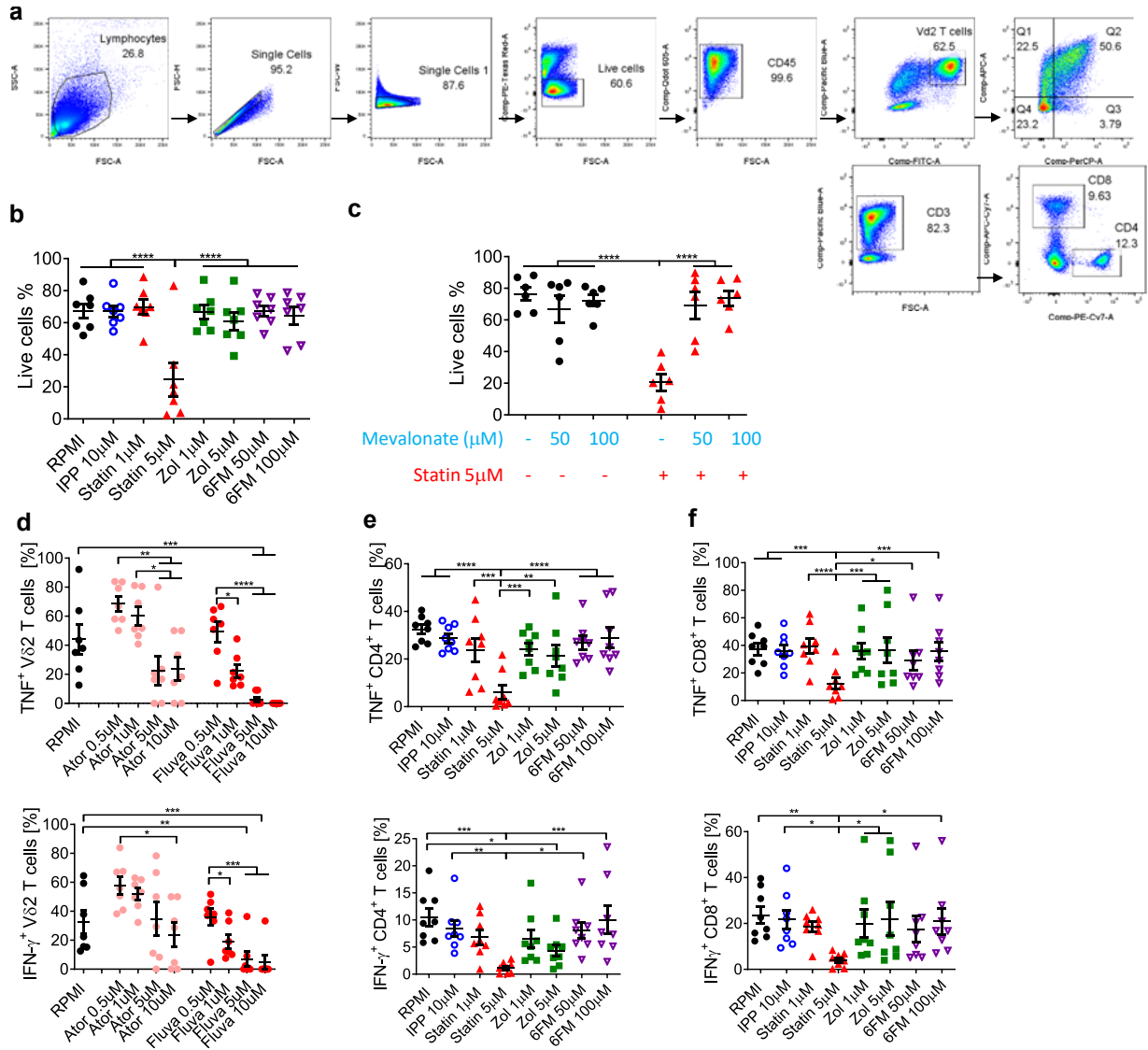
Extended Data Fig. 1: *In vitro* **mevalonate pathway inhibition impairs cytokine production by conventional T cells. a,** Schematic gating strategy of Vδ2 T cells, CD4 T cells and CD8 T cells in PBMC cultures. **b,** Cumulative % of live cells in PBMC cultures after incubation with indicated inhibitors (Mean ± SEM, n=7); and **c,** in the presence or absence of mevalonic acid (Mean ± SEM, n=6). **d,** Cumulative % of TNF⁺ and IFN-γ⁺ Vδ2 T cells in PBMC cultures treated as in Fig. 1 with atorvastatin (Ator) and Fluvastatin (Fluva) (Mean ± SEM, n=7); Cumulative % of TNF⁺ and IFN-γ⁺ **e,** CD4 T cells and **f,** CD8 T cells in PBMC cultures treated as in Fig. 1b (n=8); **b-f,** each dot represents one donor (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05).

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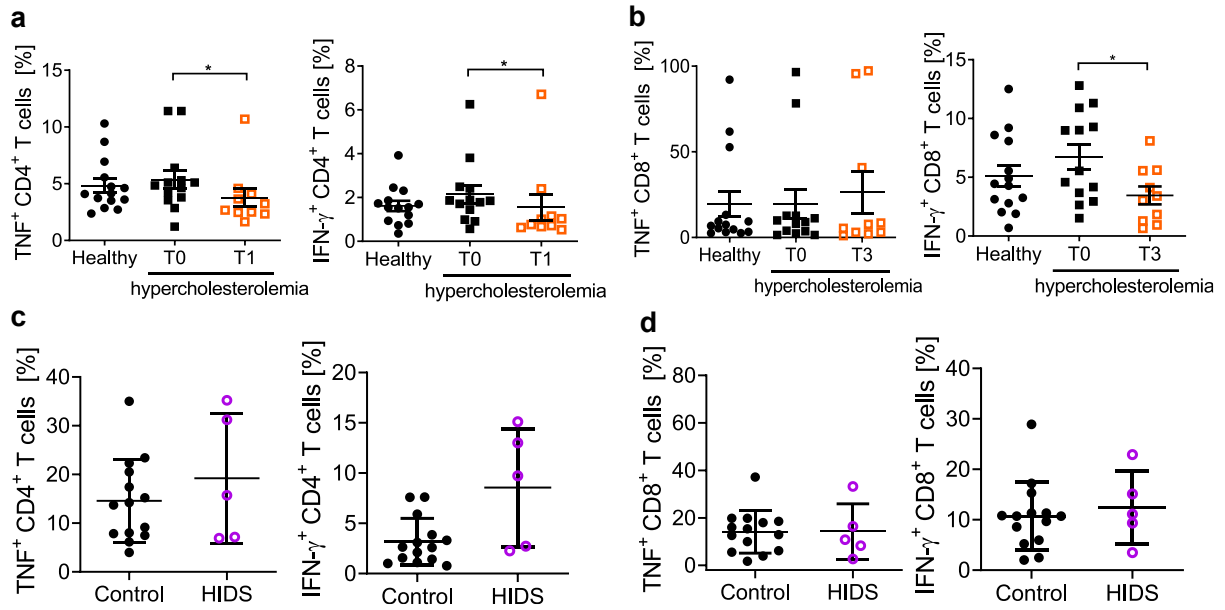
Extended Data Fig. 2: *In vivo* **statin treatment results in compromised cytokine production by CD4 and CD8 T cells. a-b,** Flow cytometry analysis of PBMCs isolated from patients with hypercholesterolemia before and after 3-months of statin treatment (Mean ± SEM, n=13: patients T0; n=10 patients T1; n=14: healthy donors); (Mann-Whitney test: patients and healthy donors; Wilcoxon test: before and after treatment, P value * < 0.05). **c-d,** from hyper-IgD patients and from healthy individuals (Mean ± SEM, n=5: patients; n=14: healthy donors); (Mann-Whitney test, P value * < 0.05); cumulative % of TNF$^+$ and IFN-γ^+ **a,c,** CD4 T cells and **b,d,** CD8 T cells. **a-d,** Each dot represents one donor.

a Number of up-regulated genes vs. RPMI

IPP   Zol
25   46   71
    1   2   1
        13
        Statin

Number of down-regulated genes vs. RPMI

IPP   Zol
28   63   76
    0   1   0
        35
        Statin

b Co-expression network coloured by cluster

c

RPMI
IPP
Zol
Statin

IFNG

RPMI
IPP
Zol
Statin

TNF
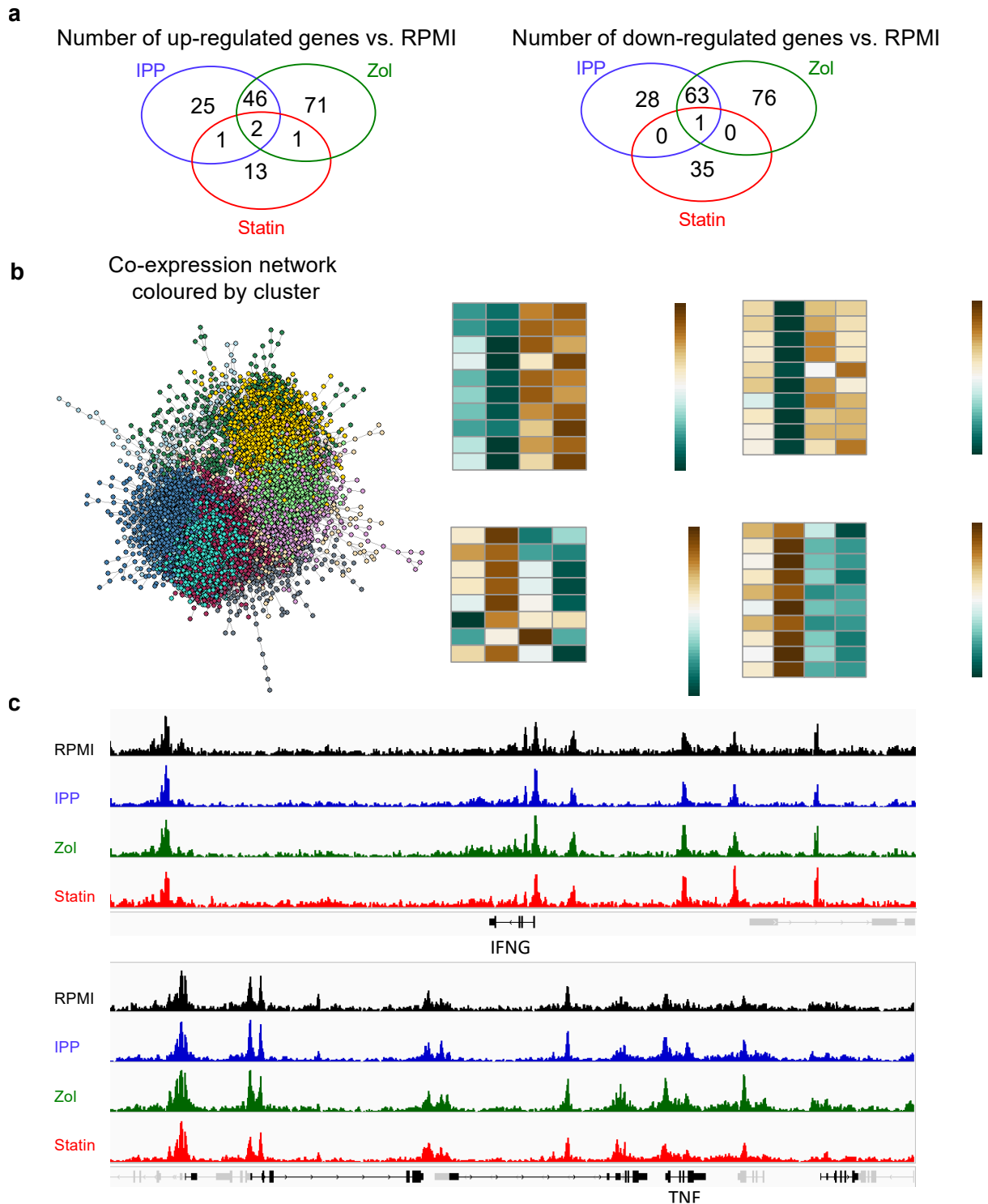
Extended Data Fig. 3: Transcript expression is differentially altered in Vδ2 T cells upon fluvastatin and zoledronate treatment. a-b, RNA-seq analysis of Vδ2 T cells isolated from 12-days-PBMC cultures treated with IPP, zoledronate, fluvastatin or RPMI alone. **a,** Venn diagram demonstrating the numbers of upregulated and downregulated transcripts in Vδ2 T cell in inhibitor-treated conditions vs. RPMI alone. **b,** hCoCena Integrated co-expression network colored by cluster and heat map showing mean expression of genes in the modules identified by hCoCena analysis for the RPMI, IPP, Zol and Statin-treated groups **c,** ATAC-seq analysis of Vδ2 T cells isolated from 12-days-PBMC cultures treated with IPP, zoledronate, fluvastatin or RPMI alone (n=3). Snapshot from the IGV browser of ATAC-seq signals around the IFNG (upper) and TNF (lower) loci (n=2).
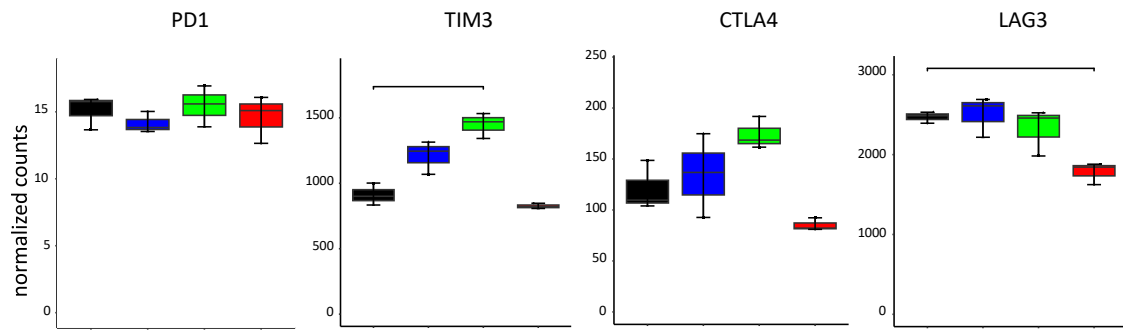
Extended Data Fig. 4: Transcript expressions of Tim3 and Lag3 are differentially altered in Vδ2 T cells upon zoledronate and Fluvastatin treatment respectively. a, RNA-seq analysis of Vδ2 T cells isolated from 12-days-PBMC cultures treated with IPP, zoledronate, fluvastatin or RPMI alone. Bar plot showing normalized count of differentially expressed transcripts of PD-1 (*PDCD1*), Tim3 (*HAVCR2*), CTLA4 and Lag3 upon IPP, Fluvastatin or zoledronate treatment in Vδ2 T cell (n=3).
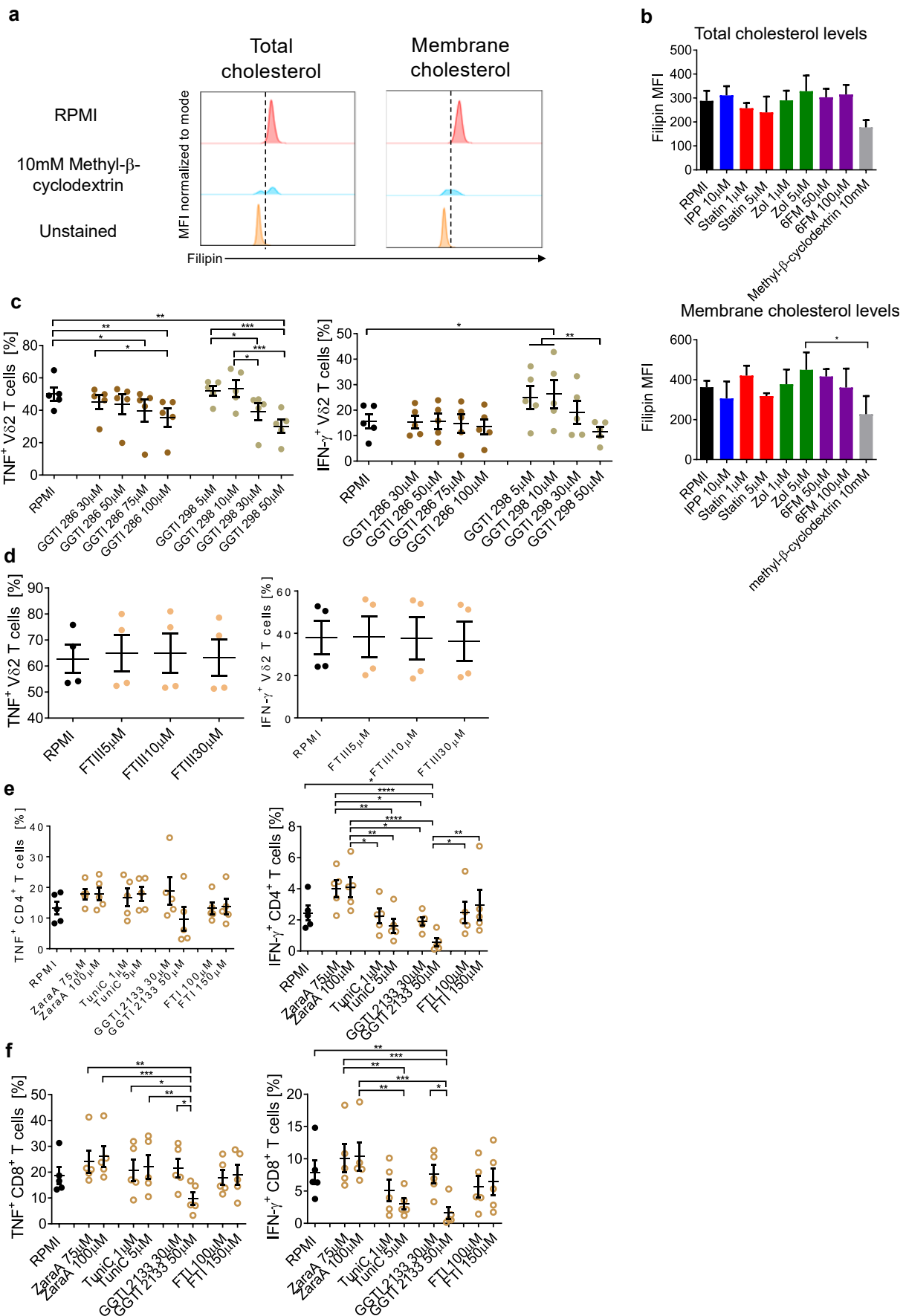
Extended Data Fig. 5: Mevalonate pathway inhibition does not affect cholesterol levels in Vδ2 T cells. Flow cytometry analysis of PBMC cultures treated as in Fig. 5a. **a,** Representative FACS histogram showing MFI of filipin in Vδ2 T cells after incubation with methyl-β-cyclodextrin in PBMC cultures overnight. **b,** Bar plot

showing MFI of filipin in Vδ2 T cells upon mevalonate pathway inhibition in PBMC cultures (Mean ± SEM, n=4; methyl-β-cyclodextrin: n=2). **c,** Cumulative % of TNF[+] and IFN-γ[+] Vδ2 T cells in PBMC cultures treated as in Fig. 6A with the following GGTIs at indicated concentrations: GGTI 286 and GGTI 298 (Mean ± SEM, n=5). **d,** Cumulative % of TNF[+] and IFN-γ[+] Vδ2 T cells in PBMC cultures treated as in Fig. 6a with the following FTI II at indicated concentrations (Mean ± SEM, n=4). Cumulative % of TNF[+] and IFN-γ[+] **e,** CD4 T cells and **f,** CD8 T cells in PBMC cultures after incubation in indicated conditions (Mean ± SEM, n=5). (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05).
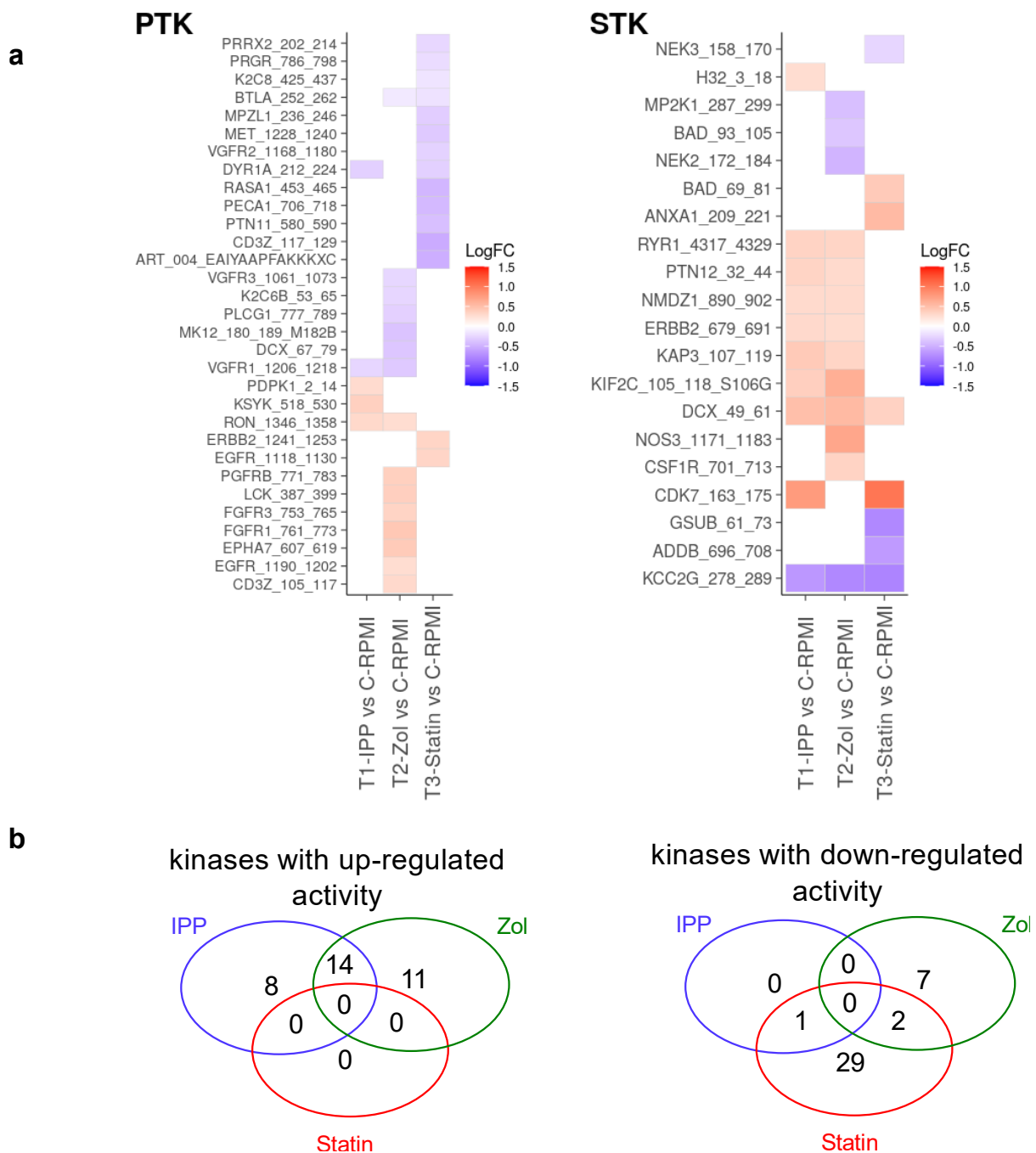
Extended Data Fig. 6: Phosphosites downstream of small G proteins show reduced phosphorylation levels in both statin- and zoledronate-treated Vδ2 T cells. a, Heatmap showing differentially affected phosphosites in IPP-, zoledronate- and fluvastatin- vs. RPMI-treated Vδ2 T cells. **b,** Venn plot showing number of kinases with up- and down-regulated activity in IPP-, zoledronate- and fluvastatin- vs. RPMI-treated Vδ2 T cells.
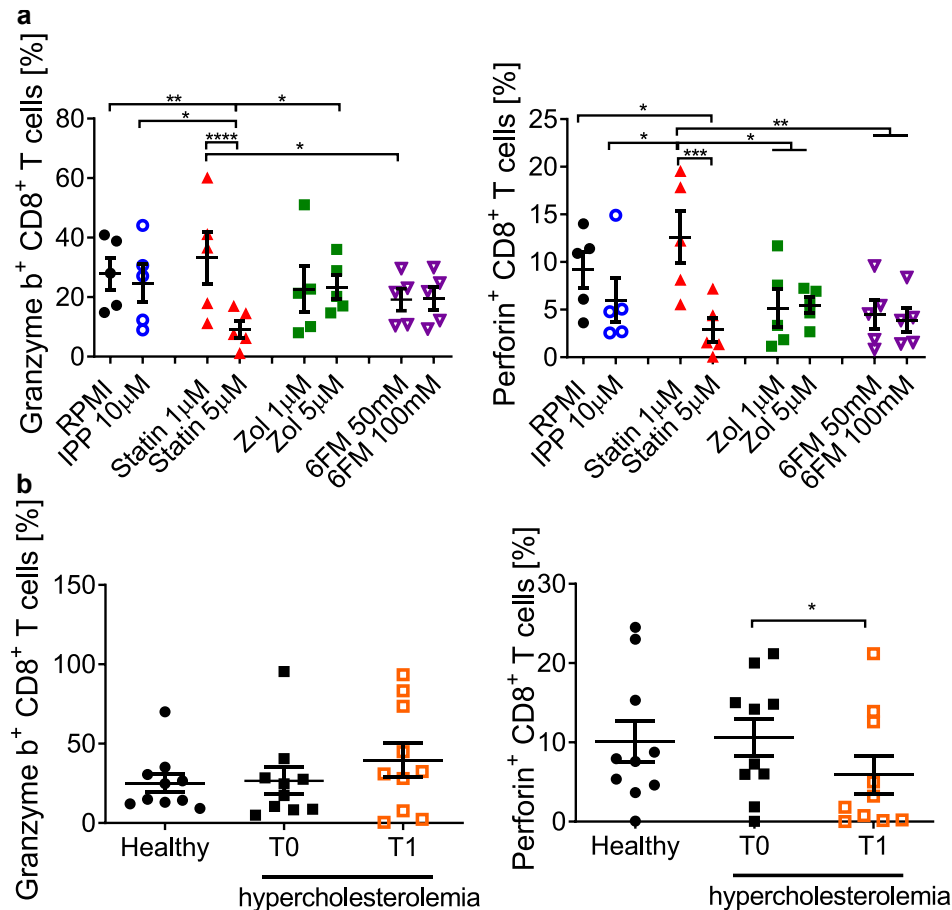
Extended Data Fig. 7: Mevalonate metabolism is important for cytotoxic properties of CD8 T cells *in vitro*. **a,** Cumulative % of granzyme B⁺ and perforin⁺ CD8 T cells in PBMC cultures after incubation with indicated inhibitors, assessed as in Fig. 7a (Mean ± SEM, n=5) (Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test, P value * < 0.05). **b,** Cumulative % of granzyme B⁺ and perforin⁺ CD8 T cells in patients with hypercholesterolemia before and after 3-months of statin treatment (Mean ± SEM, n=10: patients T0, T1; n=10: healthy donors). (Mann-Whitney test: patients and healthy donors; Wilcoxon test: before and after treatment, P value * < 0.05)

Patient ID	LDL [nmol/L] T0	LDL [nmol/L] T1	Lipid lowering medication
Eras_FH_001	5,16	2,29	Rosuvastatine 10 mg 1dd1
Radb_FH_002	5,96	2,63	Rosuvastatin 20 mg 1dd1
Eras_FH_004	8	3,61	Rosuvastatin 20 mg 1dd1; Ezetimib 10 mg 1dd1
Eras_FH_005	5,1	3,25	Rosuvastatin 10 mg 1dd1
Eras_FH_007	5,45	3,38	Rosuvastatine 20 mg 1dd1
Eras_FH_010	6,17	3,38	Atorvastatine 40 mg 1dd1
Eras_FH_011	5,4	1,77	Rosuvastatine 10 mg 1dd1
Radb_FH_001	5,21	3,92	Rosuvastatine 40mg 1dd1
Eras_FH_012	TBA	1,21	Atorvastatine 80 mg 1dd1; Ezetimib 10 mg 1dd1
Eras_FH_013	5,53	4,08	Rosuvastine 10 mg 1dd1
Eras_FH_014	5,1	N/A	Rosuvastatine 10 mg 1dd1
Eras_FH_015	5,5	2,61	Rosuvastatine 10 mg 1dd1
Eras_FH_016	5,5	2,55	Rosuvastatine 20 mg 1dd1

Supplementary Table 1. Patient information in the statin trial.

Acknowledgements

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Conceptualization, K.P.; methodology, T.K.S., B.A., J.B. and K.P.; investigation, T.K.S., B.A. and K.P.; data curation, T.K.S., B.A., N.R. and K.P.; formal analysis, T.K.S. , B.A., N.R. and K.P. ; resources, H.B., S.B., N.R., L.A.B.J., J.L.S., M.G.N. and K.P.; writing—original draft preparation, T.K.S. and K.P.; writing—review and editing K.P., M.G.N, L.A.B.J., J.L.S., N.R., J.B., S.B., H.B., N.R., B.A. and T.K.S.; supervision, M.G.N. and K.P.; project administration, K.P.; funding acquisition, M.G.N. and K.P. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

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

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Chapter 6

Additional preliminary studies:

Metabolic profile of Vδ2 T cells upon mevalonate metabolism inhibition

Chronic granulomatous disease functionally reprograms Vδ2 T cells

Metabolic and functional profile of Vδ2 T cells in Hyper-IgE Syndrome Patients

Metabolic profile of Vδ2 T cells upon mevalonate metabolism inhibition

Introduction

Many cellular processes, such as cholesterol synthesis, protein N-glycosylation and protein prenylation, require the functional mevalonate metabolism to be maintained (1,2). The mevalonate pathway also supports the vital respiration of the cells by producing an important electron carrier for oxidative phosphorylation in the mitochondria, called ubiquinone (3,4). The presence of ubiquinone ensures the continuation of redox reactions to maintain proper energy metabolism in cells (3). The deficiency of ubiquinone is associated with defective sulfide metabolism or metabolic diseases such as phenylketonuria, which is characterized by a high level of phenylalanine that inhibits the HMG-CoA reductase of the mevalonate metabolism (5,6). Similarly, certain therapies using statins or the anti-depressant amitriptyline that target the mevalonate pathway may also lead to a deficiency of ubiquinone and potentially affect the energy metabolism of the cells (7). In our previous study, we found that mevalonate pathway inhibition adversely affects cytokines and cytotoxic granule production by Vδ2 T cells. In this study, we applied single-cell energetic metabolism by profiling translation inhibition (SCENITH) technique (8) to investigate whether the metabolic status of Vδ2 T cells is altered and contributes to the compromised immune function of Vδ2 T cells upon mevalonate metabolism inhibition.

SCENITH is a flow cytometry-based method using puromycin incorporation to determine the metabolic profile of heterogenous or scarce living cell populations at single cell resolution. The methodology is based on the assumption that protein synthesis is the most energy-demanding cellular process, which consumes half of the total energy of the cells. Using puromycin, which is stably incorporated to translated proteins and a fluorescent dye-conjugated antibody against puromycin, the protein synthesis and therefore the energy consumed for this process can be determined by flow cytometry. By incubating the cells with glycolysis inhibitor 2-Deoxy-D-glucose (2-DG) and mitochondrial ATP synthase inhibitor, Oligomycin (Oligo), the level of metabolic dependencies and capacities can be calculated based on mathematical calculations (8). SCENITH allows us to determine the metabolic profile of a scarce number of Vδ2 T cells, where the traditional metabolic analyzer Seahorse is not able to. Adopting this method, a recent study demonstrated that anti-tumoral and pro-tumoral γδ T cell subsets have distinct intrinsic metabolic profiles, where the former showed higher glycolysis dependence and the latter showed higher oxidative metabolism (9).

In this study, we determined the energy metabolism profile of Vδ2 T cells upon fluvastatin, zoledronate and 6-fluoromevalonte treatment by employing SCENITH and we did not observe a coherent decrease in mitochondrial dependency or fatty acid and amino acid oxidation capacities in Vδ2 T cells upon all three inhibitor treatments. This indicated that mitochondrial function in Vδ2 T cells was selectively hindered by the mevalonate pathway inhibitors. The

altered metabolic status might be one of the causes of the compromised immune function of Vδ2 T cells upon mevalonate pathway inhibition.

Method

PBMCs isolation

Freshly collected buffy coats from healthy volunteers were transferred to a 200ml culture flask. The blood was diluted 1:4 with phosphate-buffered saline (PBS) buffer. The diluted blood was layered over Pancoll Human (Pan Biotech) and density gradient centrifuged (610 cf, 30 mins, 22°C) to separate human peripheral blood mononuclear cells (PBMCs) from red blood cells and granulocytes. The interphase rings containing the PBMCs were collected into new tubes and washed twice with cold PBS. The PBMCs were pelleted by centrifugation (1700 RPM, 15 mins, 4 °C), resolved and counted using trypan blue exclusion of dead cells on hemacytometer.

In vitro inhibition of mevalonate pathway

PBMCs reconstituted as above were incubated in RPMI alone or in the presence or absence of IPP (10 μM; Sigma), fluvastatin (1 μM, 5 μM; Sigma), zoledronate (1 μM, 5 μM; Merck) or 6-fluoromevalonate (50 μM, 100 μM; Sigma) in 96-wells plate at 37°C and 5% CO_2 for 12 days. All cultures were supplemented with IL-2 (100U; Peprotech). On day 12, the PBMC cultures were stimulated with soluble anti-CD3 (1μg/ml; HIT3A BD Bioscience), anti-CD28 (1μg/ml; CD28.2 BD Bioscience) and incubated with Golgi Plug (1μg/ml; BD Bioscience) at 37°C and 5% CO_2 for 4 hours. The cultures were harvested for surface marker and intracellular staining and analyzed by flow cytometry.

Surface marker staining

PBMCs were incubated with the following antibodies against cell surface markers: anti-human CD3 Pacific blue (UCHT1; BioLegend), anti-human CD45 Brilliant Violet 605 (HI30; BioLegend), anti-human CD45 PE/Dazzle 594 (HI30; BioLegend), anti-human CD4 PE-Cy7 (OKT4; BioLegend), anti-human CD8 APC-Cy7 (SK1; BioLegend), anti-human TCR Vδ2 APC (REA711; Miltenyi Biotec), Live-or-Dye™ Fixable stain (Biotidium) in FACS buffer (1xPBS, 5% FBS and 2mM EDTA) at 4 °C in dark for 30 mins.

SCENITH

Cells were plated at $0.3x10^6$ cells/well in 96-well plates. The cells were treated by RPMI alone or stimulated with soluble anti-CD3/anti-CD28 (1μg/ml), or IPP 10μM at 37°C, 5% CO_2 for 4 hours. Cells were treated with control, 2-deoxy-D-Glucose (2DG, final concentration 100mM), Oligomycin (O, final concentration 10 μM) and combination of 2-Deoxy-D-Glucose and Oligomycin (DGO, final concentration 100mM and 10 μM) for 30 min at 37°C. Puromycin (final concentration 10mg/ml) was added and incubated for 45 mins at 37°C. Cells were washed in cold FACS buffer and stained with a combination of Fc receptor blocker, live-dead stain and surface marker antibodies (described above) for 15 mins at 4°C. Cells were permeabilized and fixed with Cytofix/Cytoperm (BD biosciences) for 30 mins at 4°C. The cells were washed 2 times with Permwash buffer (BD biosciences) and incubated with anti-puromycin AF488

(12D10; Millipore) in Permwash buffer for 20 mins at 4°C. Color compensation was done using OneComp eBeads (BD bioscience). FACS analysis was performed on LSR II (BD bioscience). Data analysis is performed using FlowJo vX.07 software. The metabolic dependencies and capacities were calculated based on the method provided in the study of *Argüello* et al. (8).

Statistical analysis
Acquired data was analyzed statistically by Wilcoxon test on graphic analysis software GraphPad Prism 6.0 (GraphPad Software Inc.). Results were considered statistically significant with P value < 0.05 (*), < 0.01 (**), < 0.001 (***) or < 0.0001 (****).

Result

To determine the changes in energy metabolism of Vδ2 T cells upon mevalonate pathway inhibition, we applied SCENITH technique based on the protocol described in the study of Argüello et al. (8) to analyze the metabolic dependencies and capacities of Vδ2 T cells which treated with IPP, fluvastatin, zoledronate or fluoromevalonate for 12 days. We observed that while the metabolic profile of IPP-treated Vδ2 T cells is comparable to the RPMI-treated cells, not all mevalonate pathway inhibitors (Fluvastatin, zoledronate and 6-fluoromevalonate) induce changes to the metabolic profile of Vδ2 T cells **(Figure 1)**. As such, fluvastatin and 6-fluoromevalonate treatment resulted in the most changes to the metabolic profile of Vδ2 T cells, while zoledronate-treated Vδ2 T cells showed a comparable metabolic profile to RPMI-treated cells. Particularly, fluvastatin and 6-fluoromevalonate treatment reduced the glucose dependence of Vδ2 T cells **(Figure 1A)** and increased fatty acid and amino acid oxidation capacity in Vδ2 T cells **(Figure 1C)**. Yet only 6-fluoromevalonate-treated Vδ2 T cells showed a decrease in mitochondrial dependence **(Figure 1B)** and an increase in glycolytic capacity **(Figure 1D)**. All together this data shows a distinct effect of different mevalonate pathway inhibitors on energy metabolism of Vδ2 T cells.
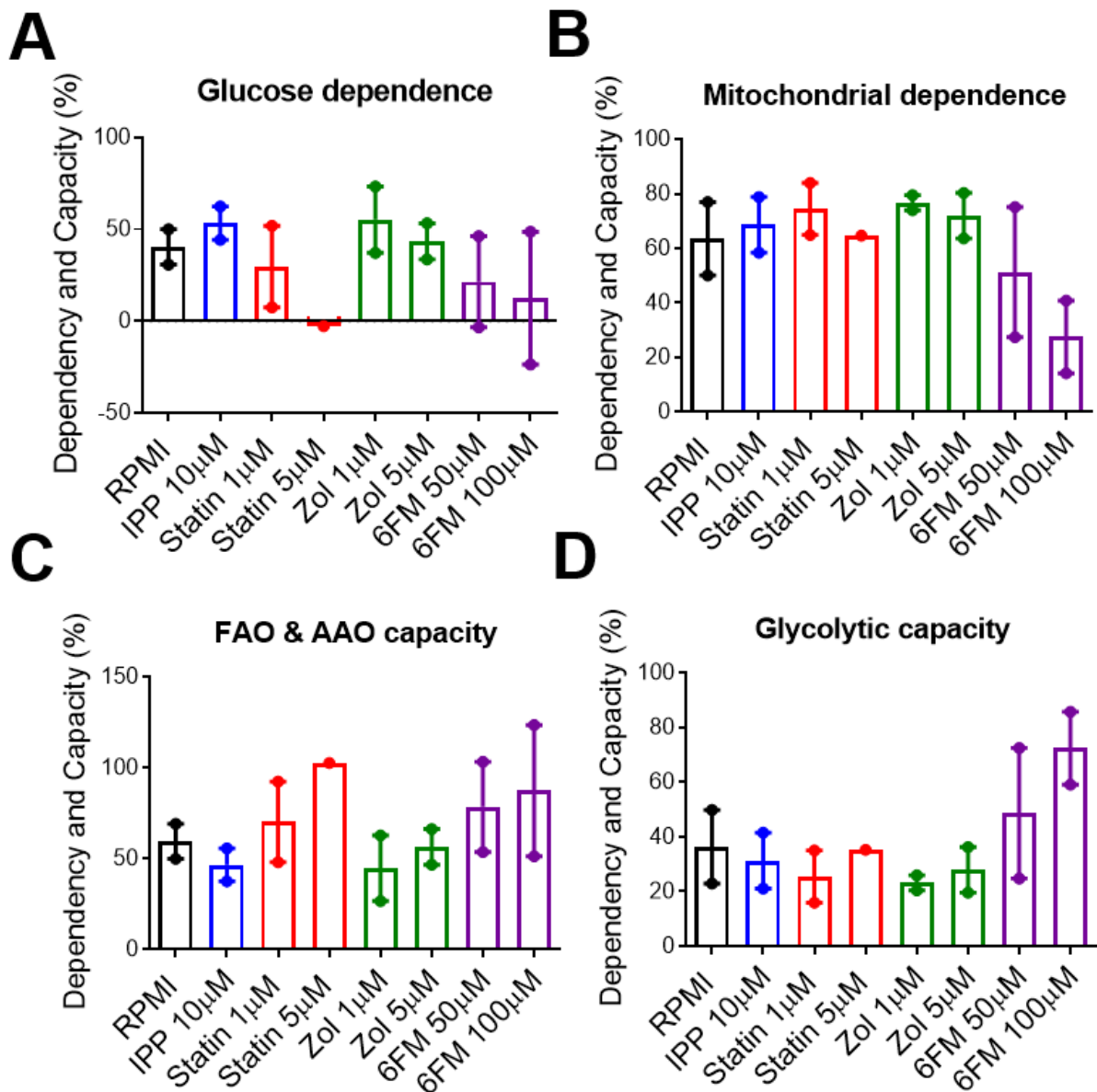
Figure 1. Mevalonate pathway inhibition alters metabolic profile of Vδ2 cells. Metabolic parameters by modified SCENITH[TM] (https://www.scenith.com) calculated as in Argüello et al. (A) glucose dependence, (B) Mitochondrial dependence, (C) FAO & AAO capacity, and (D) Glycolytic capacity of Vδ2 T cells. n=2; Statin 5μM: n=1

Discussion

The mevalonate pathway governs the energy metabolism of cells by maintaining the production of ubiquinone, which is a paramount component of the electron transport chain in mitochondria. Despite the importance of ubiquinone in maintaining the functional powerhouse of the cells, in our study, we did not observe a coherent decrease in mitochondrial dependency or fatty acid and amino acid oxidation capacities by Vδ2 T cells upon incubation with three mevalonate pathway inhibitors. The only mevalonate pathway inhibitor that caused a decrease in mitochondrial dependency was 6-fluoromevalonate. Instead, the fatty acid and amino acid oxidation capacities in Vδ2 T cells even increased upon treatment with high concentrations of fluvastatin and 6-fluoromevalonate. This observation might indicate that not all mevalonate

pathway inhibitors compromised the production of ubiquinone in Vδ2 T cells, but 6-fluoromevalonate. Nevertheless, numerous earlier studies reported reduced serum ubiquinone levels in patients undergoing statin therapy (10–14). Consistently, another study claimed that statin treatment can increase the feeling of tiredness in patients and ubiquinone supplements can replenish their energy levels(9). On the other hand, several studies reported that bisphosphonate compromised production of ubiquinone (16–19). Particularly, zoledronate was shown to reduce ubiquinone biosynthesis in hepatic and cancer cells (16,17). Similar to the inhibitory effect of 6-fluoromevalonate on the mevalonate pathway, hyper-IgD syndrome patients, with a deficiency of mevalonate kinase, also suffer from compromised mevalonate metabolism. In our study, the phenotype of increased fatty acid and amino acid oxidation capacities in 6-fluoromevalonate-treated Vδ2 T cells is consistent with an early study in which hyper-IgD syndrome patients present a higher oxidation of very long-chain fatty acids (20). However, whether such a phenotype is due to the modulation of ubiquinone production in the cells of hyper-IgD syndrome patients is not known. Further investigation is needed to determine whether the intrinsic level of ubiquinone is affected in Vδ2 T cells upon incubation with different mevalonate pathway inhibitors. In our previous study in **Chapter 5**, we found that fluvastatin treatment downregulated glucose metabolism-related transcripts. This is in line with the decreased glucose dependency in Vδ2 T cells measured by SCENITH. Likewise, 6-fluoromevalonate might induce a similar effect on the glucose metabolism of Vδ2 T cells. Further transcriptional analysis may be of interest to validate whether there are changes in glucose metabolism-related transcripts in Vδ2 T cells upon 6-fluoromevalonate treatment. However, the decrease in glucose dependency might also be an indirect effect of fluvastatin and 6-fluoromevalonate on Vδ2 T cells. Due to the limitation of the sample size, statistical analysis was ineligible in this study. In the future, it will be important to introduce more donors to confirm the metabolic changes in Vδ2 T cells.

Altogether, fluvastatin and 6-fluoromevalonate induced metabolic changes in Vδ2 T cells, while zoledronate did not, like IPP. We observed in our previous study that all three mevalonate pathway inhibitors led to a decrease in immune functions of Vδ2 T cells, such as cytokines and cytotoxic granule production. However, the lack of mutual phenotype in the metabolic profile of Vδ2 T cells upon different mevalonate pathway inhibitor treatments indicated that the inhibitors' effect on Vδ2 T cell function is complex and can be mediated by various biological pathways where energy metabolism could be one of them.

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Chronic granulomatous disease functionally reprograms Vδ2 T cells

Introduction

Chronic granulomatous disease (CGD) is an immunodeficiency with some characteristics of an autoinflammatory disease. It is caused by a diversity of genetic mutations in the subunits of the NADPH oxidase enzyme, such as $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ (1,2). Patients who suffer from CGD inherit dysfunctional phagocyte-specific NADPH-oxidase complexes that are unable to produce reactive oxygen species (ROS) to eliminate bacteria and fungi, therefore making CGD patients susceptible to infections (3). Apart from the immunodeficient phenotype, this disease also renders patients susceptible to uncontrolled hyperinflammatory symptoms such as Crohn-like colitis or lupus-like skin lesions (4,5). The underlying mechanism may be due to the failure of a process called "efferocytosis" to remove apoptotic cells and anti-inflammatory cytokine production (6–9). In addition, *in vitro* studies showed that ROS acts as an anti-inflammatory agent during acute inflammation and mice with dysfunctional ROS production were prone to proinflammatory phenotypes (10). Apart from classical phagocytes, T cells have also been shown to express functional phagocyte-type NADPH oxidase (11) whose deficiency results in enhanced Th2 responses (12) and reduced function of regulatory T cells (13). Previous studies showed that excessive production of ROS inhibits interleukin (IL)-17A$^+$ gamma delta (γδ) T cells and their immune response (14,15). In human peripheral blood, Vδ2 T cells compose the majority of γδ T cells. However, to our knowledge, whether Vδ2 T cells are affected in CGD patients and if they play a role in CGD progression is unclear. We speculate that the effector function of Vδ2 T cells and their energy metabolism may be altered in CGD patients with dysfunctional NADPH-oxidase complexes.

In this study, we showed that Vδ2 T cell numbers were reduced in CGD patients. These cells exhibited an activated phenotype, increased cytokine production but reduced cytotoxic granule production and showed marks of exhaustion such as increased expression of PD-1, Tim-3 and CTLA-4. Using single-cell energetic metabolism by profiling translation inhibition (SCENITH) (16), we observed that the protein translation levels in Vδ2 T cells of CGD patients were lower than those of healthy donors. CGD also affected the energy metabolism of Vδ2 T cells, altogether suggesting that Vδ2 T cells may also play a role in the hyper-inflammatory state of the disease.

Methods

Recruitment of healthy volunteers and participants

Inclusion of healthy controls and CGD patients was approved by the local institutional review board (CMO region Arnhem-Nijmegen, #2299 2010/104) and conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines. Inclusion of CGD patients was approved by: NL40331.078 v13 "The causes and clinical complications of Primary Immunodeficiencies. Blood was drawn from two healthy

donors and two CGD patients with gp91^{phox} and p47^{phox} subunit mutation respectively (**Supplementary Table 1**).

PBMC freezing and thawing

PBMCs isolated from donors were resuspended in ice-cold fetal bovine serum (FBS) before cryopreservation. Ice-cold DMSO was applied until a final concentration of 10% DMSO was reached. The cells were stored in CoolCell alcohol-free freezing containers (Corning) at -80 °C overnight. The vials were then transferred to freezer at -150 °C for long-term storage.

The PBMCs were retrieved from the -150 °C freezer. The vials were thawed rapidly in 37 °C water bath. The thawed cells were transferred immediately into falcon tube containing pre-warmed thawing medium (RPMI supplemented with 20% FBS and 12.5 µg/ml DNase-I). The cells were centrifuged at 500g for 10 minutes at RT and resuspended in warm thawing medium. The centrifugation was repeated once, and the cells were resuspended in cold PBS and counted using trypan blue exclusion to distinguish live and dead cells. The thawed PBMCs (0.5×10^6 cells/well) were stimulated with soluble anti-CD3/anti-CD28 (1mg/ml) and incubated with Golgi Plug (1mg/ml) in 96-well plate at 37°C, 5% CO$_2$ for 4 hours. The cultures were harvested for surface markers and intracellular staining and analyzed by flow cytometry.

Surface marker staining

PBMCs were incubated with the following antibodies against cell surface markers: anti-human CD3 Pacific blue (UCHT1; BioLegend), anti-human CD3 APC (UCHT1; BioLegend), anti-human CD45 Brilliant Violet 605 (HI30; BioLegend), anti-human CD45 PE/Dazzle 594 (HI30; BioLegend), anti-human CD4 PE-Cy7 (OKT4; BioLegend), CD4 Brilliant Violet 605 (OKT4; BioLegend), anti-human PD-1 PerCP 5.5 (A17188B; BD Bioscience), anti-human CD8 APC-Cy7 (SK1; BioLegend), anti-human Lag3 Dazzle red (11C3C65; BioLegend), anti-human Tim3 PerCP-Cy5.5 (F38-2E2; BioLegend), anti-human CTLA4 Brilliant Violet 605 (BNI3; BioLegend), anti-human TCR Vδ2 FITC (REA711; Miltenyi Biotec), anti-human CD45RA APC (HI100; BioLegend), anti-human CD27 PerCP-Cy5.5 (m T271; BD bioscience), anti-human CD314 BV605 (1D11; BioLegend), Live-or-Dye™ Fixable stain (Biotidium) and Live/dead™ Fixable Aqua Dead stain (Invitrogen) in FACS buffer (1xPBS, 5% FBS and 2mM EDTA) at 4 °C in dark for 30 mins. The cells were washed with PBS then permeabilized/fixed in Cytofix permeabilization/fixation reagent (BD biosciences) for 30 mins. The cells were washed with Cytofix permeabilization/washing buffer (BD biosciences) twice and incubated in Cytofix permeabilization/washing buffer with the following antibodies against intracellular markers: anti-human TNFα APC (MAb11; BioLegend), anti-human IFNγ PerCP-Cy5.5 (B27; BD Pharmingen), anti-human granzymeB Alexa Fluor 687 (BG11; BioLegend), anti-human Perforin PE-Cy7 (dG9; BioLegend) and anti-human CD3 Pacific blue (UCHT1; BioLegend) at 4 °C in dark for 30 mins. The cells were washed with PBS then fixed in CellFIX reagent (BD Bioscience) and stored at 4 °C in dark. Color compensation was done using OneComp eBeads (BD bioscience). FACS analysis was performed on LSR II (BD bioscience). Data analysis is performed using FlowJo vX.07 software.

SCENITH

Cells were plated at 0.3×10^6 cells/well in 96-well plates. The cells were treated by RPMI alone or stimulated with soluble anti-CD3/anti-CD28 (1μg/ml), or IPP 10μM at 37°C, 5% CO_2 for 4 hours. Cells were treated with control, 2-deoxy-D-Glucose (2-DG, final concentration 100mM), Oligomycin (Oligo, final concentration 10 μM) and combination of 2-Deoxy-D-Glucose and Oligomycin (DGO, final concentration 100mM and 10 μM) for 30 min at 37°C. Puromycin (final concentration 10mg/ml) was added, and cells were incubated for additionally 45 mins at 37°C. Cells were washed in cold FACS buffer and stained with a combination of Fc receptor blocker, live-dead stain and surface marker (described above) for 15 mins at 4°C. Cells were permeabilized and fixed with Cytofix/Cytoperm (BD biosciences) for 30 mins at 4°C. The cells were washed 2 times with Permwash buffer (BD biosciences) and incubated with anti-puromycin AF488 (12D10; Millipore) in Permwash buffer for 20 mins at 4°C. Color compensation was done using OneComp eBeads (BD bioscience). FACS analysis was performed on LSR II (BD bioscience). Data analysis is performed using FlowJo vX.07 software. The metabolic dependencies and capacities were calculated based on the method provided in the study of Argüello *et al* (16).

Results

Our previous study on immunophenotyping of Vδ2 T cells demonstrated that Vδ2 T cells in peripheral blood contributed to the pathology of 6 CGD patients (Appendix) (17) . This indicated that the Vδ2 T cell compartment was altered in CGD patients. To further characterize the immunephenotypes of Vδ2 T cells in the disease, we additionally analyzed two more patients in this study (**Figure 1** and **2**). We showed that the frequency of Vδ2 T cells in the two CGD patients was lower than that of healthy donors **(Figure 1A)**, in consistence to our previous study (Appendix) (17). Consistently, the activation markers CD69 and CD38 were higher in Vδ2 T cells of these CGD patients **(Figure 1B)** (Appendix) (17). On the other hand, the pro-inflammatory cytokine Tumor necrosis factor (TNF) and Interferon-gamma (IFN-γ) production increased **(Figure 1C)**, whereas the production of cytotoxic granules such as granzyme B and perforin was reduced compared to healthy donors **(Figure 1D)**. Additionally, we also analyzed the memory phenotype based on CD27 and CD45RA expression (18,19) and showed that the effector memory Vδ2 T cell population increased while terminally differentiated Vδ2 T cell frequency decreased in these CGD patients compared to healthy donors **(Figure 1E)**. Furthermore, a small portion of Vδ2 T cells had a higher expression of exhaustion markers such as PD-1 and CTLA4 **(Figure 1F)**, in consistent to our previous study (Appendix) (17). We observed that another exhaustion marker, Tim3, also increased in a small percentage of Vδ2 T cells.

To decipher whether the metabolic profile of Vδ2 T cells was altered in CGD patients compared with the healthy donors, we applied SCENITH to measure the protein translation level and calculated the metabolic dependencies and capacities based on the protocol described in the study of Argüello et al. (10) (Figure 3) **(Figure 2)**. By measuring puromycin incorporation, we observed that the protein translation level in CGD patients was lower than that of healthy donors in both unstimulated and stimulated Vδ2 T cells **(Figure 2A)**. The

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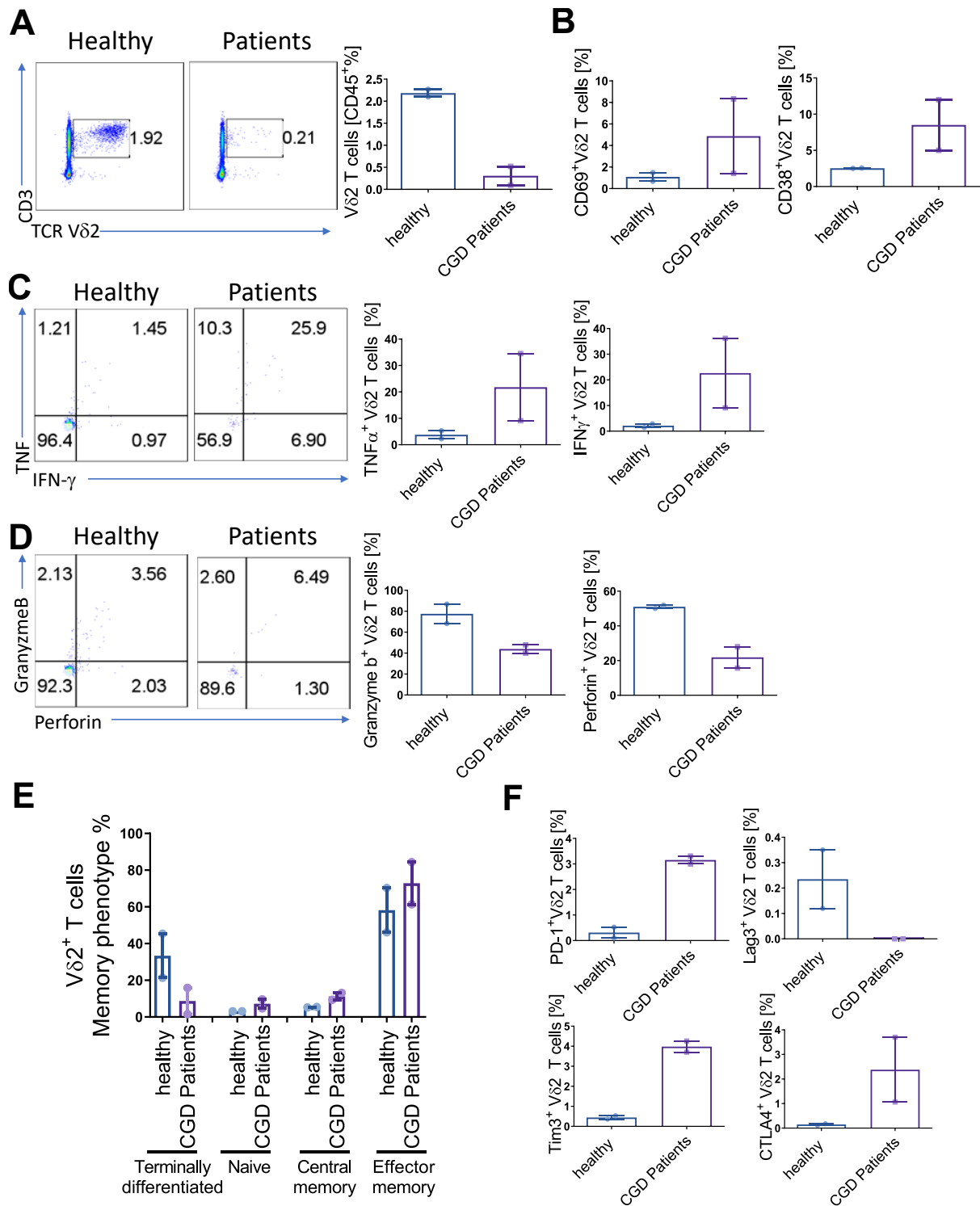
Figure 1. Immuneophenotyping revealed decreased number and functional reprogramming of Vδ2 T cells in CGD patients compared to healthy donors. Flow cytometry analysis of circulating immune cells in CGD and healthy individuals (A) top, representative dot plots showing % of Vδ2 T cells in peripheral blood of CGD patients and healthy donors. Bottom, cumulative % of Vδ2 T cells in PBMC culture. (B) Cumulative % of CD69+ and CD38+ Vδ2 T cells in PBMC culture. (C) top, representative dot plots showing % of TNF+ and IFN-γ+ Vδ2 T cells in peripheral blood of CGD patients and healthy donors. Bottom, cumulative % of TNF+ and IFN-γ+Vδ2 T cells in PBMC culture. (D) top, representative dot plots showing % of granzymeB+ and perforin+ Vδ2 T cells in PBMC culture. Bottom, cumulative % of granzymeB+ and perforin+ Vδ2 T cells in peripheral blood of CGD patients and healthy donors. (E) Cumulative % of memory compartment of Vδ2 T cells characterized by expression of CD27 and CD45RA. (F) Cumulative % of PD-1+, LAG3+, TIM3+, CTLA4+ and CD38+ Vd2 T cells in PBMC culture.

Figure 2. Metabolic analysis revealed a lower translation and an altered energy metabolism in Vδ2 cells in CGD patients compared to healthy donors. Metabolic parameters by modified SCENITH^{TM} (https://www.scenith.com) calculated as in Argüello et al. (A) puromycin incorporation of Vδ2 T cell unstimulated and stimulated with soluble anti-CD3/CD28 antibodies upon addition of 2-deoxy-D-Glucose (2-DG; 100mM), oligomycin (Oligo; 10μM) or combination of both.B) glucose dependence, Mitochondrial dependence, FAO & AAO capacity, and Glycolytic capacity of Vδ2 T cells from CGD patients and healthy donors before and after CD3/CD28 stimulation. n=2

translation level of Vδ2 T cells did not increase after TCR stimulation; instead, it decreased in both healthy donors and patients. The translation rate decreased further in both patients and healthy controls by adding the metabolic inhibitors (**Figure 2A**). We further observed that glucose dependency is reduced in CGD patients with no changes to mitochondrial dependency, while glycolytic capacity, fatty acid and amino acid oxidation capacity were increased in patients (**Figure 2B**). Soluble anti-CD3/CD28 antibodies can stimulate the TCR of Vδ2 T cells and lead to activation of effector function. Upon TCR stimulation, glucose dependency Vδ2 T cells in healthy donors reduced, while fatty acid and amino acid oxidation capacity slightly increased. These changes were not observed in CGD patients (**Figure 2B**). Instead, glucose dependency and glycolytic capacity increased in Vδ2 T cells of CGD patients upon TCR stimulation, while mitochondrial dependency and fatty acid and amino acid oxidation capacity decreased (**Figure 2B**). Thus, the causes of CGD disease affected the energy metabolism of Vδ2 T cells.

Discussion

CGD is commonly known as a disease of the phagocyte system. However, the NADPH oxidase, a mutated complex in the phagocytes of CGD patients, has also been shown to be active in T cells (11) and affect αβ T cell polarization and their immune response when it is dysfunctional

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(12,13). Particularly, the killing component produced by NADPH oxidase, ROS, is not only essential for eliminating engulfed pathogens and apoptotic cells, but also plays an important role in regulating the inflammatory state in the individual (6,7). In fact, previous studies showed that ROS can dampen inflammasome activation and, therefore IL-1β secretion (20). On the other hand, defective production of ROS can lead to a hyperinflammatory phenotype in mice (15). A recent study demonstrated that the decrease of ROS also led to blockage of tryptophan metabolism in CGD mice and resulted in dysregulated activation of γδ T cells and a high production of the proinflammatory cytokine IL-17 (15), indicating that γδ T cells may play a role in the hyperinflammatory state of CGD patients. As a result, CGD patients who suffer from NADPH oxidase mutations not only show immunodeficiency against infection but are also prone to hyperinflammatory conditions.

In this study, we showed that the CGD condition affects γδ T cells in humans. We found that the percentage of Vδ2 T cells in CGD patients was lower than that of healthy donors at steady state, in consistence to our previous findings (Appendix) (17). However, previous studies claimed inconsistent observations of T cell numbers in CGD patients when compared with healthy donors (21–24). The contradiction between the studies might be due to the difference in patients' age or mutated genes of NADPH oxidase within the cohort. Indeed, a previous study showed that CGD patients older than 3 years had lower conventional T cell numbers(24), which might be owing to their reduced proliferative capacity(22). However, whether the decrease of Vδ2 T cells in our study was also a result of the patients' age remains to be investigated. Nevertheless, Romani et al. reported higher numbers of γδ T cells in a mouse model of CGD than those of wild-type controls upon *Aspergillus fumigatus* infection in their study (15). It will be of interest to confirm whether the percentage of Vδ2 T cells in CGD patients can also surpass that of healthy donors in an infection condition.

In our study, we revealed a high expression of activation markers CD69 and CD38 on Vδ2 T cells and increased production of pro-inflammatory cytokines: TNF and IFN-γ by Vδ2 T cells in CGD patients, in consistent to our previous study (Appendix) (17), indicating that Vδ2 T cells were highly activated and may also contribute to the hyper-inflammatory condition of the patients. Previous studies showed a similar trend in CD4 T cells with increasing production of TNF and IFN-γ in CGD mouse models and CGD patients' biopsies (11,25). Romani et al. demonstrated that the acute inflammatory condition in CGD is driven by the pro-inflammatory cytokine IL-17, which is mainly produced by the γδ T cells (15). In the future, it will be of interest to investigate the correlation of pathology with IL-17 production by Vδ2 T cells in CGD patients. On the other hand, we observed decreased granzyme B and perforin production by Vδ2 T cells in CGD patients. A previous study showed that granzyme B was necessary to induce mitochondrial ROS production, but whether ROS is needed for granzyme B production is unclear (26). Further study may be of interest to dissect the association between granzyme B and ROS production. Further immune profiling indicated that CGD patients had a greater effector memory compartment of Vδ2 T cells, which was rendered to more immediate effector functions such as cytokine production, supporting the pro-inflammatory phenotype of the disease. Whereas the terminally differentiated memory compartment of Vδ2 T cells decreased

in CGD patients, showing a lower number of exhausted Vδ2 T cells. However, based on the exhaustion marker expression, we found that a small proportion of Vδ2 T cells expressed PD-1, TIM3 and CTLA4, indicating that a minority of Vδ2 T cells may be exhausted in CGD patients.

By employing SCENITH to determine the energy metabolism of Vδ2 T cells, we found that mitochondrial dependency and oxidation capacity decreased, whereas glucose dependency and glycolytic capacity increased in Vδ2 T cells of CGD patients, indicating a shift in energy metabolism in Vδ2 T cells. A previous study showed that ROS can abrogate glycolysis by inhibiting multiple glycolytic enzymes(27). Further study is required to investigate whether the elevated glycolytic capacity in Vδ2 T cells was due to the decrease in intrinsic ROS levels in CGD patients. The protein translation in Vδ2 T cells of CGD patients was lower than that of healthy donors. The underlying cause leading to such a decrease in protein translation in CGD patients warrants further investigation.

Our previous study illustrated that CGD patients were prescribed antibiotics, anti-fungal drugs and steroids to reduce infections and hyperinflammation (Appendix) (17). Here, we do not exclude the possibility that the medication also exerted side effects on the Vδ2 T cells of our patients in this study. For instance, it is known that steroids can modulate the expression of immunoregulatory proteins, inhibitory receptors, co-stimulatory molecules and the production of cytokines and cycle mediators in T cells (28). The CGD patients in our study presented defects in distinct subunits of the NADPH oxidase complex; the inter-individual heterogeneity may be due to their differences in genetic background. Our study was also limited to marginal sample size of both CGD patients and healthy controls; more samples are required to draw statistical conclusions.

All in all, our CGD patients presented a distinctive metabolic and immune profile of Vδ2 T cells compared to healthy donors. To better understand whether the pro-inflammatory phenotype and metabolic shift of Vδ2 T cells are indeed responsible for the hyperinflammatory condition and pathology of CGD, we need to include more patients in our cohort and conduct further investigation in the future.

Supplementary

Subject Name	Mutation	Age	Sex	Date Isolation
HC07	NA – healthy control	47	M	28.07.22
HC08	NA – healthy control	36	M	28.07.22
CGD01	Gp91 (*CYBB*)	44	M	28.07.22
CGD02 (HIT010; (Appendix) (17))	p47 (*NCF1*)	40	M	28.07.22

Table 1 Donors information

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Metabolic and functional profile of Vδ2 T cells in Hyper-IgE Syndrome Patients

Introduction

Hyper-IgE syndrome (HIES) consists of a group of rare immunodeficiency disorders caused by genetic defects including *DOCK8, STAT3, STAT1, SPINK5, ZNF431, PGM3, ERBIN, IL6ST* and *CARD11* mutations (1–3). Patients who are diagnosed with HIES have pulmonary infections and recurrent skin infections such as eczema and increased serum IgE (4). HIES patients with *CARD11* mutations have defective caspase recruitment domain family member 11 (CARD11) activity (5,6). CARD11 is essential for the assembly of the multimeric complex CARMA1-BCL-9-MALT1 which drives many downstream signaling events (6,7). Particularly, CARD11 is important for NF-kB and mTORC signaling upon B and T cell receptor (TCR) activation, leading to proliferation, differentiation, survival and cytokine production by T and B cells (8–11). Genetic inactivation of CARD11 in mice and humans results in a complete block of B and T cell immunity (12,13). Patients who suffer from the *CARD11* mutation also present a defect in IFN-γ production by T cells (5). In addition, CARD11 signaling plays a role in alanine-serine-cysteine transporter 2 (ASCT2) regulation, which is an essential glutamine transporter facilitating the active uptake of glutamine (14). Supplementation of glutamine could partially rescue the mTORC1 and IFN-γ production by conventional T cells in patients with the *CARD11* mutation (5). IFN-γ treatment could also improve clinical scores and reduce severe atopic dermatitis in HIES patients (15). However, the mechanism causing such an improvement is not clear. A recent study showed that a HIES patient presented a clonal expansion of γδ T cells and an elevated level of IL-4 contributing to the high IgE levels in serum (16). Therefore, whether γδ T cells promote the disease pathology has yet to be examined. In our study, we aimed to investigate whether the defect of CARD11 causes a shift in the metabolic and immune profile of a predominant subset of γδ T cells, namely Vδ2 T cells, in the peripheral blood of HIES patients, and whether IFN-γ treatment improves the disease outcome by acting on the Vδ2 T cell population.

We found reduced frequency of Vδ2 T cells together with decreased production of cytotoxic molecules in two families with the HIES history caused by the *CARD11* mutation. IFN-γ treatment further decreased the Vδ2 T cell numbers and effector molecule production by these cells. We found that Vδ2 T cells from HIES patients showed a decrease in glutaminolysis capacity, which was improved after IFN-γ treatment. This indicates that glutamine uptake in Vδ2 T cells might be interrupted due to the CARD11 defect, and IFN-γ treatment can revert this defect. All these observations indicated that the immune response of Vδ2 T cells was affected in HIES patients. While some aspects of the beneficial effect of the IFN-γ treatment might be mediated by Vδ2 T cells, it could not be fully explained by the improved Vδ2 T cell function.

Methods

Sample collection from healthy volunteers and participants of clinical trial.

Inclusion of healthy controls was approved by the local institutional review board (CMO region Arnhem-Nijmegen, #2299 2010/104) and conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines. Inclusion of CARD11 patients was approved by: NL40331.078 v13 "The causes and clinical complications of Primary Immunodeficiencies. PBMCs were isolated from healthy donors (one member is related to family 1), three related HIES patients from family 1 (c.127A>C;p.(Thr43Pro) variant) and two related HIES patients from family 2 (c.358+1G>C variant), before IFN-γ injection (T1) and 4 hours after IFN-γ injection (T2) (**Supplementary Figure 1; Table 1**). IFN-γ dosage is applied according to the patient body surface to weight ratio.

PBMC freezing and thawing

PBMCs isolated were resuspended in ice-cold fetal bovine serum (FBS) before cryopreservation. Ice-cold DMSO was applied until a final concentration of 10% DMSO was reached. The cells were stored in CoolCell alcohol-free freezing containers (Corning) at -80 °C overnight. The vials were then transferred to freezer at -150 °C for long-term storage. The PBMCs were retrieved from the -150 °C freezer. The vials were thawed rapidly in 37 °C water bath. The thawed cells were transferred immediately into falcon tube containing pre-warmed thawing medium (RPMI supplemented with 20% FBS and 12.5 µg/ml DNase-I). The cells were centrifuged at 500g for 10 minutes at RT and resuspended in warm thawing medium. The centrifugation was repeated once, and the cells were resuspended in cold PBS and counted using trypan blue exclusion to distinguish live and dead cells. The thawed PBMCs (0.5×10^6 cells/well) were stimulated with soluble anti-CD3/anti-CD28 (1mg/ml; HIT3A/ CD28.2 BD Bioscience) and incubated with Golgi Plug (1mg/ml; BD Bioscience) in 96-well plate at 37°C, 5% CO_2 for 4 hours. The cultures were harvested for surface markers and intracellular staining and analyzed by flow cytometry.

Surface marker staining

PBMCs were incubated with the following antibodies against cell surface markers: anti-human CD3 Pacific blue (UCHT1; BioLegend), anti-human CD3 APC (UCHT1; BioLegend), anti-human CD45 Brilliant Violet 605 (HI30; BioLegend), anti-human CD45 PE/Dazzle 594 (HI30; BioLegend), anti-human CD4 PE-Cy7 (OKT4; BioLegend), CD4 Brilliant Violet 605 (OKT4; BioLegend), anti-human CD8 APC-Cy7 (SK1; BioLegend), anti-human Lag3 Dazzle red (11C3C65; BioLegend), anti-human TCR Vδ2 FITC (REA711; Miltenyi Biotec), Live-or-Dye™ Fixable stain (Biotidium) and Live/dead™ Fixable Aqua Dead stain (Invitrogen) in FACS buffer (1xPBS, 5% FBS and 2mM EDTA) at 4 °C in dark for 30 mins. The cells were washed with PBS then permeabilized/fixed in Cytofix permeabilization/fixation reagent (BD biosciences) for 30 mins. The cells were washed with Cytofix permeabilization/washing buffer (BD biosciences) twice and incubated in Cytofix permeabilization/washing buffer with the following antibodies against intracellular markers: anti-human TNFα APC (MAb11; BioLegend), anti-human IFNγ PerCP-Cy5.5 (B27; BD Pharmingen), anti-human granzymeB Alexa Fluor 687 (BG11; BioLegend), anti-human Perforin PE-Cy7 (dG9; BioLegend) and anti-human CD3 Pacific blue (UCHT1; BioLegend) at 4 °C in dark for 30 mins. The cells were

washed with PBS then fixed in CellFIX reagent (BD Bioscience) and stored at 4 °C in dark. Color compensation was done using OneComp eBeads (BD bioscience). FACS analysis was performed on LSR II (BD bioscience). Data analysis is performed using FlowJo vX.07 software.

SCENITH

SCENITH was performed as described previously (17,18) . Cells were plated at 0.3×10^6 cells/well in 96-well plates. The cells were treated by RPMI alone or stimulated with soluble anti-CD3/anti-CD28 (1µg/ml; HIT3A/ CD28.2 BD Bioscience), or IPP 10mM at 37°C, 5% CO_2 for 4 hours. Cells were untreated (control) or treated with 2-deoxy-D-Glucose (2DG, final concentration 100mM, Sigma), Oligomycin (O, final concentration 10 µM, Sigma), combination of 2-Deoxy-D-Glucose and Oligomycin (DGO, final concentration 100mM and 10 µM, Sigma) or CB-839 (5 µM; Selleckchem) for 30 min at 37°C. Puromycin (final concentration 10mg/ml, Sigma) was added and incubated for 45 mins at 37°C. Cells were washed in cold FACS buffer and stained with a combination of Fc receptor blocker (Biolegend), live-dead stain and surface marker (described above) for 15 mins at 4°C. Cells were permeabilized and fixed with Cytofix/Cytoperm (BD biosciences) for 30 mins at 4°C. The cells were washed 2 times with Permwash buffer (BD biosciences) and incubated with anti-puromycin AF488 (12D10; Millipore) in Permwash buffer for 20 mins at 4°C. Color compensation was done using OneComp eBeads (BD bioscience). FACS analysis was performed on LSR II (BD bioscience). Data analysis is performed using FlowJo vX.07 software. The metabolic dependencies and capacities were calculated according to the method provided in the study of *Argüello et al* (17) and *Adamik et. al* (18).

Statistical analysis

Acquired data was analyzed statistically by Wilcoxon test on graphic analysis software GraphPad Prism 6.0 (GraphPad Software Inc.). Result considered as significant with P value < 0.05 (*), < 0.01 (**), < 0.001 (***) or < 0.0001 (****).

Result

In order to determine the phenotypical changes in Vδ2 T cells in HIES patients, we analyzed the PBMC of healthy donors and two families of *CARD11* mutated HIES patients (c.127A>C;p.(Thr43Pro) variant and c.358+1G>C variant; **Supplementary Figure 1, Table 1**) before IFN-γ treatment and 4 hours after treatment by flow cytometry (**Figure 1A**). We found a trend towards reduced percentages of Vδ2 T cells in HIES patients compared to healthy donors (**Figure 1B**). The IFN-γ treatment further decreased these percentages (**Figure 1B**). Furthermore, we observed that TNF and IFN-γ production by Vδ2 T cells were comparable between healthy donors and HIES patients (**Figure 1C**). However, granzyme B and perforin production by Vδ2 T cells tended to decrease in HIES patients compared to that of healthy donors (**Figure 1D**). IFN-γ treatment further decreased granzyme B and perforin but also TNF production by Vδ2 T cells (**Figure 1C** and **D**). To determine whether the metabolic profile of Vδ2 T cells was also altered in *CARD11* mutated HIES patients, we applied SCENITH (17) and further adapted the protocol from *Adamik et. al* (18) to assess the glutaminolysis capacity

by incubating the cells with glutaminase inhibitor CB-839 (**Figure 2**). We calculated the metabolic capacity and dependency of Vδ2 T cells in accordance with the SCENITH protocol (17,18). Despite the high heterogeneity of results and limited sample size, we observed a trend towards decreased glutaminolysis capacity in Vδ2 T cells from HIES patients, whereas the glycolysis and oxidative capacities of the cells were comparable **(Figure 2A)**. After IFN-γ treatment, the glutaminolysis capacity of Vδ2 T cells increased **(Figure 2A)**. Because CARD11 is essential for the activation of T cells (12), we compared the metabolic profile of Vδ2 T cells before and after CD3/CD28 stimulation among healthy donors, HIES patients before and after IFN-γ treatment **(Figure 2B)**. TCR stimulation resulted in a decrease in glucose dependence, mitochondrial dependence and glutaminolysis capacity in healthy donors and to the same extent in HIES patients before IFN-γ therapy, indicating that activation metabolically reprogramed Vδ2 T cells independently of the CARD11 mutation. IFN-γ treatment blunted the effect of TCR stimulation on the metabolic requirements of Vδ2 T cells. Yet, these results need to be confirmed in a larger and not related cohort of patients.

Discussion

CARD11 mutations in HIES affect the signaling events downstream of the multimeric complex CARMA1-BCL-9-MALT1, leading to the dysregulation of ASCT2 (8,10,14). ASCT2 is an important glutamine transporter that is responsible for glutamine uptake by cells. Lymphocytes utilize glutamine in a process called glutaminolysis to generate energy in order to sustain the effector functions such as proliferation and cytokine production (19,20). A previous study showed that knockout of ASCT2 can lead to decreased glutaminolysis in mice (21). Consistent with this observation, we found that the glutaminolysis capacity of Vδ2 T cells in HIES patients was reduced compared to healthy donors, indicating that the glutamine import may be abrogated in Vδ2 T cells. A previous study showed that supplementation of glutamine could partially rescue the mTORC1 and IFN-γ production by conventional T cells in patients with *CARD11* mutation. It may be of interest to supplement glutamine to Vδ2 T cells to determine whether glutaminolysis capacity can be rescued. The shift of glutaminolysis was not accompanied by changes in other metabolic parameters, such as glycolysis and mitochondrial oxidation in Vδ2 T cells.

Moreover, we found a trend towards decreased Vδ2 T cell numbers in the peripheral blood of HIES patients. This is not consistent with the study by Simon et al., where the authors observed elevated numbers of γδ T cells in the blood in one HIES patient (16). As this report described only one patient, it is difficult to translate it to all HIES patients. The study further reported the patient received treatment of flucloxacillin and intravenous immunoglobulin preparations for years, therefore it may also influence the frequency of immune cells in the blood. In contrast to the study of Ma et al. (5) where they observed a decrease of IFN-γ production by conventional T cells in *CARD11* mutated patients, we did not observe a difference in the production capacity of proinflammatory cytokines TNF and IFN-γ by Vδ2 T cells between HIES patients and healthy donors. In the study of Dadi et al. (6), they also found a decrease of IL-2 production in addition to IFN-γ by conventional T cells in members of a *CARD11* mutated

family. Although the published studies did not show specific data on Vδ2 T cells but only on conventional T cells, the difference between our and their results may not only due to cell specificity, other factors such as patients age, and mutation background may also play a role. First, participants in our studies present a large age variation (8 to 62 years old, **Supplementary Table 1**), the participants of the previous study were between 1 to 37 (5,6). Second, while our patients carry the c.127A>C;p.(Thr43Pro) and c.358+1G>C variant, patients in the previous studies carry L194P, R975W, E57D, dup186_196 and R30W variant (5,6). All these factors can contribute to the discrepancy of the findings. To further dissect the association of Vδ2 T
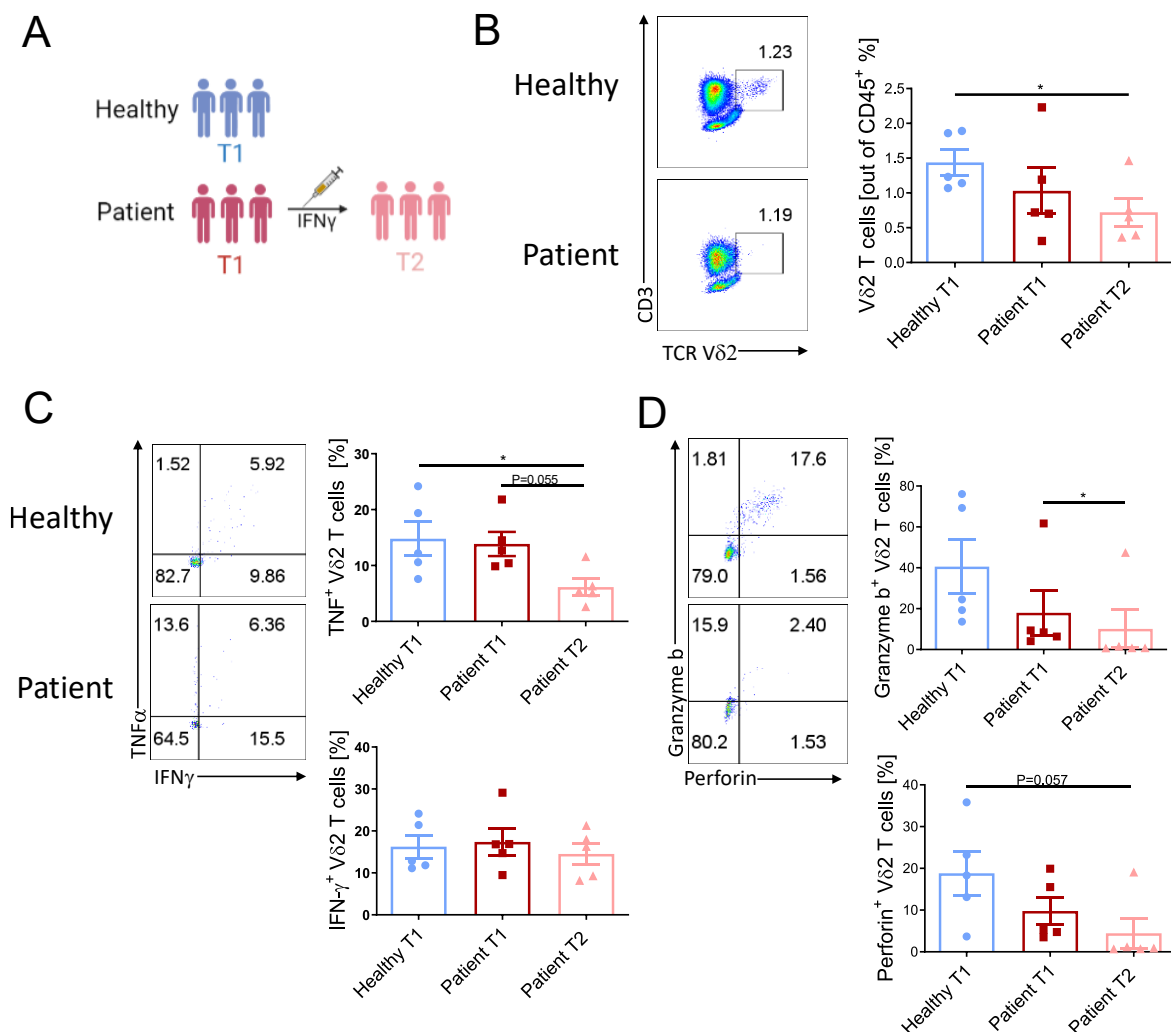
Figure 1. Hyper-IgE syndrome patients display decreased Vδ2 cell numbers and cytotoxic granule production compared to healthy donors. (A) schematic representation of the cohort study. PBMCs were isolated from healthy donors and HIES patients before IFN-γ injection (T1) and 4 hours after IFN-γ injection (T2) (B) top, representative dot plots showing % of Vδ2 T cells in peripheral blood of HIES patients and healthy donors. Bottom, cumulative % of Vδ2 T cells in PBMCs (C) top, representative dot plots showing % of TNF+ and IFN-γ+ Vδ2 T cells in peripheral blood of HIES patients and healthy donors. Bottom, cumulative % of TNF+ and IFN-γ+Vδ2 T cells in in peripheral blood of HIES patients and healthy donors. (D) top, representative dot plots showing % of granzyme B+ and perforin+ Vδ2 T cells in peripheral blood of HIES patients and healthy donors. Bottom, cumulative % of granzyme B+ and perforin+Vδ2 T cells in in peripheral blood of HIES patients and healthy donors. (Paired and unpaired t test, P value * < 0.05).
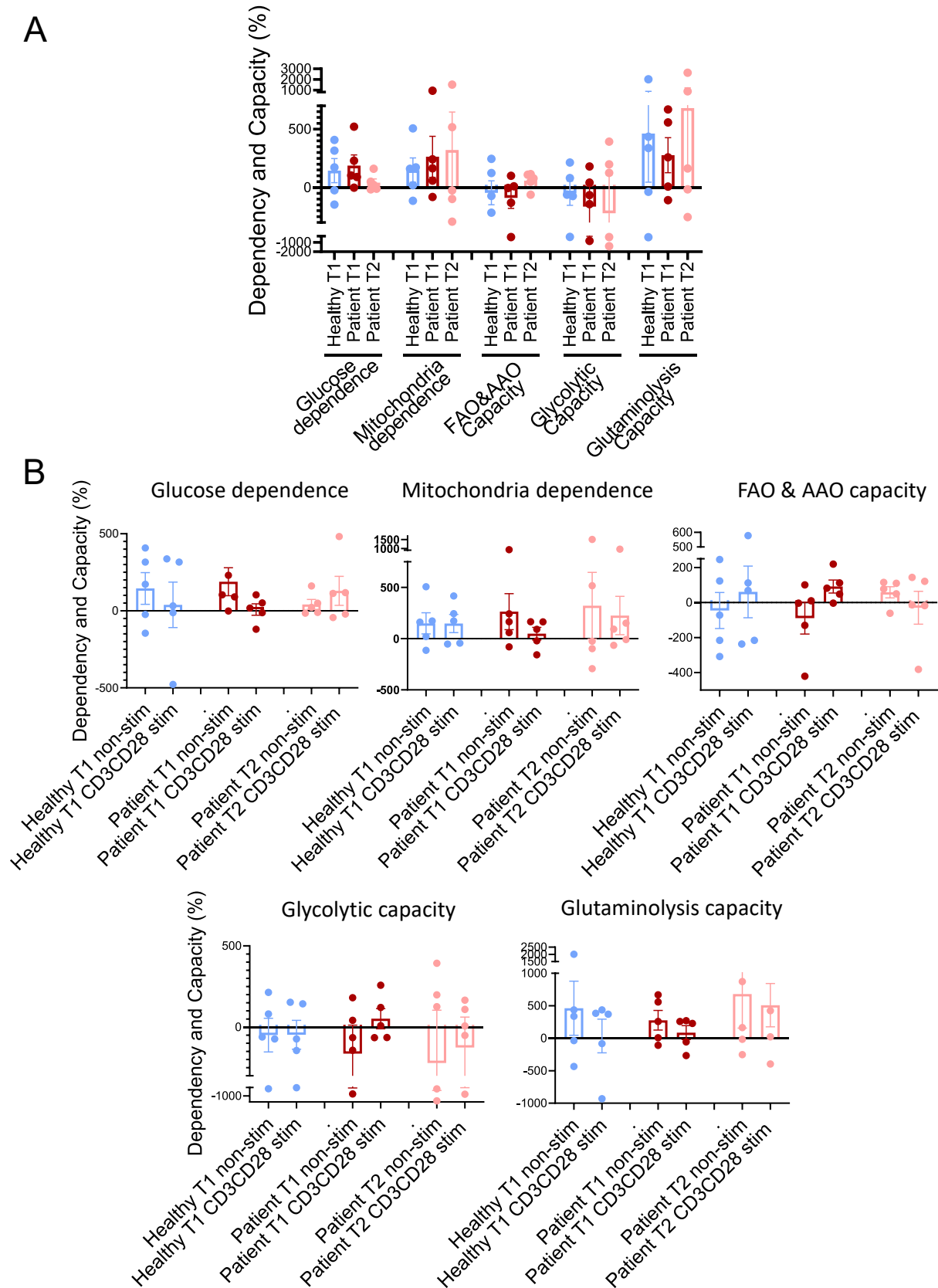
Figure 2. Glutaminolysis capacity by Vδ2 cells in a decreased in HIES patients and can be rescued by the IFN-γ treatment. Metabolic parameters by modified SCENITH[TM] (https://www.scenith.com) calculated as in Argüello et al. (A) glucose dependence, mitochondrial dependence, FAO & AAO capacity, glycolytic capacity and glutaminolysis capacity of Vδ2 T cells in healthy donors, HIES patients before IFN-γ treatment and after IFN-γ treatment. (B) glucose dependence, mitochondrial dependence, FAO & AAO capacity, glycolytic capacity and and glutaminolysis capacity of Vδ2 T cells in HIES patients and healthy donors before and after CD3/CD28 stimulation.

cells with immunodeficient phenotypes of HIES, it may be of interest to measure other cytokines produced by Vδ2 T cells, such as IL-2. Although the cytokine production by Vδ2 T cells was not affected in our patients, we observed a decrease in granzyme B and perforin production, which may lead to inefficiency in infected cell killing. We speculated that the cause of the decrease in cytotoxic properties of Vδ2 T cells may contribute to the immunodeficiency of the disease and render the patient susceptible to recurrent infections. However, IFN-γ treatment, which was known to provide positive clinical outcomes such as improvement of recurrent skin infections in HIES patients (15), did not restore granzyme B and perforin production by Vδ2 T cells in our study; instead, it further decreased their expression. We also observed a significant decrease in Vδ2 T cell percentages and TNF production in HIES patients after IFN-γ treatment. Therefore, we concluded that the clinical improvement after IFN-γ treatment is unlikely to be mediated by the improvement in Vδ2 T cell function assessed by cytokine and cytotoxic molecule production. Interestingly, the glutaminolysis capacity of Vδ2 T cells increased in HIES patients after IFN-γ treatment, indicating that IFN-γ treatment may restore the distorted metabolic state caused by the deficiency of CARD11 in these cells. Future studies are needed to explore the molecular mechanism of how IFN-γ treatment leads to the rescue of glutaminolysis capacity in Vδ2 T cells, whether other unexamined effector molecules produced by Vδ2 T cells contribute to the phenotype of HIES and IFN-γ treatment and to test the Vδ2 T cells in functional assays.

As HIES is a rare immunodeficiency disease with an annual incidence of 1 out of 1,000,000 (22), it is difficult to obtain a large cohort size to perform the study. We focused on two families with *CARD11* mutations in which the sample size was limited. We also found high inter-individual heterogeneity in our result. It may be due to the variability in age and sex. Thus, a study with the inclusion of a more homogenous population will be required in the future to draw further conclusions. Furthermore, our study encompasses two families whose members' genetic backgrounds are similar. Inclusions or other unrelated patients are necessary to confirm whether our observations are limited to these two particular families or can be found in the other ones. It is of note that one of our HIES patients was prescribed medications such as Dupilumab to treat eczema, which may play a role in modulating the immune and metabolic profiles of the Vδ2 T cells. We do not exclude the possibility that the prescribed drugs can influence our results. In the future, it is important to recruit more patients to validate our results and reach statistical significance.

**Pedigree chart of the CARD
11 mutated family patients**

Figure 1. A pedigree chart demonstrating inheritance of the c.127A>C;p.(Thr43Pro) and c.358+1G>C variants in *CARD11* mutated family.
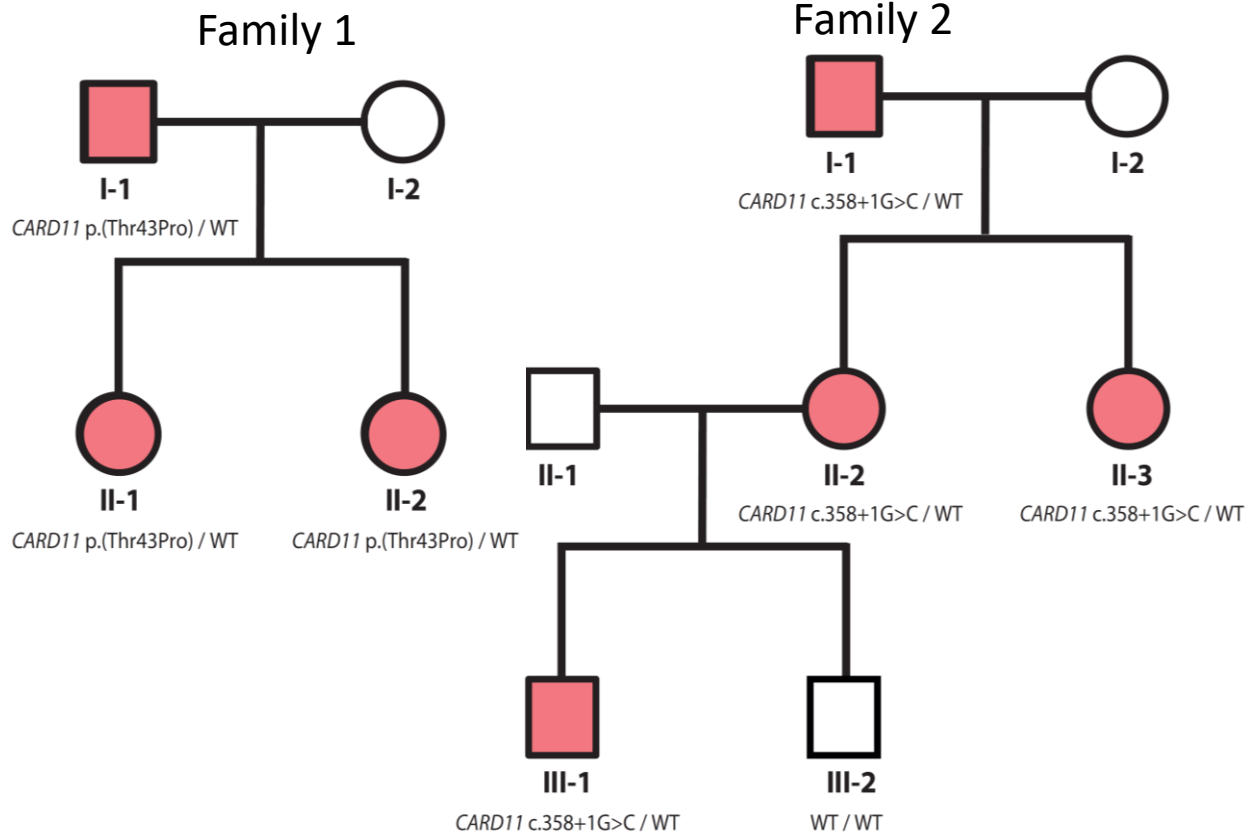
	Family 1	Family 2
Genotype	**c.127A>C; p.(Thr43Pro)**	**c.358+1G>C**
Effect prediction	**CADD: 27.2 SIFT/PolyPhen: Deleterious**	**Loss of donor splice site, skip of exon 5**
Amino acid conservation	**PhyloP 4.56**	**-**

Donors ID	Age	Sex	Date of experiment	Venipuncture time (T1)	Venipuncture time (T2)	Kinship
CARD01	8	M	27.07.22	11:08	15:40	Family 1
CARD02	37	F	27.07.22	10:48	15:32	Family 1
CARD03	63	M	27.07.22	10:42	15:26	Family 1
CARD04	22	F	28.07.22	10:36	15:10	Family 2
CARD05	24	F	28.07.22	10:26	15:18	Family 2
HC01	8	M	27.07.22	10:32	-	Family 1
HC02	37	F	27.07.22	10:12	-	Non-related
HC03	62	M	27.07.22	10:00	-	Non-related
HC04	22	F	28.07.22	10:05	-	Non-related
HC05	25	F	28.07.22	10:11	-	Non-related

Table 1. Donor information.

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Chapter 7

Summary and discussion

Summary

γδ T cells are one of the most mysterious cells in the human immune system. Although γδ T cells have a smaller number of segments for somatic recombinants than conventional T cells, alteration of coupling sites during gene segment rearrangement allows a wide spectrum of γδ repertoire to be developed in the thymus (1,2). Furthermore, based on the TCR composition, many γδ subsets can be identified in different tissues of the body with distinctive functions (3). With specific cytokine and chemokine stimulations, γδ T cells can polarize towards different functional subsets exerting either pro-inflammatory or anti-inflammatory properties (4–7). The discovery of the phagocytotic feature and professional antigen-presenting property of γδ T cells demonstrates their strong ability to orchestrate with other components of the host defense (8–11). Although their TCR repertoire is more restricted than that of conventional T cells, the ligands recognized by γδ TCR are still largely unknown. For instance, one of the most researched γδ T cell subsets in humans, the Vδ2 T cell, expresses innate-like and MHC-unrestricted TCR. This receptor recognizes non-peptide phosphoantigens produced by microbes or eukaryotic cells in isoprenoid synthesis (12,13).

Immune memory is a feature of immune cells that allows them to exert a more rapid and robust immune response upon reinfection. Recently, an increasing body of studies has shown that the development of immune memory is not only exclusive to the adaptive arm, but also to the innate arm of immunity (14–16). γδ T cells, which share features of both adaptive and innate immunity, also showed signs of induction of both adaptive immune memory and innate immune memory responses in several studies (14,17–21), sparking interest in investigating the contribution of γδ T cells in vaccinations and reinfections. Although much is yet to be learned about these multi-functional cells, general conclusions from recent studies suggest that γδ T cells play indispensable roles in host defense against infection and immune surveillance against cancer. Considering the extraordinary potential and some advantages over conventional T cells, many studies have focused on expanding γδ T cells as an immunotherapeutic tool to tackle cancer and infectious diseases. However, many clinical trials demonstrated the limited efficiency of γδ T cells in cancer remission. The underlying mechanisms leading to such a deficiency may resort to the lack of target specificity of γδ T cells, exhaustion of γδ T cells in long-term expanded cultures, modulatory or inhibitory effects of the expanding agents or the unfavorable microenvironment created by tumors (22–26).

In my thesis, I aimed to investigate the induction of innate immune memory in γδ T cells and the modulatory effect of mevalonate metabolism inhibition on the immune functions of γδ T cells, as well as the role of these cells in certain immunodeficiencies. First, I reviewed the current discoveries and the underlying molecular mechanisms of trained immunity in different immune cells. Second, I used commonly administered vaccines such as BCG and MMR vaccines, which have been shown to induce trained immunity in their myeloid counterparts, to examine whether trained immunity can also be induced in human γδ T cells. Next, I investigated the influence of mevalonate pathway inhibition on the immune functions of γδ T cells, which may be one of the main causes leading to the limited efficiency in cancer therapy

of expanded γδ T cells. Finally, I applied a flow cytometric-based method to profile the functional and metabolic status of γδ T cells upon inhibition of mevalonate metabolism as well as autoinflammatory and immunodeficient diseases such as chronic granulomatous disease and hyper-IgE syndrome. In the final chapter, I summarize all the findings from my thesis and discuss the future direction of my study.

Part I: **Innate immune memory in γδ T cells**
This part of my thesis aims to review the current knowledge of trained immunity and suggested that the BCG and MMR vaccines induce trained immunity in γδ T cells. The summary can be found in each chapter (**Chapter 2**, **Chapter 3** and **Chapter 4**) separately. The chapters are accompanied by a reprint publication in the Appendix.

Part II: **Effect of modulating mevalonate metabolism on the immune function of γδ T cells**
This part of the thesis aims to decipher the role of mevalonate metabolism in the immune function of Vδ2 T cells. Mevalonate metabolism is an important metabolic pathway that provides thousands of initial building blocks for many downstream biological processes such as cholesterol synthesis, N-glycosylation and prenylation of proteins (86, 87). Interestingly, an intermediate metabolite of this pathway, IPP, is a potent activator of Vδ2 T cells (29–31). Taking advantage of the drug zoledronate, which inhibits the mevalonate pathway and leads to the accumulation of IPP, Vδ2 T cells can be efficiently expanded in PBMC cultures *in vitro* (91, 92). However, inhibition of the mevalonate pathway by zoledronate may also pose a side effect on the immune function of Vδ2 T cells. Indeed, many clinical trials adopting zoledronate-expanded Vδ2 T cells show limited efficiency in cancer therapies (22, 93). In **Chapter 5**, we combine immune functional assays, next-generation sequencing techniques, and kinase activity assays to investigate the consequences of mevalonate metabolism inhibition on the immune functions of Vδ2 T cells. In this study, we showed that TNF and IFN-γ production by Vδ2 T cells were compromised upon mevalonate inhibition by zoledronate, fluvastatin and 6-FM *in vitro*. A similar phenotype was observed in the hypercholesteremia patients who underwent 3-month statin treatment and in the hyper-IgD syndrome patients who suffer from mevalonate kinase deficiency. These observations indicated that the mevalonate pathway is important for cytokine production by Vδ2 T cells. We further showed that zoledronate and fluvastatin treatment *in vitro* reduced the production of the degranulation marker CD107a and the cytotoxic molecule granzyme B. Consistently, patients who participated in statin therapy also displayed a diminished proportion of granzyme B- and perforin-producing Vδ2 T cells. Our *in vitro* cytotoxic assay further demonstrated that the cytotoxic function of zoledronate- and fluvastatin-treated Vδ2 T cells is decreased.

We then applied RNAseq and ATACseq to investigate the transcriptomic and epigenetic changes in Vδ2 T cells upon mevalonate pathway inhibition. We found that IPP and zoledronate induced the most changes to the transcriptome of Vδ2 T cells, where genes regulating cell cycle, proliferation and replication as well as immune effector processes, cell adhesion and adaptive immune response, were highly upregulated. Fluvastatin caused the downregulation of transcripts largely related to metabolic processes and the upregulation of genes related to negative regulation of immune effector processes, including leukocyte-mediated immunity and cytotoxicity. All in all, the RNA-seq data showed the modulatory effect of mevalonate pathway inhibitors on the transcriptome of Vδ2 T cells. On the other hand, the ATAC-seq analysis showed that the chromatin accessibility of the differentially expressed transcripts from RNA-seq, including *TNF* and *IFNG* loci, was not affected. Exhaustion might

be one of the reasons why we observed an impairment of Vδ2 T cell function in long-term cultures. However, the exhaustion markers: TIM3 and LAG3 were significantly upregulated only on 6-fluoromevalonate-treated Vδ2 T cells and none of the ligands were upregulated in our cultures. We therefore concluded that exhaustion may not be the main cause of the impaired cytokine production by Vδ2 T cells. We further investigated whether biological processes downstream of the mevalonate pathway, such as post-translational modifications, affect TNF and IFN-γ production by Vδ2 T cells. By applying different inhibitors downstream of the mevalonate pathway, we found that inhibition of protein geranylgeranylation and farnesylation led to a decrease in TNF and IFN-γ production by Vδ2 T cells, indicating that compromised protein prenylation may be the main cause of cytokine arrest in Vδ2 T cells upon mevalonate pathway inhibition. We further observed an accumulation of unprenylated forms of small G proteins such as RAC, RAP1A/B and RAS in the cytosol fraction of inhibitor-treated Vδ2 T cells. Protein prenylation plays an important role in signaling events downstream of TCR (35–37). When small G proteins are prenylated, they can easily anchor to the cellular membranes, where they can interact with various receptors. To investigate whether signaling downstream of small G proteins is affected upon mevalonate pathway inhibition, we applied a global kinase activity assay by PamGene to assess the intracellular signaling in Vδ2 T cells. We observed that fluvastatin-treated Vδ2 T cells presented the highest number of kinases with downregulated activity, while IPP- and zoledronate-treated Vδ2 T cells showed the highest number of kinases with upregulated activity. In IPP- and zoledronate-treated Vδ2 T cells, the kinases with upregulated activity were related to proliferation, differentiation, and cell cycle regulation, consistent with our RNAseq data and previous studies. Whereas activities of serine threonine kinases downstream of small GTPases, such as ASK/MAP3K5 and SEK1/MAP2K4, were downregulated upon zoledronate treatment. Similarly, we also observed a downregulated activity of kinases such as ERK, P38 and JNK in fluvstatin-treated cells, indicating that effector signaling cascades downstream of GTPases were impaired upon mevalonate pathway inhibition. Furthermore, the activity of kinases related to TCR signaling, such as LCK, LTK and MET were also downregulated in fluvastatin-treated Vδ2 T cells. This indicated that inhibition of the mevalonate pathway resulted in dysregulation of signaling events in Vδ2 T cells. Although we observed that comprised protein prenylation was the main cause of our phenotype, the effects of different mevalonate pathway inhibitors were not coherent, pointing to a certain level of inhibitor specificity. Altogether, our results show that mevalonate metabolism is essential for stabilizing the intracellular signaling of Vδ2 T cells and ensuring the proper immune function of these cells.

The mevalonate pathway also provides the metabolites for ubiquinone production, an important electron carrier for the respiratory chain at the mitochondria (38,39). In **Chapter 6**, we aim to investigate whether the energy status of Vδ2 T cells is altered upon mevalonate pathway inhibition. To examine the metabolic profile of Vδ2 T cells, we applied the newly developed SCENITH technique (40). We observed that fluvastatin and 6-fluoromevalonate treatment altered the energy metabolism of Vδ2 T cells, while zoledronate treatment did not. Particularly, the glucose dependence of Vδ2 T cells decreased upon fluvastatin and 6-fluoromevalonate treatment, while the fatty acid and amino acid oxidation capacities were higher. Furthermore, 6-fluoromevalonate decreased the mitochondrial dependence but increased the glycolytic capacity of Vδ2 T cells. Altogether, we did not observe a unanimous change in metabolism profile upon all three inhibitor treatments. Our result indicated that ubiquinone production, and therefore mitochondrial function, in Vδ2 T cells was only selectively hindered by certain mevalonate pathway inhibitors. Such a change in metabolic profile induced by these inhibitors might be a cause of the compromised immune function of Vδ2 T cells.

Applying the same technique, we investigated the energy metabolism of Vδ2 T cells in autoinflammatory and immunodeficient diseases, namely CGD and HIES. CGD patients suffer from mutations in NADPH oxidase complexes, which therefore lead to a decrease of ROS production (41). The reduced ROS production in phagocytes renders these cells ineffective in clearing pathogens and apoptotic cells. As a result, CGD patients are prone to severe recurrent infections and unexplained inflammation (41). Previous studies showed that γδ T cells are also affected in CGD patients (Appendix) (42–44). To our knowledge, whether Vδ2 T cells play a role in CGD progression is unclear. This study aimed to determine the metabolic and functional profiles of Vδ2 T cells in CGD patients. We found that the Vδ2 T cell percentage decreased in CGD patients, along with their capacity to produce cytotoxic molecules. The expressions of activation markers: CD38 and CD69, pro-inflammatory cytokines: TNF and IFN-γ and exhaustion markers: PD-1, Tim3 and CTLA4 by Vδ2 T cells were elevated, in line with the inflammatory phenotype of this disease. We further observed that the translation level of Vδ2 T cells in CGD patients was lower than that of healthy donors. The mitochondrial dependency, fatty acid and amino acid oxidation capacities of Vδ2 T cells decreased, while glucose dependency and glycolytic capacity increased in CGD patients compared to healthy donors upon CD3 and CD28 stimulation. The glucose dependency was lower, whereas fatty acid and amino acid oxidation capacities increased in Vδ2 T cells of CGD patients without CD3/CD28 stimulation. Altogether this indicates a distinctive metabolic and immune profile of Vδ2 T cells in CGD patients. The pro-inflammatory phenotype and metabolic shift of Vδ2 T cells might contribute to the hyperinflammatory condition and pathology of CGD. However, due to the limited number of examined patients, these data require further confirmation in a larger cohort.

Another group of immunodeficiencies with autoinflammatory characteristics is hyper-IgE syndrome. Patients who suffer from CARD11 deficiency present common pathologies such as recurrent skin infections and high serum IgE levels (45) and show dysfunctional downstream signaling pathways and glutamine metabolism (46–49). IFN-γ treatment was reported to improve the clinical scores of these patients and reduce severe atopic dermatitis (50). A previous study showed that the clonal expansion of γδ T cells and their IL-4 production contribute to the high IgE production in a patient's serum (51). In this study, we investigate whether the CARD11 deficiency results in dysregulated glutamine metabolism and other metabolic pathways in Vδ2 T cells and whether Vδ2 T cells contribute to the disease's progression. We observed that the glutaminolysis capacity of Vδ2 T cells in *CARD11*-mutated hyper-IgE syndrome patients is lower than that of healthy donors and the IFN-γ treatment enhanced it. However, the IFN-γ treatment resulted in a significant decrease in Vδ2 T cell numbers and their cytokine and cytotoxic molecule production capacity. Therefore, even though IFN-γ therapy was able to rescue the glutaminolysis capacity of Vδ2 T cells, it did not improve cell numbers and their function. Altogether, our result indicated that the immune profile of Vδ2 T cells was affected in *CARD11*-mutated hyper-IgE syndrome patients. IFN-γ treatment induced beneficial effects on patients, but not necessarily due to the improvement of Vδ2 T cell function.

Altogether, the second part of the thesis revealed that mevalonate metabolism ensures the functional immune response of Vδ2 T cells. Furthermore, immunodeficiencies such as CGD and HIES affect Vδ2 T cell function and metabolism.

General Discussion

With increasing technological and methodological advances, we are now able to identify and explore different subsets of γδ T cells and decipher their immunological functions and activation mechanisms. However, much is still yet to be discovered underlying the immune response of γδ T cells. Particularly, the unknown repertoire of activation ligands for the γδ T cell TCRs and its activation mechanism in the context of butyrophilin dimerization. On top of that, many immune functions of these cells were recently discovered, and the nature of their immune memory remains one of the unclear areas sparking great interest for investigation. As γδ T cells have long been considered the bridge between the innate and adaptive arms of immunity, they are so far one of the few cell types that potentially possess the ability to mount both adaptive and innate memory. Previous studies have shown that BCG-vaccinated donors show secondary expansion and effector molecule production by γδ T cells (14,17,18), indicating that γδ T cells may play a role in acquired immunity. Understanding the nature of immunological memory in γδ T cells may be beneficial for the design and development of the next effective vaccines.

In **Chapter 3** and **Chapter 4**, we showed evidence that two live attenuated vaccines: BCG and MMR can induce the innate memory phenotype in γδ T cells. According to past studies, it is well-known that γδ T cells, in particular Vδ2 T cells, mount adaptive immune memory upon re-infection (14,17,18). To our knowledge, we were the first to discover that BCG-exposed γδ T cells can exert an enhanced heterologous immune response upon rechallenge by a non-mycobacterial pathogen like *C. albicans*. Although we observed enhanced cytokine production by BCG- and MMR-trained Vδ2 T cells upon restimulation, the cytotoxic granule production was differentially regulated by both vaccines. While BCG boosted perforin expression, the MMR vaccine did not, indicating that the cytotoxic function of Vδ2 T cells may be selectively affected by the two vaccines. It may be of interest to decipher how these processes are regulated. Our cell-cell interaction analysis also provided a list of specific ligands, showcasing the strong interaction between monocytes and γδ T cells during BCG training. A previous study showed that antigen presentation and co-stimulation mediated by monocytes were responsible for triggering IFN-γ production by γδ T cells (52). This indicates that the memory induction in γδ T cell subsets may involve the participation of monocytes. Interestingly, while MMR can also induce trained immunity phenotypes in γδ T cells, it does not induce trained immunity in monocytes. This indicates that different vaccines may induce different trained immunity programs in distinct cell types. It is therefore critical to further dissect the interaction between both cell types in the context of trained immunity induction in the future.

The molecular mechanisms underlying the trained immunity formation in γδ T cells remain a paramount unanswered question. Vδ2 T cells can be activated by intermediate metabolites, such as HMBPP and IPP, produced by eukaryotic cells and microbes (29–31). These metabolites have also been shown to be responsible for Vδ2 T cell activation by mycobacteria (53) and thus can explain immune memory responses towards other HMBPP- or IPP-producing pathogens. However, MMR vaccine is a viral vaccine. Although previous studies showed that

γδ T cells respond vigorously to acute and chronic viral infections (54–60), little is known regarding which viral ligands can directly activate the Vδ2 T cells. We speculate on three possibilities: 1) Specific ligands in the MMR vaccine may directly stimulate the TLRs or other PRRs of the Vδ2 T cells and induce innate immune memory; 2) The attenuated virus in the MMR vaccine alters the metabolism of certain cell populations, which then leads to upregulation of intermediate metabolites as potent activators of Vδ2 T cells and induces training; 3) Other cell responses to the MMR vaccine may mediate the induction of trained immunity in Vδ2 T cells, but the mediators were not detected in our experiment. To this end, it is important to further investigate the antigen-recognition mechanisms of trained immunity induction in Vδ2 T cells.

In our BCG-vaccination trial, only about half of the donors showed immune memory responses by γδ T cells upon heterologous rechallenge by non-mycobacterial pathogens such as *S. aurues* and *E. coli*. This heterogeneity of the response among individuals is consistent with previous studies that showed a large interindividual variation of the BCG-induced trained immunity phenotype in other innate immune cells (61,62). Furthermore, interindividual differences in the human peripheral γδ T cell population are well documented (63–65). Interestingly, besides interindividual variation, we also found intercellular variation of the trained immunity phenotype in γδ T cells. Based on our scRNAseq data, only two of the five γδ T cell subsets showed an upregulation of a prominent number of trained immunity response genes, comprising less than half of the γδ T cells. The limited induction of trained immunity in γδ T cells by BCG vaccination may be due to the age of the participants in this clinical study. The BCG vaccine poses a weaker effect in adults, as a previous study showed that the γδ T cell memory response is more prominent when individuals receive the vaccine at neonatal age (66). In fact, most of the γδ T cells in the peripheral blood of adults belong to the effector and terminally differentiated memory compartments. These cells may be susceptible to prolonged activation and, therefore, exhaustion. We speculate that memory formation by γδ T cells is more efficient in newborns, whose T cell population is mainly naïve. In addition, different strains of BCG vaccine are known to exert distinct immunomodulatory effects, with BCG-Denmark and BCG-Japan being the most immunogenic (67,68). Our study used BCG-Bulgaria, which was previously shown to exert a weaker immunomodulatory effect when compared to other studies using BCG-Denmark. Therefore, further investigation regarding different strains of BCG in training γδ T cells should be granted. γδ T cells present a promising target for the next-generation vaccines. However, the intraindividual differences in γδ T cell populations and heterogeneous responses to vaccinations within a population urge a personalized approach to vaccine design.

Finally, the observation of *S. aureus* and *C. albicans* as potent activators of γδ T cells also raises interest in using γδ T cells as a new therapeutic tool against multi-drug-resistant candidiasis and *Staphylococcus* infection. Indeed, γδ T cells are a strong candidate for immune therapy against not only infectious diseases but also cancer because they are easily expandable, eligible for allogeneic adoptive transfer, and exert proinflammatory and cytotoxic functions

against cancer and infected cells. However, the limited success of the recent clinical trials using γδ T cells suggested that, to be able to apply γδ T cells as a potential therapeutic tool against cancer and infectious diseases, the efficiency and effectiveness of γδ T cell functions require optimization (22). In **Chapter 5** and **Chapter 6**, we uncovered one of the main reasons leading to such limitations in the therapy when using aminobisphosphonates such as zoledronate to expand γδ T cells *in vitro*. Aminobisphosphonates not only inhibit the mevalonate metabolism of antigen-presenting cells, which make them activate Vδ2 T cells, but also the mevalonate metabolism in γδ T cells in the PBMC culture. Using various pharmacological inhibitors: statin, zoledronate and 6-fluoromevalonate, in *in vitro* cultures, we showed that the deficient mevalonate metabolism resulted in impaired Vδ2 T cell function, such as cytokine and cytotoxic molecule production. This phenomenon was consistent with the *in vivo* study of hypercholesterolemic patients undergoing statin therapy and hyper-IgD syndrome patients with mevalonate kinase deficiency. Among various biological processes downstream of the mevalonate pathway, we found that both TNF and IFN-γ production were sensitive to protein prenylation inhibition. Inhibiting N-glycosylation, another mevalonate metabolism-dependent process, also resulted in a decrease in IFN-γ but not TNF production, consistent with the observation that IFN-γ protein undergoes N-glycosylation (69) while TNF did not. Impaired prenylation processes upon mevalonate pathway inhibition likely result in the dysregulation of intracellular signaling, which relies on prenylated proteins such as small GTPases. Indeed, our western blot analysis demonstrated that unprenylated forms of small G proteins such as RAC, RHOA, RAP1 and RAS were accumulated in the cytosol fraction of Vδ2 T cells upon zoledronate or statin treatment. As this is an indirect way of assessing the defect of protein prenylation and we also observed an increase of unprenylated GTPases in IPP-treated cells, more elaborated techniques, such as assessment of prenylated forms in the cellular membrane, need to be employed but were not available at the time. Our global kinase activity assay indeed demonstrated that the activity of signaling kinases downstream of GTPases was affected in Vδ2 T cells by mevalonate pathway inhibition. Yet, zoledronate and fluvastatin exerted a differential impact on the kinome. Such as zoledronate reduced activities of MAPK kinases downstream GTPases: MAP3K5 and MAP3K6, while fluvastatin reduced activities of JNK1,2,3, P38δ, and EKR1,2 kinases downstream of small GTPases. We did not find any kinases downstream of small GTPase whose activities were mutually affected by zoledronate- and fluvastatin; therefore, these inhibitors likely affect the signaling in Vδ2 T cells at a specific and distinctive level. Consistently, our next-generation sequencing analysis showed that both IPP and mevalonate pathway inhibitors induced differential changes in the transcriptome of Vδ2 T cells.

The transcriptome analysis revealed that genes related to cell division and glucose metabolism were downregulated in Vδ2 T cells from fluvastatin-treated PBMC cultures. This is in line with the observation that Vδ2 T cells failed to proliferate and with the SCENITH analysis, which revealed a huge decrease in glucose dependence of Vδ2 T cells upon fluvastatin treatment. In the future, it may be of interest to further investigate whether such a decrease in glucose metabolism is due to the direct effect of mevalonate pathway inhibition or an off-target effect,

which consequently leads to compromised immune function of Vδ2 T cells. Besides, according to the kinome analysis, the activities of many kinases related to TCR activation and proliferation, such as Src, Lck, TYRO3 and MER, were downregulated in fluvastatin-treated Vδ2 T cells. We also observed increased cell death upon fluvastatin treatment, which could be rescued by supplementing mevalonate to the cultures. This indicates that statins affect the proliferation and viability of cells, most likely by inhibiting the mevalonate metabolism.

On the other hand, genes related to cell cycle regulation, proliferation and DNA replication were highly up-regulated in IPP- and zoledronate-treated Vδ2 T cells, which was consistent with the observation that Vδ2 T cells rapidly proliferate upon these treatments. Interestingly, we observed an up-regulated transcript expression of TNF in zoledronate-treated Vδ2 T cells, contradicting what we observed in the functional assay, where TNF production by Vδ2 T cells decreased. There may be two possible explanations for such a discrepancy: 1) RNA-seq analysis was performed on unstimulated Vδ2 T cells, while immune functional assays were performed on Vδ2 T cells that were stimulated by CD3/CD28 for 4 hours; 2) The decrease in TNF production by Vδ2 T cells was not directly caused by the change in its transcript expression but by other post-transcriptional processes. Without further evidence, we do not exclude the possibility that other differentially expressed genes may have indirect effects on the immune function of Vδ2 T cells. Further investigation is warranted to investigate these possible effects. Early studies showed that statins were associated with histone acetylation modifications and DNA methylation in blood cells (70–72). It was also shown that the effector functions of Vδ2 T cells were repressed by deacetylase inhibitors (73). However, our ATAC-seq analysis showed no significant alterations in chromatin accessibility at the loci of *TNF* and *IFNG*. Investigating histone modifications and DNA methylation may provide a well-rounded aspect of epigenetic changes in Vδ2 T cells upon mevalonate metabolism inhibition in the future.

Although the exhaustion of immune cells is a leading cause of the failure of cancer control (74–77), our data suggest that the exhaustion of Vδ2 T cells over a long-term culture might not occur. Although some check-point receptors were upregulated upon mevalonate metabolism inhibition at the transcript and protein levels, their corresponding suppressive ligands were not upregulated in the cultures. Indeed, a previous study showed that γδ T cells exhibit an exhaustion phenotype with higher PD-1 expression after antigenic stimulation (78,79). However, the inhibitory effect requires receptor and ligand interaction, which was supported by the observation that PD-L1+ Daudi cells reduced IFN-γ production and cytotoxicity of γδ T cells to a greater extent than PD-L1- Daudi cells (78). As a result, we concluded that T cell exhaustion may not be the main driving cause of the compromised Vδ2 T cell function in our cultures. Yet, to further confirm such speculation, it may be of interest to apply check-point inhibitors to our culture to confirm the effect on cytokine production of Vδ2 T cells (77).

Our global kinase activity assay indeed demonstrated that the activity of signaling kinases downstream of GTPases, such as MAPK kinases: MAP3K5 and MAP3K6, was downregulated in zoledronate-treated Vδ2 T cells. These signaling cascades are essential to activate JNK and

P38 kinase signaling, which are known to be involved in T cell cytokine production and proliferation in mice (80). These findings indicate that the signaling pathways of JUN and P38 might be compromised in Vδ2 T cells when exposed to zoledronate treatment, which aligns with our Western Blot result that the unprenylated forms of RAC and RHOA increased, respectively. Similarly, kinase activities downstream of small GTPases such as JNK1,2,3, P38δ, and EKR1,2 were reduced in Vδ2 T cells upon fluvastatin treatment. Besides, the activities of many kinases related to TCR activation and proliferation were also downregulated in fluvastatin-treated Vδ2 T cells.

Interestingly, a phosphosite of the BTLN protein that is known to control lymphocyte proliferation and cytokine production, was commonly regulated in both zoledronate- and fluvastatin-treated Vδ2 T cells. It will be of interest to examine whether BTLN plays a crucial role in the immune function of Vδ2 T cells. Finally, we also demonstrated that the cytotoxic properties of Vδ2 T cells also diminished upon mevalonate pathway inhibition, indicating that mevalonate metabolism not only governs cytokine production, but also the cytotoxic ability of Vδ2 T cells. Taken together, our study showed that mevalonate metabolism is important for the proper effector functions of Vδ2 T cells. In future studies, several areas may be worth further investigation. For example, it is of interest to investigate whether mevalonate inhibitors affect the expression levels of BTN2A1 and BTN3A1 molecules in our PBMC cultures as Vδ2 T cells require recognition of BTN molecules for activation. Since Vδ2 T cells also show other immune functions such as professional antigen-presenting properties and phagocytosis, it is necessary to determine whether these functions are also driven by the mevalonate metabolism in the cells. Our study showed that effector molecule production and proliferation of Vδ2 T cells were uncoupled, which is consistent with the study by Ryan et al. (81). In the future, it may be of interest to investigate the regulatory mechanisms that mediate these differential functions in Vδ2 T cells.

In our follow-up study in **Chapter 6**, we speculated that inhibition of mevalonate metabolism may affect the energy usage in Vδ2 T cells. It is known that energy is not only consumed to support rapid proliferation of Vδ2 T cells during expansion but also to support effector molecule production upon restimulation (82–84). The decrease in effector molecule production by Vδ2 T cells in **Chapter 5** may be due to the exhaustion of energy after long-term cell proliferation. In **Chapter 6**, we evaluated the metabolic profile of Vδ2 T cells upon mevalonate pathway inhibition and identified the changes in their energetic state. First, we found that mevalonate inhibitors, particularly fluvastatin and 6-fluoromevalonate, induced lower mitochondrial dependence of Vδ2 T cells but higher fatty acid and amino acid oxidation capacities, indicating that the energy dependency of Vδ2 T cells is altered. A previous study showed that activation of T cells as well as IFN-γ production by mouse $\gamma\delta$ T cells highly depend on energy derived from aerobic glycolysis (85–87). Inconsistently, 6-fluoromevalonate-treated Vδ2 T cells, which showed lower IFN-γ production, have a higher glycolytic capacity than other conditions. This indicates that the decrease in cytokine production by Vδ2 T cells may not be entirely due to the change in energy dependency but, instead driven by other important

factors. Different mevalonate pathway inhibitors may regulate the metabolic profile of Vδ2 T cells distinctively. Further investigation is needed to explore the balance of energy expenditure between expansion capacity and effector function of Vδ2 T cells in order to create a more functionally balanced tool for cancer and infection treatment.

We also explored the metabolic profile of Vδ2 T cells in two immunodeficiencies with autoinflammatory characteristics: CGD and HIES. A recent study demonstrated that the decrease of ROS, which is found in CGD patients due to the defect in NADPH-oxidase complexes (88), also led to blockage of tryptophan metabolism in CGD mice and resulted in dysregulated activation of γδ T cells and a high production of the proinflammatory cytokine IL-17 (43). Another study showed that a HIES patient featured a clonal expansion of γδ T cells and their elevated IL-4 production, contributing to the high IgE levels in serum (51). These studies indicate that γδ T cells may play a role in the pathology of both diseases.

First, we found diminished mitochondrial dependency, fatty acid and amino acid oxidation capacities of Vδ2 T cells in CGD patients. It is estimated that 45% to 90% of cellular ROS is generated by mitochondria. (89). A decrease in mitochondrial dependency may reflect the dysfunction of mitochondria in generating ROS in these patients. The elevation of activation markers and pro-inflammatory cytokines measured in Vδ2 T cells is in line with the hyper-inflammatory state of these patients. Further investigation is needed to examine whether the proinflammatory phenotype of Vδ2 T cells is one of the causes of the disease progression in these patients. In our study on hyper-IgE syndrome patients, we found that the glutaminolysis capacity of Vδ2 T cells decreased in patients compared with healthy donors, while other metabolic dependencies were not affected. This may be the first evidence of defective glutamine metabolism in hyper-IgE patients and can act as a biomarker for the disease's diagnosis. Previous studies suggested that IFN-γ treatment had a positive effect on patient clinical scores (50). We showed that the glutaminolysis capacity of Vδ2 T cells in patients was restored to a level similar to that of healthy donors after IFN-γ treatment, showing that IFN-γ treatment may improve the glutamine metabolism of Vδ2 T cells. Further studies are required to determine whether the rescue of glutamine metabolism in Vδ2 T cells leads to this positive effect and the reduction of IgE in serum. However, immunoprofiling showed that there was no difference in TNF and IFN-γ production but only in cytotoxic granule production by Vδ2 T cells between patients and healthy donors. Interestingly, TNF, granzyme B and perforin production by Vδ2 T cells decreased further after IFN-γ treatment. More studies are required to determine whether Vδ2 T cells contribute to the disease pathology and to the clinical improvement of the patients under IFN-γ treatment. Our data also presents a large inter-individual variation among donors. Therefore, more donors may be required to validate our finding, and further investigation is needed to determine whether Vδ2 T cells play a significant role in hyper-IgE syndrome.

Conclusion

In my thesis, I studied the biology of unconventional γδ T cells. I showed that BCG and MMR vaccines can induce trained immunity in human γδ T cells. This is the first demonstration that γδ T cells can mount innate immune memory. Further investigation is required to reveal the mechanistic nature of the trained immunity induction in γδ T cells and their direct contribution to protection against heterologous infections in order to inform the design of more effective and efficient vaccines in the future. My thesis also emphasizes the importance of mevalonate metabolism for the proper functioning of Vδ2 T cells. Particularly, I showed that bisphosphonates, the widely applied agent for *in vitro* expansion of γδ T cell, and statins, the widely prescribed cholesterol-lowering drugs, modulate the immune functions and alter the transcriptomic and metabolic profiles of human γδ T cells. This study showcases the adverse effects of these drugs on γδ T cells, which should be taken into consideration while treating patients. Our finding also provides a possible explanation for the limited effectiveness and efficiency of current γδ T cell-based therapy. Furthermore, I showed that γδ T cells can also play a role in CGD and hyper-IgE syndromes, the findings of which urge confirmation in functional assays in a larger cohort study. Hopefully, my thesis can act as additional knowledge to facilitate the development of a better γδ T cell-based therapy for cancer and infectious diseases in the future.

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Chapter 8

Appendix

Reprinted Publications

Innate (learned) memory

Check for updates

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With the growing body of evidence, it is now clear that not only adaptive immune cells but also innate immune cells can mount a more rapid and potent nonspecific immune response to subsequent exposures. This process is known as trained immunity or innate (learned) immune memory. This review discusses the different immune and nonimmune cell types of the central and peripheral immune systems that can develop trained immunity. This review highlights the intracellular signaling and metabolic and epigenetic mechanisms underlying the formation of innate immune memory. Finally, this review explores the health implications together with the potential therapeutic interventions harnessing trained immunity. (J Allergy Clin Immunol 2023;152:551-66.)

The survival of an organism depends on its adaptation to the surrounding environment, with infections representing one of the most important factors that can impact survival. The development of mechanisms to store information and to use it subsequently to mount a more effective adaptive response is crucial, with an efficient defense against infections relying on the ability of the immune system to mount memory responses. Immune responses have been traditionally divided into antigen-independent innate immunity, which is rapidly activated in minutes and hours and provides broad protection against microorganisms at the beginning of an infection, and antigen-dependent adaptive immune responses, which are triggered later in days and weeks in case the infection has not been eliminated.[1] Adaptive immune responses are mediated by specialized immune cells that recognize specific antigens from the invading pathogens and that could subsequently build specific memory against that infection through memory B and T cells.[2] However, recent studies have shown that innate immune cells can also develop

Abbreviations used	
ac:	Acetylation
AKT:	Protein kinase B
AMs:	Alveolar macrophages
BECs:	Bronchial epithelial cells
COVID-19:	Coronavirus infectious disease 2019
H3:	Histone 3
HDL:	High-density lipoprotein
HSCs:	Hematopoietic stem cells
ILCs:	Innate lymphoid cells
ILC2s:	Group 2 innate lymphoid cells
K9:	Lysine 9
IPLs:	Immune priming long noncoding RNAs
me:	Methylation
mTOR:	Mammalian target of rapamycin
NK:	Natural killer
oxLDL:	Oxidized low-density lipoprotein
OXPHOS:	Oxidative phosphorylation
PI3K:	Phosphatidylinositol 3-kinase
PRRs:	Pattern recognition receptors
ROS:	Reactive oxygen species
Syk:	Spleen tyrosine kinase
TADs:	Topologically associating domains
TCA:	Tricarboxylic acid
TCR:	T-cell receptor
TLR:	Toll-like receptor

immune memory mechanisms (also termed "trained immunity") through antigen-independent epigenetic reprogramming.

For the last decade, a large body of work has broadened our understanding of the innate immune memory/trained immunity, which is defined as an enhanced but nonspecific immune response to reinfection (Table I).[3-29] Trained immunity is classically characterized by the increased production of particular proinflammatory cytokines such as TNF-α, IL-6, and IL-1β,[3] an enhanced antimicrobial activity[4] accompanied by the increased reactive oxygen species (ROS) production,[30,31] and antitumor activity[9] on a secondary challenge. This more effective innate memory response, which we refer to as trained immunity phenotype, is achieved by alterations in metabolic and epigenetic mechanisms induced on the initial stimulation (Fig 1).[3,5,6,25] Trained immunity is mounted by the immune cells that mediate the "first line of defense" and is distinct from the adaptive immune memory responses through several important characteristics: (1) it is antigen-independent and provides heterologous protection; (2) it is mediated by epigenetic reprogramming of precursor cells; (3) it is induced in different cell types and receptors involved in ligand recognition; and (4) it has a shorter duration of the memory phenotype. The receptors of T and B cells undergo a complex series of DNA rearrangements that give rise to countless unique

TABLE I. Trained immunity features of immune and nonimmune cells

Cell type	Training agent	Altered immune function	Molecular mechanisms	References
Myeloid lineage				
Monocytes and macrophages	ß-glucan	Increased TNF-α and IL-6 production	Increased H3K4me and H3K27ac marks Increased metabolic enzyme production	Saeed et al[3]
		Increased proinflammatory cytokine production IL-1–dependent protection	Altered histone modifications Increased myelopoiesis	Moorlag et al[4]
		Enhanced TNF-α production	Increased dectin-1/AKT/ HIF1A–dependent aerobic glycolysis	Cheng et al[5]
			Altered glycolysis, glutaminolysis, and cholesterol synthesis pathways Fumarate induced epigenetic changes	Arts et al[6]
Monocytes	BCG	Upregulated IL-1ß production	Genome-wide epigenetic reprograming	Arts et al[7]
Monocytes	oxLDL	Pro-inflammatory phenotype	Increased glycolysis Histone methylation remodeling	Bekkering et al[8]
Neutrophils	ß-glucan	Antitumor phenotype	Type-1 interferon signaling dependent Transcriptomic and epigenetic rewiring of granulopoiesis	Kalafati et al[9]
Neutrophils	BCG	Increased nonspecific antimicrobial capacity (eg, ROS production, phagocytosis) Increased expression of activation markers Proinflammatory phenotype	Increased H3K4me3	Moorlag et al[10]
Dendritic cells	*C neoformans*	Proinflammatory phenotype Higher levels of IL-2, IFN-γ, IL-4, and TNF-α production	Histone mark rearrangements	Hole et al[11]
Lymphoid lineage				
NK cells	BCG; *C albicans*	Increased heterologous protection against infection		Kleinnijenhuis et al[12]
	IL-12, IL-15, and IL-18	Increased proliferation Increased IFN-γ production and cytotoxicity		Uppendahl et al[13] Zieleniewska et al[14] Duivenvoorden et al[15] Romee et al[16]
		Increased cytotoxicity Increased TNF-α production	Increased glycolysis and OXPHOS	Uppendahl et al[13] Kedia-Mehta et al[17]
ILCs	Fungal protease allergen, papain, IL-22	Increased expansion Increased IL-5 and IL-13 production		Martinez-Gonzalez et al[18]
	IL-33	Increased allergic response	Epigenetic reprogramming	Fu et al[19]
	C rodentium	Mucosal defense with increased expansion Increased IL-22 production	Metabolic rewiring	Serafini et al[20]
γδ−T cells	PHA, mycobacteria	Increased lysis toward herpes simplex virus– and vaccinia-infected cells		Bukowski et al[21]
	BCG	Secondary expansion of γδ-T cells		Suliman et al[22] Shen et al[23]
Nonimmune cells				
Mesenchymal stem cell	LPS	Increased inducible nitric oxide synthase expression		Domínguez-Andrés et al[24]
Long-term hematopoietic stem cells	LPS	Enhanced myeloid differentiation Increased response of myeloid cells to secondary stimulation	Increased chromatin accessibility of myeloid enhancers Decreased chromatin accessibility of erythroid enhancers	de Laval et al[25]
Epithelial cell	Imiquimod	Escalated wound healing	Changes in chromatin accessibility at genes related to inflammation and hyperproliferation Epigenetic remodeling	Naik et al[26]
	Pseudomonas aeruginosa	Increased IL-8 production on LPS restimulation		Bigot et al[27]
Fibroblast	Interferon	Enhanced transcription on interferon-induced genes		Kamada et al[28]
	TNF	Increased proinflammatory signaling components	Epigenetic reprogramming	Gangishetti et al[29]
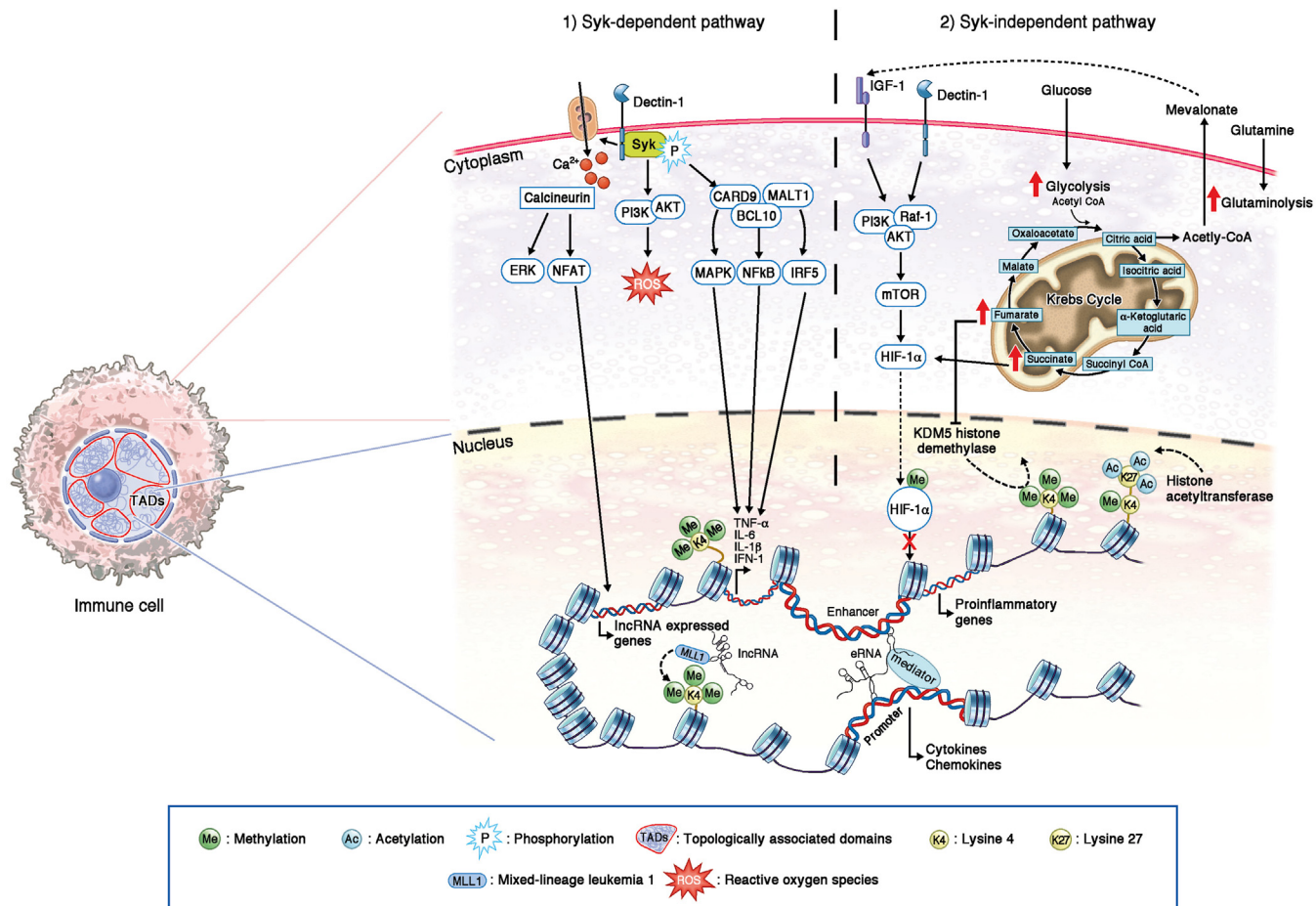
FIG 1. Molecular basis of trained immunity formation via dectin-1 and IGF1. Dectin-1 receptor engagement triggers 2 signaling pathways. **(1)** Syk kinase activation leads to an influx of calcium into the cell. The increase in intracellular calcium activates the nuclear factor of activated T cells (*NFAT*), which translocates to the nucleus, where it upregulates the long noncoding RNAs (*lncRNAs*) including enhancer RNAs (*eRNAs*) and guides histone-modifying enzymes to gene promoters. eRNAs further contribute to the DNA looping to bring distal DNA sites nearby to enhance the transcription of certain cytokines and chemokines within a TAD. Concomitantly, Syk kinase activates PI3K and the downstream AKT signaling pathway to support ROS production and CARD9 to promote proinflammatory gene expression. **(2)** Syk-independent pathway is initiated by the Raf-1 kinase and consequently triggers a cascade of events involving PI3K, AKT, and mTOR. This ultimately leads to the activation of HIF1A, which blocks the conversion of pyruvate to acetyl-coenzyme A (*acetyl-CoA*) and increases the expression of lactate dehydrogenase A. It induces glycolysis, glutaminolysis, and the accumulation of intermediate metabolites, which activate or inhibit epigenetic enzymes such as lysine-specific demethylase (KDM5) or histone acetyltransferases. This leads to an increase of transcriptional activation-associated marks (H3K4me1, H3K4me3, and H3K27ac) at the regulatory elements of proinflammatory genes such as *TNFA* and *IL6. ERK*, Extracellular signal-regulated kinase; *MAPK*, mitogen-activated protein kinases; *MLL-1,* mixed-lineage leukemia 1; *NF-κB*, nuclear factor-κB.

receptors needed for the recognition of numerous potential pathogens one can be exposed to throughout life. The adaptive immune memory responses rely on these highly specific receptors that allow more efficient reactions to the same reoccurring environmental threats. By contrast, trained immunity mainly relies on pattern recognition receptors (PRRs) that recognize evolutionarily conserved structures in pathogens, suggesting a limited repertoire to mount memory responses. Although originally described in innate immune cells of the myeloid lineage, trained immunity is now also acknowledged in some innate lymphoid cells (ILCs) and nonimmune cells.

Pioneering observations, suggesting immune memory mechanisms other than solely adaptive immune memory ones, were first documented in plants and invertebrates.[32] These lower organisms do not harbor an adaptive immune system, yet they can adapt their immune responses to a primary challenge.[33] More recent observations coming from large human cohort studies of BCG-vaccinated individuals and from experimental mouse models suggested that vertebrates also have the capability of enhanced immune responses to reinfection with a heterologous pathogen.[7,34-36] The same nonspecific benefits of live vaccines have been described for smallpox,[37,38] measles,[39] and oral polio vaccines.[40,41] The heterologous effects of the vaccines could not be explained by T-cell–dependent cross-reactivity, and a study performed on athymic mice showed increased immune response on second *Candida albicans* infection, implying a T-cell–independent mechanism of

the improved immunity.[42] Moreover, mice with dysfunctional T and B cells are protected from the *C albicans* reinfection where reprogrammed monocytes and an elevated cytokine production play a central role in this protective mechanism.[43,44] Furthermore, an increased expression of PRRs, such as Toll-like receptors (TLRs) and dectin-1, on infection demonstrates the adaptation capability of innate immune cells that can augment their affinity to pathogens during possible reinfection.[45,46] Taken together, these findings convey the concept that cells belonging to the myeloid lineage also develop memory mechanisms that shape immunological responses in light of the previous pathogen encounters.

TRAINED IMMUNITY IN MYELOID CELLS

Trained immunity has been first described in monocytes, macrophages, and natural killer (NK) cells.[3,12,44,47,48] The primary stimulation of these cells with certain pathogens amplifies the secondary immune response to the same or different stimuli via metabolic and epigenetic reprogramming.[44] Recently, other immune cells, including neutrophils,[10,49] eosinophils,[50] and dendritic cells[51-53] have been shown to mount memory responses. On BCG vaccination, not only monocyte and NK cell recall responses were enhanced, but also human neutrophils acquired a long-term immunophenotypic activation and an increased antimicrobial capacity such as ROS production against various pathogens.[10]

These enhanced secondary responses were accompanied by changes in the histone modification landscape in both monocytes and neutrophils.[10] Histone modifications have been suggested to underlie also the fungal vaccine-induced trained immunity phenotype in dendritic cells.[11] The increased interferon response gene expression and the enhanced *Cryptococcus*-specific cytokine recall responses were abrogated by the specific histone modification inhibitors.[11] Enhanced secondary immune responses of eosinophils were reported in house dust mite–sensitized mice.[50] The improved protection against subsequent helminth infection in these mice was a result of an interplay of various immune components including T_H2 cells, macrophages, group 2 ILCs (ILC2s), IL-33, IL-4, IL-13, and an increase in eosinophil activity, highlighting the fact that trained immunity acts at the systemic level. In LPS-primed mast cells, an elevated TNF response was observed on fungal infection such as live *C albicans* as the secondary challenge.[54]

Apart from circulating myeloid cells, tissue-resident immune cells have the ability to enhance secondary responses. Microglia, which are tissue-resident macrophages in the central nervous system, develop trained immunity after being systemically challenged with ß-glucan as evidenced by the enhanced TNF-α, IL-6, and CCL3 expression, morphological alterations, and an epigenetic rearrangement.[55,56] In alveolar macrophages, which are the lung-resident macrophages, trained immunity is established on the intranasal LPS exposure followed by a bacterial infection. It results in an increased immune response and boosted bacterial clearance as a result of the epigenetic modifications and metabolic rewiring.[57] Macrophages are also involved in the type 2 immune responses induced by high levels of IL-4 and IL-13, which support the resolution of inflammation and ensure a proper healing of the tissue but when dysregulated, might lead to allergic responses and asthma. The role of trained immunity in the context of type 2 immunity is still largely unknown, yet in recent reports,

transcriptional, epigenetic, and metabolic changes in macrophages,[58,59] ILC2s,[18,19] and epithelial cells[60] have been demonstrated in the presence of allergic and nonallergic asthma as well as other allergy-dependent inflammatory reactions. Recently, Lechner et al suggested that monocytes and macrophages develop trained immunity phenotype on allergen sensitization.[58] As such, macrophages from the house dust mite allergens–sensitized mice and allergic individuals express greatly higher levels of CCL17, IL-6, and cysteinyl leukotrienes on reexposure, implying the memory responses resulted from metabolic reprogramming and histone demethylation.[58] Trained immunity might also contribute to allergic responses in children. Recent studies have demonstrated that cord blood and peripheral blood monocytes from food-allergic infants display increased production of proinflammatory cytokines and chemokines such as TNF, IL-1β, IL-6, IL-8, and innate cell recruiting factors such as macrophage inflammatory protein 1α on endotoxin challenge.[61,62] The hyperresponsiveness of myeloid cells was accompanied by a dysregulated T_H cell activity, suggesting a likely interplay between trained innate immune cells and the adaptive immune system.

Beyond pathogenic encounters and allergen reactions, endogenous ligands and some sterile conditions can also induce trained immunity. For example, the Western-type diet induces systemic inflammation and trained immunity phenotype in circulating myeloid cells and their precursors through transcriptomic and epigenetic reprogramming.[63] Furthermore, endogenous ligands such as oxidized low-density lipoprotein (oxLDL),[64] uric acid,[65] heme,[66] vimentin, and HMGB1[67] have been well documented to promote trained immunity. Recently, it has been revealed that urate- and urate crystal–dependent inflammatory responses share signaling pathways with trained immunity, inducing the same phenotypic changes and epigenetic rewiring.[65,68]

INNATE IMMUNE MEMORY IN LYMPHOID CELLS

An increasing body of studies shows that innate memory can be acquired by innate-like lymphoid cells such as NK cells, group 1 and 2 ILCs, and γδ T cells.

NK cells

NK cells bridge myeloid and lymphoid lineages due to their expression of innate-like receptors such as NK group 2 C and acquisition of an adaptive-like memory.[69-73] Despite the innate character of NK cell receptors, their memory responses have been mainly shown in the context of the same pathogen: several viruses and bacteria[22,74-77] as well as cytokines.[75,76] Reactivation of the human CMV induces the secondary expansion of NK group 2 C–expressing NK cells and increases their IFN-γ production.[78,79] Vaccination with BCG increases the number of BCG-reactive NK cells in the peripheral blood.[22] Moreover, a unique (FcεRIγ⁻) NK cell population is enriched on the exposure to *Plasmodium falciparum*–infected red blood cells and shows the enhanced killing capacity of infected red blood cells on restimulation.[77] Evidence of memory responses against heterologous stimuli was at last identified in the NK cells of BCG-vaccinated people. The NK cells from these individuals showed traits of the classical trained immunity phenotype on *C albicans* stimulation.[12] Interestingly, cytokine-exposed NK cells also show

enhanced killing capacity against ovarian cancer.[13] Such increased effector activity is accompanied by metabolic shifts including the upregulation of glycolysis and oxidative phosphorylation (OXPHOS).[17] Altogether, these studies indicate the generation of trained immunity in NK cells.

ILCs

ILCs are innate immune cells that carry the characteristics of T cells. Although they resemble T cells in terms of phenotype and function, ILCs do not express somatically rearranged antigen receptors and do not undergo clonal expansion.[80] ILCs can be separated into 3 groups, namely: ILC1s, ILC2s, and ILC3s that resemble T_H1, T_H2, and T_H17 cell subsets, respectively.[81] Recent studies have shown that some ILC subpopulations can retain immunological memory. For example, ILC2s, which play an important role in the type 2 immunity against allergen- and helminth-induced infection, can undergo the secondary expansion and produce higher amounts of IL-5 and IL-13, leading to a more severe allergic inflammation on rechallenge by unrelated allergens.[18] These cells do not sense an allergen directly but through IL-33 produced by damaged epithelium. The lung ILC2s can retain immunological memory after primary exposure to an allergen or IL-33. Epigenetic remodeling and activator of transcription 3 (STAT3)-dependent serine727 phosphorylation constitute the molecular basis of this innate-like memory formation in ILC2s.[19] Furthermore, ILC2s can acquire trained immunity characteristics on helminth infections. *Strongyloides venezuelensis*–exposed ILC2s rapidly expanded and produced IL-5 and IL-13 on reinfection with *Nippostrongylus brasiliensis* in mice.[82]

Deprived of pattern recognition receptors, ILC3s are also indirectly activated by soluble factors such as IL-1β and IL-23 derived from epithelial and hematopoietic sentinel cells. Recently, they have been shown to acquire trained immunity phenotype on *Citrobacter rodentium* exposure.[20] ILC3s maintained an activated state for months after the initial encounter with the pathogen and when rechalled they expanded and upregulated IL-22 production to a higher extent than naïve cells, ensuring long-term defense. The improved protection provided by the *C rodentium*–trained ILC3s is not specific to the pathogen. Interestingly, cytokines alone were not able to induce memory phenotype in ILC3s, suggesting that memory formation in these cells requires other tissue-derived factors beyond IL-1β and IL-23.[20]

γδ-T cells

The γδ-T cells are unconventional T cells that express innate-like receptors, such as NK group 2 D and TLRs,[83-85] in addition to the T-cell receptor (TCR), which is composed of γ and δ chains. They can be activated by unspecific pathogen- or stress-related signals resembling the characteristics of the innate immune cells. The memory responses of γδ-T cells have been reported in humans and mice, yet they were mainly addressed in the pathogen-specific context.[86,87] For example, the antigen-specific recall responses of Vδ2 T cells, the most prevalent γδ–T cell subset in human peripheral blood, on BCG vaccination or *Mycobacterium tuberculosis* infection were shown in humans and nonhuman primates.[22,23] The TCR of Vδ2 T cells is composed of Vγ9 and Vδ2 chains and recognizes unspecific nonpeptide pyrophosphate molecules produced by many microbes.[88-91] The innate character of Vδ2 TCR implies heterologous immune memory responses of these cells. Indeed, an early study reported that Vδ2 T cells previously stimulated with PHA or mycobacteria showed an increased lysing ability of herpes simplex virus– and vaccinia-infected cells.[21] Further studies are required to determine the immunological memory characteristics of these innate-like lymphoid cells.

MEMORY PROPERTIES OF NONIMMUNE CELLS

A growing body of evidence supports the induction of trained immunity not only in immune cells but also in other cell types such as mesenchymal stem cells, epithelial cells, or fibroblasts.[26,28,29,92]

Mesenchymal stem cells

LPS-trained mesenchymal stem cells, when restimulated with IFN-γ and TNF-α showed enhanced expression of inducible nitric oxide synthase and anti-inflammatory markers: arginase 1 and CD206. This functional program was accompanied by the decreased expression of the proinflammatory markers: TNF-α, IL-1β, inducible nitric oxide synthase, and IL-6 in the mesenchymal stem cells–macrophages cocultures. The LPS-induced immunomodulatory effect is driven by the activation of c-Jun N-terminal kinase pathway and histone methylation.[92]

Epithelial cells

Epithelial stem cells acquire memory phenotype on exposure to a TLR7 agonist, imiquimod.[26] This allows the escalated wound healing on unrelated secondary challenges such as physical damage. The innate memory phenotype is associated with changes in chromatin accessibility in the epithelial progenitors at genes related to inflammation and hyperproliferation.[26] Innate immune memory can be also acquired by bronchial epithelial cells.[27] Pre-exposure of bronchial epithelial cells to *Pseudomonas aeruginosa* leads to an increased IL-8 production on restimulation with non-related ligands and pathogens such as LPS and live *Aspergillus fumigatus*. The use of agents that affect histone modification status reversed the phenotype, implying epigenetic regulation of this process.[27]

Fibroblasts

Long-term memory has been reported in various types of fibroblasts.[28,29] For example, on exposure to IFN-β embryonic fibroblasts mounted a memory response toward the same mediators. Half of the IFN-induced genes exhibited greater and faster transcription on restimulation. This IFN-induced memory was retained through cell divisions. The improved antiviral protection was attributed to the increased recruitment of transcriptional machinery that was accompanied by the acquisition of activating histone marks.[28] Similarly, fibroblast-like synoviocytes chronically exposed to TNF established inflammatory memory associated with the remodeling of chromatin accessibility that reprogrammed cell signaling pathways.[29]

CENTRAL VERSUS PERIPHERAL TRAINED IMMUNITY

Unlike adaptive memory, which can last for years and decades due to the long survival of adaptive memory cells, the innate memory phenotype has been shown to last up to 1 year in the peripheral innate immune cells, while the heterologous protection of live vaccines has been reported to endure for up to 5 years.[93] Given that the early discovery of trained immunity was made in circulating macrophages and monocytes with a short life span of 1 to 7 days,[94,95] the concept of retaining innate immune memory, which can last for months or years, cannot be explained unless it is induced in progenitor cells in the bone marrow.[96,97] Induction of innate immune memory at the level of bone marrow progenitors was termed "central trained immunity."

Consistent with early reports on trained immunity, BCG and β-glucan were the first agents shown to induce central trained immunity in mice and humans.[4,49,98,99] Intravenous administration of BCG to mice reprograms hematopoietic stem cells (HSCs) in an IFN-γ–dependent manner.[98] The development of innate immune memory, which conferred better protection against a *Myobacterium tuberculosis* infection even 5 months after the vaccination, led to an increased myelopoiesis and was accompanied by the transcriptomic and epigenetic reprogramming of HSCs, multipotent progenitors, and macrophages. Consistently, BCG vaccination induces transcriptional shifts that can be epigenetically imparted from HSCs to peripheral CD14^{+} monocytes in humans.[49] Similarly, the administration of β-glucan in mice led to the expansion of myeloid-biased MPP3 in the bone marrow, which was still detected 28 days postadministration.[99] The β-glucan–enhanced myelopoiesis was accompanied by a specific transcriptome signature related to glycolysis and cholesterol synthesis and contributed to a protection against pulmonary *M tuberculosis* infection and a chemotherapy-induced immunosuppression.[4] This β-glucan–mediated central trained immunity required IL-1β signaling. The adoptive transfer of β-glucan-trained HSCs to native mouse recipients can suppress tumor growth in a ROS-dependent manner.[9] BCG and β-glucan are not the only triggers of trained immunity in HSCs. Exposure to the TLR4 ligand, LPS, also leads to an enhanced myeloid differentiation accompanied by an increased chromatin accessibility at myeloid enhancers and increased transcriptome signature of oxidative metabolism.[25] Mice transplanted with these LPS-trained HSCs exerted increased survival on *P aeruginosa* infection.[25]

Apart from infectious agents, damage-associated molecular patterns can induce central trained immunity. Heme, which is released during infection, has been recently reported to mediate trained immunity in murine HSCs.[66] Heme-induced trained immunity depends on the spleen tyrosine kinase (Syk) signal transduction pathways and is associated with an increased chromatin accessibility at transcription factor binding sites that favor the myeloid cell expansion. The enhanced myelopoiesis is protective against polymicrobial sepsis. Altogether, these studies demonstrate that central trained immunity developed in the immune progenitor cells plays an important role in maintaining the innate immune memory in their progeny and confers a host defense against infection and an anticancer immune surveillance.[66]

Trained immunity can also be retained in peripheral tissue- and organ-resident cells of the lungs, intestine, and skin that frequently encounter immune insults. These cells can self-renew and have a life span ranging from months to years.[100,101] The innate memory developed in the innate immune cells in the tissues is termed "peripheral trained immunity."

Peripheral trained immunity is well described in lung-resident alveolar macrophages (AMs). A respiratory adenoviral infection was shown to induce innate immune memory in AMs in an IFN-γ–dependent manner.[102] The adenovirus-trained AMs protect the lung from *Streptococcus pneumoniae* infection even after 16 weeks.[102] The improved protection was achieved by the increased MHC class II expression, enhanced glycolytic metabolism, chemokine production, and neutrophilia. Moreover, chronic infection with a gammaherpesvirus promoted trained immunity in AMs that protected the host from acquiring the house dust mite–induced asthma.[103] The enhanced proinflammatory phenotype of trained AMs might also have a detrimental role in lung immunity. For example, CD11b^{+} AMs from neonatal mice infected with the pneumonia virus of mice acquired innate immune memory and mediated the allergic asthma phenotype.[104]

Muscularis macrophages, which are present at the ileum of the intestine, also exhibit trained immunity traits. On a *Yersinia pseudotuberculosis* infection, muscularis macrophages acquired innate immune memory that enabled them to protect against a neuronal loss and dysmotility after subsequent challenges with *Salmonella typhimurium*.[105] A duodenal helminth infection can also induce distal neuroprotection by muscularis macrophages in the ileum and colon on a heterologous infection. Helminth-triggered trained immunity relies on eosinophil and T$_H$2 responses.[105] Dermal macrophages develop memory characteristics on local *Staphylococcus aureus* infection and confer faster monocyte recruitment, increased bacterial killing, and improved healing on a secondary infection. The duration of the innate immune memory in dermal macrophages is limited by their life span.[106]

Altogether, these studies highlight the induction of trained immunity in the tissue- and organ-resident immune cells, showing that innate immune memory can be maintained at local sites independently of the bone marrow progenitors.

METABOLIC REPROGRAMMING

Cells depend on several metabolic pathways such as glycolysis, tricarboxylic acid (TCA) cycle, OXPHOS, the pentose phosphate pathway, and fatty acid and amino acid oxidation to sustain their energy usage in adaptation to microenvironmental changes and to mediate appropriate responses.[107] These metabolic pathways can regulate the availability of intermediate metabolites that serve as substrates of epigenetic enzymes, such as histone methyl and acetyltransferases, deacetylases and demethylases, as well as co-activators or corepressors of epigenetic editors, which can alter the epigenetic landscape in a way that promotes innate immune memory formation.[108]

Glycolysis is among the most important metabolic pathways for trained immunity. This process involves the conversion of glucose to pyruvate that is then either imported into the TCA cycle or used for lactate production. The latter process usually occurs in hypoxic conditions. However, it can also occur in normal oxygenation conditions when the immune cells are activated and in urgent need of producing more energy for the inflammatory response. Such reaction is called aerobic glycolysis. The typical characteristic of β-glucan–, BCG-, and

oxLDL-induced trained immunity in monocytes, macrophages, and in progenitor cells is an increased aerobic glycolysis.[5,6,99,109] The increase in aerobic glycolysis in trained-macrophages is accompanied by increased lactate production and the NAD^+/NADH ratio.[110] During training with β-glucan and BCG, the up-regulation of glycolysis drives the activation of phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/ mTOR)/HIF1A pathway, which is important for cytokine production.[9,110] Pharmacological inhibition of glycolysis or the PI3K/AKT/mTOR/HIF1A signaling pathway abrogates the induction of trained immunity.[111]

Glycolysis also produces intermediate products such as glucose-6-phosphate to fuel the pentose phosphate pathway, which is essential for amino acid and nucleotide production. Although pentose phosphate pathway is highly activated in BCG and β-glucan–trained macrophages, inhibition of this pathway did not compromise the induction of trained immunity, indicating that this pathway may not be essential for trained immunity.[6,110]

The TCA cycle and OXPHOS, which take place in mitochondria, are the most efficient pathways for energy production by consuming glycolysis-derived pyruvate, amino acids, and fatty acids. Meanwhile, the shift in the pathway provides intermediate metabolites for biosynthetic pathways through processes called cataplerosis and anaplerosis that replenish the TCA cycle by metabolites to support the induction of trained immunity.[107,112] Concomitantly with increased glycolysis, OXPHOS is suppressed in monocytes trained with a high concentration of β-glucan.[5] Interestingly, training with low concentrations of β-glucan or BCG or with oxLDL not only increases the glycolysis rate but also the OXPHOS potential.[113,114] This dose-dependent mechanism awaits further investigation. Yet, the inhibition of mitochondrial oxidation by oligomycin in β-glucan–trained cells abrogates cytokine production on LPS-restimulation, demonstrating that energy produced by OXPHOS is important for the trained immunity.[6]

PI3K/AKT/mTOR/HIF1A pathway activated by glycolysis can inhibit pyruvate dehydrogenase in the mitochondria of immune cells and therefore the conversion of pyruvate to acetyl-coenzyme A.[115] This results in the enhancement of glutaminolysis during which glutamine is actively metabolized to glutamate as an alternative to pyruvate to support the TCA cycle.[6] Replenishing the TCA cycle via glutaminolysis leads to the accumulation of intermediate metabolites such as succinate and fumarate in trained cells.[6] Pharmacological inhibition of the glutamine transporter prevents trained immunity, indicating that a functional TCA cycle sustained by glutaminolysis is essential for trained immunity induction.[6] Interestingly, accumulated fumarate inhibits the KDM5 histone demethylase that elicits epigenetic remodeling,[116] whereas accumulation of succinate stabilizes HIF1A by preventing it from prolyl hydroxylase degradation.[117] This results in an amplification loop of trained immunity induction.

On the other hand, itaconate, another metabolite of the TCA cycle, has been shown to play a role in immune tolerance, the opposite functional program of trained immunity.[24] Itaconate can induce anti-inflammatory activity in LPS-stimulated macrophages by inhibiting the oxidation of succinate to fumarate and by supporting the activity of the anti-inflammatory transcription factors ATF3[118] and NFE2L2.[119,120] ATF3 acts as negative regulator of nuclear factor-κB seta while NFE2L2 in turn enhances the expression of antioxidant and anti-inflammatory genes. It can also increase the succinate level by the inhibition of succinate

dehydrogenase.[24] Yet, training monocytes with β-glucan can inhibit the expression of itaconate and promote the accumulation of fumarate.[6] Therefore, the wrestling of itaconate and fumarate determines the fate of macrophage functional programs either toward training-induced proinflammatory function or tolerance-induced anti-inflammatory activity.

The citrate produced in the TCA cycle is converted to acetyl-coenzyme A in cytosol, which is used in many biochemical reactions and metabolic processes. Among others, it fuels cholesterol synthesis via the mevalonate pathway, whose intermediate metabolite, mevalonate, promotes trained immunity in monocytes by activating insulin-like growth factor 1 receptor, and the mTOR and glycolysis pathways.[121] Furthermore, cholesterol accumulation has been shown to promote myelopoiesis as a result of trained immunity in HSCs.[99]

Besides, fatty acids synthesized from acetyl-coenzyme A have been recently found to regulate trained immunity by affecting aldosterone secretion.[112,122] Aldosterone enhances cytokine and ROS production by monocytes on restimulation with LPS and tri-palmitoyl-S-glyceryl-cysteine. The aldosterone effect is independent of the activation of glycolysis- or OXPHOS-induced[122] epigenetic changes.[123]

Apart from metabolic reprogramming induced by dectin-1 and β-glucan through PI3K/AKT/mTOR/HIF1A signaling, metabolic rewiring is also mediated by TLR4-STAT3 downstream signaling in microglia and mast cells. STAT3 phosphorylation at serine 727 in the mitochondria of trained ILC2s fuels the methionine cycle.[19] Methionine, as a precursor of S-adenosylmethionine, can act as a methyl donor for histone methylation. Interestingly, methionine consumption increases when monocytes are exposed to β-glucan,[6] suggesting a change in S-adenosylmethionine levels, which supports epigenetic reprogramming. Furthermore, a recent study has shown that BCG vaccination results in changes in circulating metabolites.[124] This study revealed that other, so far little-studied metabolic pathways, such as taurine metabolism, may also have a pronounced impact on BCG-induced trained immunity.

EPIGENETIC MECHANISMS UNDERLYING TRAINED IMMUNITY

Epigenetic enzymes responsible for the deposition, removal, and reading of chemical marks on histone proteins and DNA are affected by the metabolic changes in a cell. During primary stimulation of immune cells, metabolic changes and epigenetic remodeling take place, resulting in altered gene expression and immune response. Among the major layers where epigenetic remodeling occurs are the chemical modifications of histone tails, which are recognized by the components of transcriptional machinery and cause chromatin reorganization to enable or suppress gene expression. For example, repressed genes are often characterized by methylated lysine 9 (K9) and K27 residues on histone 3 (H3). On the contrary, methylation of K4 and K36 residues on the same histone correlates with transcriptionally accessible genes. Furthermore, acetylation of H3K9 and H3K27 promotes transcription.[125] Initial studies revealed that in ß-glucan–trained monocytes certain histone marks such as histone 3 lysine 4 mono-methylation (H3K4me1), H3K4 trimethylation (H3K4me3), and H3K27 acetylation (H3K27ac) are increased at the gene regulatory elements of proinflammatory genes such as *TNFA* and *IL6*, resulting in more accessible DNA for the transcriptional machinery.[44] Even though most of the H3K27ac marks are lost during cell

differentiation processes, H3K4me is maintained, implying the conservation of the open chromatin structure as a consequence of the initial stimulation.[3] Similarly, the analysis performed on BCG-vaccinated individuals demonstrated that the training effect of the vaccine is linked to the enrichment of H3K4me3 and H3K27ac at the promoters of *TNFA*-, *IL6*-, and *TLR4*-encoding genes.[35]

The second layer of epigenetic remodeling is DNA methylation, which is best known to suppress gene expression by, for example, recruiting transcriptional repressors and establishing transcriptionally incompetent chromatin. Although more in-depth analyses are required to decipher the relationship between DNA methylation and trained immunity, a recent study demonstrated that BCG vaccination induces persistent DNA-methylation signatures in circulating monocytes in neonates.[126] Furthermore, human monocytes trained with ß-glucan have elevated concentrations of certain metabolites, which further leads to a decrease in DNA methylation and higher expression of proinflammatory genes.[127]

The third layer that regulates epigenetic changes and trained immunity formation consists of long noncoding RNAs. This specific type of RNA is not translated into proteins; instead, it plays a role in activating or suppressing gene expression by, for example, helping DNA looping.[128] By doing so, distally located gene regulatory elements and their target genes are brought into close proximity. Such chromosomal looping occurs in topologically associating domains (TADs) where several genes with relevant functions and their regulatory elements are packed together and isolated from neighboring TADs. Thus, TADs form the fourth layer where epigenetic alterations take place.[129] A novel class of long noncoding RNAs, called "immune priming long noncoding RNAs" (IPLs), can guide histone-modifying enzymes such as mixed-lineage leukemia 1 within a TAD to gene promoters. This facilitates H3K4me3 and subsequently opens up the chromatin structure of the immune genes enabling quicker transcription on secondary stimulus.[130] IPL production was found to be increased during trained immunity formation in monocytes, leading to robust transcription of trained immunity-related genes as a result of the accumulation of H3K4me3 marks at the gene promoters.[127,130] Similarly, the level of particular IPLs that direct H3K4me3 on the promoters of *IL1B* and *IL8* was heightened in human neutrophils after BCG vaccination.[10] Another class of noncoding RNAs with regulatory functions are enhancer RNAs.[131,132] They are involved in modulation of chromatin architecture by looping, recruiting specific proteins to enhancers and promoters, and regulating transcription in neighboring genes.[133,134] Emerging evidence implies various roles for these transcripts in regulating inflammatory responses,[135] yet their mechanisms and functions in the frame of trained immunity awaits investigation.

RECEPTORS AND SIGNAL TRANSDUCTION INVOLVED IN THE INDUCTION OF TRAINED IMMUNITY

Unlike the adaptive immune memory, which develops on engagement of highly antigen-specific receptors, trained immunity is triggered by the activation of PRRs, which recognize common molecular structures on the surface of pathogens and apoptotic and damaged cells. PRRs sense pathogen-associated molecular patterns such as β-glucan, LPS, and damage-associated molecular patterns such as oxLDL or fatty acids. All these agents and triggered signaling pathways have been shown to induce the trained immunity phenotype.

β-glucans and dectin-1 signaling

β-glucans, the polysaccharides that are components of the cell wall of certain pathogens including fungi, are recognized by a C-type lectin receptor dectin-1.[136,137] The receptor is largely expressed by myeloid cells,[138-140] but recently, its expression has also been reported in cells of the epithelial tissues.[141-143] The receptor-ligand interaction can lead to the activation of 2 distinct signaling pathways that play indispensable roles in initiating innate immune memory formation.[144] The Syk-dependent pathway is activated on the engagement of dectin-1 and involves Src kinases phosphorylation of the hemi-immunoreceptor tyrosine-based activation (hemITAM) motifs on dectin-1 that enables Syk kinase docking.[145-147] The Syk kinase activation leads to the influx of Ca^{2+} into the cell through PLCG2.[148] The increased intracellular levels of $Ca2^{+}$ activate the nuclear factor of activated T cells. The dephosphorylated nuclear factor of activated T cell translocate to the nucleus where it upregulates the IPLs and increases H3K4me3 at the promoters of genes regulating trained immunity.[44,130] Nuclear factor of activated T cells can directly bind and mediate transcriptional suppression of genes responsible for glycine, serine, and threonine metabolism to enhance metabolic reprogramming, which is the hallmark of trained immunity.[149] Concomitantly, the Syk kinase activates the PI3K and the downstream AKT/PKB signal transduction pathway[150,151] to support ROS production[123] and the CARD9 activation and proinflammatory function.[152,153]

Dectin-1 signaling can be also transduced in the Syk-independent way. As such, Raf-1 kinase initiates the signaling cascade by triggering PI3K/AKT/mTOR pathway that further activates HIF1A. HIF1A induces glycolysis by modulating glycolysis-related genes expression and their epigenetic status.[5] Consistently, the inhibition of the PI3k/AKT/mTOR/HIF1A pathway compromises the trained immunity responses.[5] Similarly, the myeloid-specific knockout of Hif1a in mice and inhibition of mTOR by metformin in humans attenuate the secondary response to infection and restimulation.[5,6]

TLR signaling

Another important class of PRRs are the highly conserved TLRs.[154,155] On receptor-ligand interaction, TLRs, except for TLR3, mediate downstream signaling cascades by recruiting MYD88 to the receptor. Serine kinases IRAK1 and IRAK4 are then recruited into the Myddosome, a large intracellular oligomeric signaling complex[156,157] that interacts with TRAF6 and activates nuclear factor-κB, leading to proinflammatory cytokine production.[158] Recent studies have shown that TLRs play a role in trained immunity formation in microglia and mast cells.[54,159]

Other signaling pathways

Apart from infectious agents, some metabolites, such as mevalonate, lipids (such as oxLDL), immune mediators including cytokines (eg, IFN-β, IFN-γ),[9,28] growth factors (eg, GM-CSF),[160] and hormones (eg, aldosterone, insulin)[113,161,162] have also been shown to induce trained immunity traits. Mevalonate, an intermediate metabolite of isoprenoid biosynthesis triggers

the trained immunity in monocytes through binding to IGF1R, which stimulates the PI3K/AKT/mTOR pathway and amplifies the innate immune memory response.[121] OxLDL-induced trained immunity in monocytes, macrophages,[31,163] and coronary smooth muscle cells in humans[164] involves TLR2 and TLR4 signaling, which concomitantly activates the mTOR pathway and induces metabolic remodeling.[164] The oxLDL-induced trained immunity can be reversed by the histone methyltransferase inhibitor, indicating that oxLDL stimulation implicates epigenetic reprogramming.[165,166] The signaling pathways triggered by other agents awaits further investigation.

CONSEQUENCE OF THE INDUCTION OF TRAINED IMMUNITY IN HEALTH AND DISEASE

Immune memory serves to minimize the damage and maximize the survival of the host on reexposure to the exogenous invasion. Dysregulation of these processes may have severe consequences on the ability of an organism to cope with infections and hyperinflammation.

Host defense

Induction of trained immunity has been shown to enable innate immune cells to be more effective during infection with pathogens such as viruses (respiratory syncytial virus, influenza A virus, and herpes simplex virus),[167] bacteria, (eg, *M tuberculosis*, *S aureus*), or fungi (such as *C albicans*).[168] As discussed above, BCG is among the best-known vaccines that induces heterologous protection against infections, an effect that is thought to be at least partially mediated by the induction of trained immunity. During the pandemic with severe acute respiratory syndrome coronavirus 2 infection, the effectiveness of the BCG vaccine has been intensively assessed in combating the coronavirus infectious disease 2019 (COVID-19). A number of epidemiological studies indicated a negative correlation between national BCG vaccination programs and the prevalence and mortality of COVID-19, hypothesizing that BCG vaccination could be a potential preventive measure against COVID-19.[169,170] Moreover, observational studies performed on health workers showed a reduced severity of COVID-19 on the BCG vaccination.[171] Some clinical trials have subsequently reported a reduction in the incidence of COVID-19 in individuals revaccinated with BCG.[172-174] However, the majority of clinical trials with BCG vaccination for the reduction of COVID-19 incidence failed to observe a decrease in disease incidence,[175-178] arguing against an effect of BCG on COVID-19 susceptibility. While these studies did not have the statistical power to determine the effect on severity and mortality due to COVID-19, a summary analysis of the mortality in these trials suggested a possible beneficial effect of BCG.[179] A potential beneficial effect of induction of trained immunity on COVID-19 severity is supported by a study using VPM-1002, a recombinant BCG,[180] and the measles, mumps, rubella, or MMR, vaccine.[181]

Tissue repair

Immune cells such as macrophages and neutrophils can release growth factors to stimulate angiogenesis and proliferation, as well as rapidly remove pathogens at the inflammation site to facilitate wound healing.[182-184] Interestingly, repeated *S aureus* soft tissue infection decreases skin lesion severity.[185-187] These studies suggest the beneficial role of trained immune cells in the healing of lesions. Moreover, trained immunity in nonimmune cells has been shown to play a role in tissue repair.[26,188,189] For example, injecting subcutaneous adipose tissue mesenchymal stem cells preprimed with LPS in diabetic rats improved skin flap survival.[188] Furthermore, trained epithelial stem cells recovered from inflammation heal faster on secondary challenges such as wounding and *C albicans* infection.[26] These studies suggest that trained immunity developed in immune and nonimmune cells delivers great benefits in tissue repair leading to the proposed term "trained tissue repair."[189]

Transgenerational trained immunity

Trained immunity not only brings survival advantages to individuals but also to their progenies. A recent study demonstrated that children whose mothers had immune dysfunction, characterized by a lower prenatal IFN-γ/IL-13 production ratio, were significantly more likely to develop asthma during their childhood.[190] It was further suggested that maternal prenatal immune status altered the epigenome and trained immunity of neonates and determined the child's susceptibility to asthma. As the immune imprint of children might result from *in utero* exposure, a recent study suggested that trained immunity can also be transmitted through generations.[190-192] Offspring of *C albicans*–infected male mice have been shown to exhibit a stronger immune response to endotoxin challenge and pose better protection against infection caused by *Escherichia coli* and *Listeria monocytogenes*.[191] The improved immune response in offspring is associated with cellular, developmental, and transcriptional changes in the bone marrow myeloid and progenitor cells. While another study did not confirm these observations,[193] further investigation is required to scrutinize the external factors such as diet, microbiome, and other environmental influences that may play roles in inducing the transgenerational transmission of immune memory traits.[192,193]

Defective trained immunity

Defective trained immunity can lead to detrimental effects such as compromised host defense against infection, immunodepression in sepsis, or disease progression such as cancer. Compromised trained immunity responses and STAT1 signaling have been associated with fungal infections in mucocutaneous candidiasis.[194] Interestingly and unlike BCG, *M tuberculosis* can impair trained immunity formation by inducing necroptosis of myeloid progenitors via an IFN-I/iron axis.[195] This leads to the decreased generation of neutrophils and monocytes exposing the host to the risk of reinfection.

In the context of cancer, defective trained immunity can induce a protumorigenic profile in myeloid-derived suppressor cells. Myeloid-derived suppressor cells further promote a tumor-favored environment by producing essential growth factors such as vascular endothelial cell growth factor and β-fibroblast growth factor to facilitate tumor progression.[196] Moreover, thyroid cancer-derived lactate induces increase in aerobic glycolysis of tumor-associated macrophages followed by epigenetic reprogramming, leading to the upregulation of cytokines that facilitate tumor development.[6] Indeed, cytokines such as TNF-α and IL-6 that are produced by trained cells have been linked

to an increase in the growth and spread of certain types of tumors such as lung, breast, and kidney cancers.[197,198]

Consequences of excessive trained immunity

Trained immunity is among the host defense mechanisms that are undoubtedly beneficial during infection. Nevertheless, triggering the immune system with endogenous ligands can lead to inappropriate activation of trained immunity. With the heightened responsiveness of the trained cells, inflammation can develop further, which consequently contributes to the progression of pathology and the chronicity of inflammatory diseases. Autoimmune diseases, autoinflammatory diseases, allergies, cardiovascular complications, and neurodegenerative diseases are only some examples where an excessive trained immunity response can play a role in the pathophysiology. SLE and systemic sclerosis are autoimmune diseases in which inappropriately activated trained macrophages have been shown to play a crucial role in the exaggerated immune response. Even though the exact trigger to initiate these immunopathologies remains unclear, macrophages are found to bear metabolic and epigenetic changes that lead to an enhanced proinflammatory phenotype and self-antigen presenting capacity, causing the perpetuation of the disease.[199-201]

The link between trained immunity and cardiovascular conditions is another emerging research topic. Various studies have shown that monocytes and macrophages are the predominant cell population in atherosclerotic lesions and have critical roles in regulating this condition. Damage-associated molecular patterns, such as oxLDL, which is among the well-established endogenous inducers of trained immunity,[202] are released in an atherosclerotic environment and stimulate immune cells via PRRs. This stimulation causes long-lasting epigenetic reprogramming and increased proinflammatory cytokine production by monocytes and macrophages.[8,64] In that way, the persistent inflammation observed in atherosclerosis is further fueled and leads to the development of the disease. Not only oxLDL, but also other metabolites, namely uric acid and free fatty acids, might contribute to the trained immunity phenotype in the context of cardiovascular complications.[63,203]

Constant triggering of the immune system and induction of inappropriate trained immunity in circulating cells and the hematopoietic stem cell progenitors can be the source of the proinflammatory state that is observed in the elderly. This phenomenon is called "inflammaging" and could be the link between trained immunity and aging.[204,205] Another example of destructive trained immunity is an autoinflammatory disease called "hyper IgD syndrome." Monocytes from patients with hyper IgD syndrome show a trained immunity profile including an H3K27ac signature and increased production of proinflammatory cytokines at both the basal level and during infection. The accumulation of mevalonate in these patients activates the AKT/mTOR pathway and mevalonate itself can induce trained immunity.[121] Misguided trained immunity responses may also contribute to the progression of neurodegenerative diseases such as Alzheimer's disease. Microglial priming and trained immunity have strikingly parallel features including epigenetic reprogramming, altered inflammatory pathways, and long-term memory capacity.[159,206,207]

Excessive trained immunity responses might play important roles in allergen-driven inflammatory conditions as well. Most recently, Li et al[104] discovered that the development of allergic asthma in childhood is linked to trained macrophages through proline metabolism reprogrammed by early respiratory infection in combination with allergen sensitization. Through therapeutic approaches, metabolic and epigenetic processes involved in the trained immunity can be adequately manipulated to either enhance the immune response against infections and malignancies or inhibit the excessive responses that drive various autoimmune and cardiovascular diseases as well as allergies.

HARNESSING TRAINED IMMUNITY FOR THERAPEUTIC USE

The efficiency of trained immunity in host defense fuels the idea of harnessing this defense mechanism as a prophylactic and therapeutic tool to prevent and treat infections, immunodeficiencies, or cancer. As BCG has been shown to induce protection against unrelated bacterial and viral infections,[167,168] other live attenuated vaccines, such as measles and polio, have also been reported to confer protection against heterologous infections.[208] Moreover, BCG can potentially restore the immune response in immunodeficient individuals.[209] The inactivated polybacterial mucosal vaccine MV130 has also been shown to induce trained immunity in human monocytes and mouse bone marrow cells, ensuring protection against unrelated viral respiratory infections in mice and human.[210,211] The protective effect of MV130 has been attributed not only to the innate immune cells but also to boosted adaptive responses.[210] Naturally, there is a growing effort to harness the trained immunity mechanisms in the design of the new generation of vaccines.[212]

Trained immunity can also be harnessed to treat cancer.[213,214] β-glucan–trained monocytes and macrophages exhibit increased cytotoxicity against pancreatic tumor cells, leading to an overall reduction in tumor burden and prolonged survival in mice.[215] Influenza A virus–trained alveolar macrophages can infiltrate lung tumor lesions and exert phagocytic and cytotoxic functions against the tumor.[216] In fact, some nanomaterials have already been developed to accommodate trained immunity as a therapeutic tool against cancers. As such Ajit et al[217] have developed a controlled release of nanoparticles encapsulating microdosages of β-glucan to sustain the temporal control of trained immunity in humans and achieve prophylactic host defense. Furthermore, Priem et al[218] invented a bone marrow-avid high density lipoprotein (HDL) nanobiologic, MTP$_{10}$-HDL, that can induce epigenetic rewiring of multipotent progenitors in bone marrow. In combination with anti-programmed cell death protein 1 and anti-CTLA4 treatment, the trained immunity-induced myelopoiesis generates myeloid cells that overcome the immunosuppressive environment of the tumor. These promising developments pave the way for harnessing trained immunity as a therapeutic tool to boost anti-cancer defense and immune responses in immunocompromised patients.

Suppressing trained immunity for therapeutic use

As previously discussed, trained immunity might cause excessive inflammation in certain conditions such as atherosclerosis, allergies, autoimmune diseases, and organ transplantation.[213] Therefore, it seems beneficial to inhibit the mechanisms that promote trained immunity to improve the outcome of these diseases. There are various strategies to reduce chronic inflammation and tissue damage associated with trained immunity, including using

drugs that prevent the activation of NOD2 or dectin-1, mTOR inhibitors such as rapamycin, and inhibitors of epigenetic modifiers such as DNA methyltransferases and histone deacetylases.[219] Additionally, the use of nanocarriers to deliver these compounds to specific cells or their progenitors is proposed as a promising alternative to minimize any negative effects associated with blocking these pathways. For example, to achieve better organ transplantation acceptance, Braza et al[220] developed mTOR inhibitor–HDL nanobiologics that inhibit trained immunity associated cytokine production in myeloid cells. The study showed that mTOR inhibitor–HDL treatment during the β-glucan training period prevented trained immunity–mediated epigenetic changes and decreased cytokine and lactate production on LPS restimulation in human monocytes. Thus, the study provides a promising avenue for developing targeted trained immunity–suppressive therapies that can increase organ transplant acceptance.[220]

Hyperinflammatory states associated with trained immunity have been suggested to contribute to the pathogenesis of SLE, a chronic autoimmune disease, characterized, for example, by activated mTOR pathway, enrichment of certain epigenetic marks, and functional reprogramming at the hematopoietic stem cell level, all of which are well-known hallmarks of trained immunity. Therefore, de Graaf et al[221] suggested that IL-38, which prevents the induction of trained immunity by inhibiting the mTOR pathway, might be therapeutically used to suppress the inflammation in SLE. With similar therapeutic strategies, the enhanced inflammatory state of the immune system could be dampened in other autoimmune and autoinflammatory diseases such as rheumatoid arthritis,[222] hyper IgD syndrome,[199,219] or inflammatory bowel disease.[161,223]

Trained immunity–associated chronic inflammation is among the key factors in the development and progression of atherosclerosis. Therefore, inhibiting trained immunity by pharmacological interventions may be a promising therapy to reduce ongoing inflammation in patients with atherosclerosis.[14] Indeed, the statin-loaded reconstituted HDL nanoparticles have been shown recently to effectively inhibit atherosclerotic plaque inflammation in mice by modulating trained immunity–associated pathways such as the mevalonate pathway.[15]

As shown in different examples above, potential immunotherapeutic interventions can target trained immunity by blocking main mediators of the hyperinflammation such as IL-1β as well as using small-molecule inhibitors of epigenetic pathways, RNA interference technology, nano-immunotherapy, and vaccination.

Conclusions

In this review, we summarized recent discoveries in the field of trained immunity in immune and nonimmune cells of the central system and peripheral tissues. Trained immunity is a process of long-term functional programming of innate immune cells that is mediated by epigenetic rewiring at the level of immune cell progenitors in the bone marrow or long-lived tissue macrophages. The induction of trained immunity is an evolutionarily protective process against infections, but new studies indicate that it may also be involved in many physiological processes, including the induction of an effective antitumoral immune response and promoting tissue regeneration. However, dysregulated trained immunity likely contributes to the pathophysiology of inflammatory and autoimmune diseases. While much has been learned about innate immune memory in the past decade, the remaining molecular mechanisms

and therapeutic potential of trained immunity remain to be discovered. Modulation of trained immunity either by enhancing it in infections and cancer or by inhibiting it in inflammatory diseases is a promising therapeutic target and is likely to become an area of intense interest in the coming years.

DISCLOSURE STATEMENT

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BCG vaccination induces innate immune memory in γδ T cells in humans

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Abstract

Bacillus Calmette–Guérin vaccine is well known for inducing trained immunity in myeloid and natural killer cells, which can explain its cross-protective effect against heterologous infections. Although displaying functional characteristics of both adaptive and innate immunity, γδ T-cell memory has been only addressed in a pathogen-specific context. In this study, we aimed to determine whether human γδ T cells can mount trained immunity and therefore contribute to the cross-protective effect of the Bacillus Calmette–Guérin vaccine. We investigated in vivo induction of innate memory in γδ T cells by Bacillus Calmette–Guérin vaccination in healthy human volunteers by combining single-cell RNA sequencing technology with immune functional assays. The total number of γδ T cells and membrane markers of activation was not influenced by Bacillus Calmette–Guérin vaccination. In contrast, Bacillus Calmette–Guérin changed γδ T cells' transcriptional programs and increased their responsiveness to heterologous bacterial and fungal stimuli, including lipopolysaccharide and *Candida albicans*, as simultaneously characterized by higher tumor necrosis factor and interferon γ production, weeks after vaccination. Human γδ T cells in adults display the potential to develop a trained immunity phenotype after Bacillus Calmette–Guérin vaccination.

Keywords: γδ T cells, BCG vaccine, immune memory, trained immunity

Abbreviations: BCG, Bacillus Calmette–Guérin; CFU, colony-forming unit; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor.

1 Introduction

Immunologic memory is a feature of immune cells that allows them to mount a more robust and efficient immune response upon reencountering a pathogen. Until recently, immune memory was attributed solely to adaptive immune cells such as T and B lymphocytes. However, the striking observation in many epidemiologic studies that Bacillus Calmette–Guérin (BCG) vaccine, a live attenuated vaccine developed to protect against tuberculosis caused by *Mycobacterium tuberculosis* infections, results in an overall increased survival rate of vaccinated children,[1–7] suggested that BCG may induce heterologous protective effects (cross-protection) against other types of infections through innate

immune mechanisms. This, and similar observations in experimental models, led to breakthrough findings showing that innate immune cells such as monocytes, macrophages, and natural killer (NK) cells also "remember" a primary activation during infection and respond more effectively during reinfection with different antigens.[8–12] This property has been termed the innate immune cell memory or *trained immunity*. Innate and adaptive immune cell memory differ, however, in their ability to respond to a secondary infection: while adaptive immune memory is antigen specific, innate immune memory is antigen independent.[8,13]

Induction of trained immunity primarily involves metabolic and epigenetic reprogramming, leading to stronger immune response to secondary stimuli in the myeloid cell compartment, their progenitors, and NK cells.[8,14–19] While the effect of BCG-induced

trained immunity persists in the human monocyte/macrophage compartment for at least 3 mo,[8] long-term in vivo studies showed that BCG-induced cross-protection 1 yr after vaccination is most likely driven by heterologous interferon gamma (IFN-γ) and interleukin (IL) 17 production.[9,20] BCG vaccination also generates long-lived NK memory cells capable of producing elevated levels of IFN-γ upon rechallenge,[21] yet the role of NK cells in BCG-induced cross-protection is still unclear.[9]

Somatic rearrangement of T-cell receptors (TCRs) during T-cell development in the thymus leads to the generation of major histocompatibility complex (MHC)–restricted and highly diverse αβ T cells, as well as MHC-unrestricted, less diverse unconventional γδ T cells. γδ T cells recognize nonpeptide antigens via butyrophilin molecules[22,23] and respond to stimulation in a faster manner than conventional T cells. Interestingly, γδ T cells also possess features of innate immune cells such as expression of innate-like receptors (e.g. natural killer group 2 member D and Toll-like receptors [TLRs]).[24,25] Responsiveness to nonspecific pathogen- or stress-related signals allows a rapid activation of γδ T cells, an additional characteristic reminiscent of innate immune cells. Despite these unique characteristics, so far, the capacity of γδ T cells to mount immune memory has been mainly addressed in a pathogen-specific context,[26–28] with no reports addressing the induction of innate immune memory in these cells.

Human γδ T cells are classified based on the Vδ chain variant of the TCR in Vδ1, Vδ2, and Vδ3 T cells. In adult human peripheral blood, γδ T cells represent 1% to 10% of leukocytes. The Vδ2 T-cell subset predominates and represents 50% to 95% of the whole γδ T-cell pool in human peripheral blood.[29] Vδ2 chain mainly pairs with Vγ9 and such composed TCR recognizes microbial-derived phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and isopentyl pyrophosphate,[30] an intermediate of cholesterol synthesis in the eukaryotes. HMBPP is a product of isoprenoid biosynthesis in the nonmevalonate pathway used by most eubacteria (including all mycobacteria) and some protozoa (e.g. *Plasmodium falciparum*) but not by mammalian cells. It is also a well-known antigen in *M. tuberculosis* infection,[31] and many microorganisms that produce HMBPP can activate Vδ2 T cells.[30] Considering the fact that the TCR of Vδ2 cells recognizes pathogen-associated molecular patterns, rather than specific antigens, the TCRs of these cells resemble innate immune receptors. Consistent with that, the early study by Munk et al. reported that human peripheral γδ T cells preexpanded with *M. tuberculosis* in vitro were able to proliferate in response to rechallenge with unrelated pathogens such as *Listeria monocytogenes*, group A streptococci, or *Staphylococcus aureus*,[32] indicating the pathogen-unspecific γδ T-cell proliferating responses.

BCG vaccination induces a rapid proliferation of Vδ2 T cells with a well-established role in protection against *M. tuberculosis*. In nonhuman primates, the secondary administration of BCG results in enhanced proliferation of Vδ2 T cells.[26] *M. tuberculosis* stimulation of peripheral blood mononuclear cells (PBMCs) from BCG-vaccinated individuals resulted in greater expansion of Vδ2 T cells compared to nonvaccinated controls.[33] Moreover, γδ T cells (most likely Vδ2 T cells) from BCG-vaccinated individuals showed increased production of IFN-γ upon in vitro stimulation with BCG.[21] Taken together, these reports imply the generation of immune memory by γδ T cells during mycobacterial infection. Not only γδ T cells develop immune memory characteristics, but they are also suggested, together with NK cells, to be the main producers of IFN-γ during memory responses to

secondary challenge in vaccinated infants.[34] However, whether memory γδ T cells exhibit an enhanced response to heterologous stimuli needs to be investigated. In this study, we assessed whether BCG vaccination can induce heterologous immunologic memory and increased responsiveness to nonmycobacterial stimuli in Vδ2 T cells.

2 Methods

2.1 Sample collection from healthy volunteers and participants of clinical trial

BCG-vaccinated volunteers were a part of the 300BCG clinical trial approved by the Arnhem-Nijmegen Ethical Committee (approval number NL58553.091.16), performed at Radboud University Medical Center, Nijmegen, the Netherlands, according to the Declaration of Helsinki and Good Clinical Practice. Prior to inclusion, study volunteers were medically screened and provided written informed consent (Supplementary Table 1). Volunteers were not previously vaccinated with BCG before the study and did not receive any other vaccination 3 mo prior to the start of the study. All participants were vaccinated with 1 standard dose of BCG vaccination (BCG Bulgaria strain, Intervax; 0.1 mL intradermally), and blood was collected before vaccination and postvaccination (i.e. after 2 and 12 wk).

2.2 PBMC isolation and stimulation

Human PBMCs were isolated from peripheral blood of healthy volunteers by Ficoll (GE Healthcare) density gradient centrifuged. PBMC cultures were stimulated with heat-inactivated infectious agents: *M. tuberculosis* (2 μg/mL), *Escherichia coli* (10^6 colony-forming units [CFU]/mL), *S. aureus* (10^6 CFU/mL), *Candida albicans* (10^6 CFU/mL), and BCG (10^6 CFU/mL) reconstituted from lyophilized vials of commercial BCG vaccine provided by InterVax Ltd. For single-cell RNA sequencing (scRNA-seq) analysis, isolated PBMCs were stimulated for 4 h with either RPMI (control) or lipopolysaccharide (LPS) 10 ng/mL (serotype 055: B5; Sigma).

2.3 Flow cytometry

Cell surface marker staining was performed with the following antibodies: anti-human CD45 (HI30; BioLegend) Brilliant Violet 605, anti-human CD3 (UCHT1; BioLegend) Pacific Blue, anti-human TCRγδ (11F2; BD Bioscience) PE-Cy7 or PE, anti-human CD69 (FN50; BioLegend) APC-Cy7 or PE-Cy7, anti-human CD25 (M-A251; BioLegend) APC, and anti-human TCRαβ (IP26; BioLegend) APC-Cy7 at 4 °C in the dark for 30 min. The cells were then washed with phosphate-buffered saline (PBS) followed by fixation (CellFIX; BD Bioscience). Intracellular cytokine staining was performed on the cells, which were stimulated for 4 h with anti-human CD3 (1 μg/mL; BD Bioscience) and anti-human CD28 (1 μg/mL; BD Bioscience) in the presence of GolgiPlug (BD Bioscience; 1:1,000) prior to staining. PBMCs were then incubated for 30 min in cold PBS in the dark with the following antibodies against cell surface markers. Permeabilization and fixation were performed with the commercially available kit according to the manufacturer's instructions (eBioscience). After fixation and permeabilization, intracellular staining was performed with the following antibodies: anti-human perforin (dG9; BioLegend), anti-human-IL17A (BL168; BioLegend), anti-human TNF (MAb11; BioLegend) APC, and anti-human IFN-γ (B27; BD Pharmingen) PerCP-Cy5.5. After a 30-min incubation, the cells were washed with PBS and stored in CellFIX reagent (BD Bioscience) until

analysis. Cells were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software version 10.8.0.

2.4 Single-cell library preparation and RNA sequencing of 300BCG cohort

Blood samples collected on day 0 and 3 mo post-BCG vaccination were used for the scRNA-seq analysis. In total, 156 samples were mixed into 32 pooled libraries. In each pool, an equal number of cells from 3 or 5 different donors were pooled together. Single-cell gene expression libraries were generated on the 10× Genomics Chromium platform using the Chromium Next GEM Single Cell 3′ Library & Gel Bead Kit v3.1 and Chromium Next GEM Chip G Single Cell Kit (10× Genomics) according to the manufacturer's protocol. Libraries were sequenced on a NovaSeq 6000 S4 flow cell using v1 chemistry (Illumina) with 28-bp R1 and 90-bp R2 run settings. These pools were sequenced in 3 batches in which conditions and time points were mixed to minimize potential batch effects. Sequencing data are deposited in the European Genome-Phenome Archive under the accession number EGAS00001006990.

2.5 Single-cell data preprocessing and demultiplexing

In each library, the bcf2fastq Conversion Software (Illumina) was used to convert BCL files to FASTQ files, along with a sample sheet including 10× barcodes. The proprietary 10× Genomics STAR in CellRanger pipeline (v3.1.0) was used to align read data to GRCh38/b38 (downloaded from 10× Genomics). We set the expected cell parameter to 2,000. This generated a gene expression matrix that recorded Unique Molecular Identifier (UMI) count of each gene in each cell.

To remove duplicates, cells were assigned to their origin using the souporcell (v2.0) tool using premapped bamfiles.[35] In total, 91.98% cells were retrieved for downstream analysis. Since the T0 data (before vaccination) of 1 individual contained few cells at T3m (3 mo postvaccination), we discarded them from downstream analysis. Subsequently, the souporcell tool was employed to cluster cells based on allele counts using a hierarchical clustering strategy and assign individuals to clusters. Further, the consistency between the assigned individuals via souporcell and individual phenotypes' ID was cross-checked using the genotype data set of each individual.

2.6 Single-cell data quality control, integration, and clustering

Cells were removed if they had (i) a percentage of mitochondrial genes more than 25% and (ii) a number of detected genes fewer than 100 or more than 5,000. Only genes that were expressed in at least 5 cells were considered for downstream analysis, resulting in approximately 200,000 cells and 21,975 genes.

In total, cells were collected from 3 batches. There are 10 pools in batch 1, 10 pools in batch 2, and 12 pools in batch 3. In each pool, 5 samples from different individuals at different time points with different stimulations were pooled randomly. Seurat (v4.0.0)[36] package of R (v4.0.2) was used to integrate and analyze all data together. In brief, for each independent data set from each pool, UMI counts were normalized and the top 2,000 variable features were selected using the NormalizeData and FindVariableFeatures function with default parameters, respectively. Later, repeated features were then identified across all independent data sets and utilized for scaling and principal component analysis (PCA) analysis on each data set. Instead of

canonical correlation analysis, reciprocal PCA was used via the SelectIntegrationFeatures function to detect integration anchors in order to speed up calculation for integration and avoid overcorrection.[37] The integrated data set was then scaled and clustered using default parameters. Cell clusters were further annotated by combining the results from the SingleR[38] package of R (HumanPrimaryCellAtlasData, BlueprintEncodeData, MonacoImmuneData, DatabaseImmuneCellExpressionData, and NovershternHematopoieticData were selected as reference) and the expression level of known cell markers (CD4 T cells: *IL7R*, *CD3D*; CD8 T cells: *CD8A*, *CD8B*, *CD3D*; NK cells: *GNLY*, *NKG7*).

2.7 Identification and clustering of γδ T cells from the 300BCG data set

After cell-type annotation, we first selected T cells and NK cells from the scRNA-seq data set and extracted γδ T cells from these populations due to their overlapping gene expression profiles (Reference to PNAS paper).[39] The γδ T cells were annotated based on known their markers, including *TRDC*, *TRGC1*, *TRGC2*, *CD3D*, *CD3E*, *GZMB*, and *GZMK*.

The FindVariableFeatures function of the Seurat was employed to detect the top variable genes in γδ T cells and perform unsupervised clustering. In order to test the heterogeneity of γδ T cells leading by trained immunity, we calculated the changes of each gene between after being trained (3 mo after BCG vaccination, with LPS restimulation, T3m_LPS) and before begin trained (before BCG vaccination, with LPS stimulation, T0_LPS). Initially, we calculated the average expression level of each gene at T0_LPS for each individual. Then the expression level of each gene in each γδ T cell of each individual at T3m_LPS was subtracted from the corresponding average expression of that gene in the γδ T cell at T0_LPS, and this difference was defined as "TI changes." Next, based on TI changes, unsupervised clustering employing FindNeighbors and FindClusters in the Seurat package was performed to estimate the similarity between cells. The γδ T cells were clustered into 5 clusters using the K-nearest neighbor graph and Louvain algorithm.

2.8 Identification of trained immunity response genes and enrichment analysis

Differential gene expression analysis was performed using the FindMarkers/FindAllMarkers function with Wilcoxon rank-sum test in the Seurat package. Genes that were expressed in at least 10% cells and having a *P* value less than 0.05 (after Bonferroni correction) were considered significant. Trained immunity response genes (TIGs) were identified by the gene expression levels at T3m_LPS and T0_LPS.

For the enrichment test, sets of significant genes were analyzed using enrichGO and enrichKEGG function (with the pAdjustMethod set to "B" and a *q* value cutoff of 0.05) of the ClusterProfiler (v3.18.1)[40] package in R.

2.9 Cell–cell interaction analysis

NicheNet (v1.0.0)[41] was used to infer cell–cell interaction in the T3m_LPS based on aggregated prior information of ligand–receptor, signaling, and gene regulatory data. In brief, communications analysis was performed between monocytes and trained γδ T subpopulations, and one-time monocytes and the γδ T subpopulation were set as "receiver" and "sender," respectively, and other time vice versa. In each analysis, TIGs were regarded as sets of interest (targets) and ligands were identified from
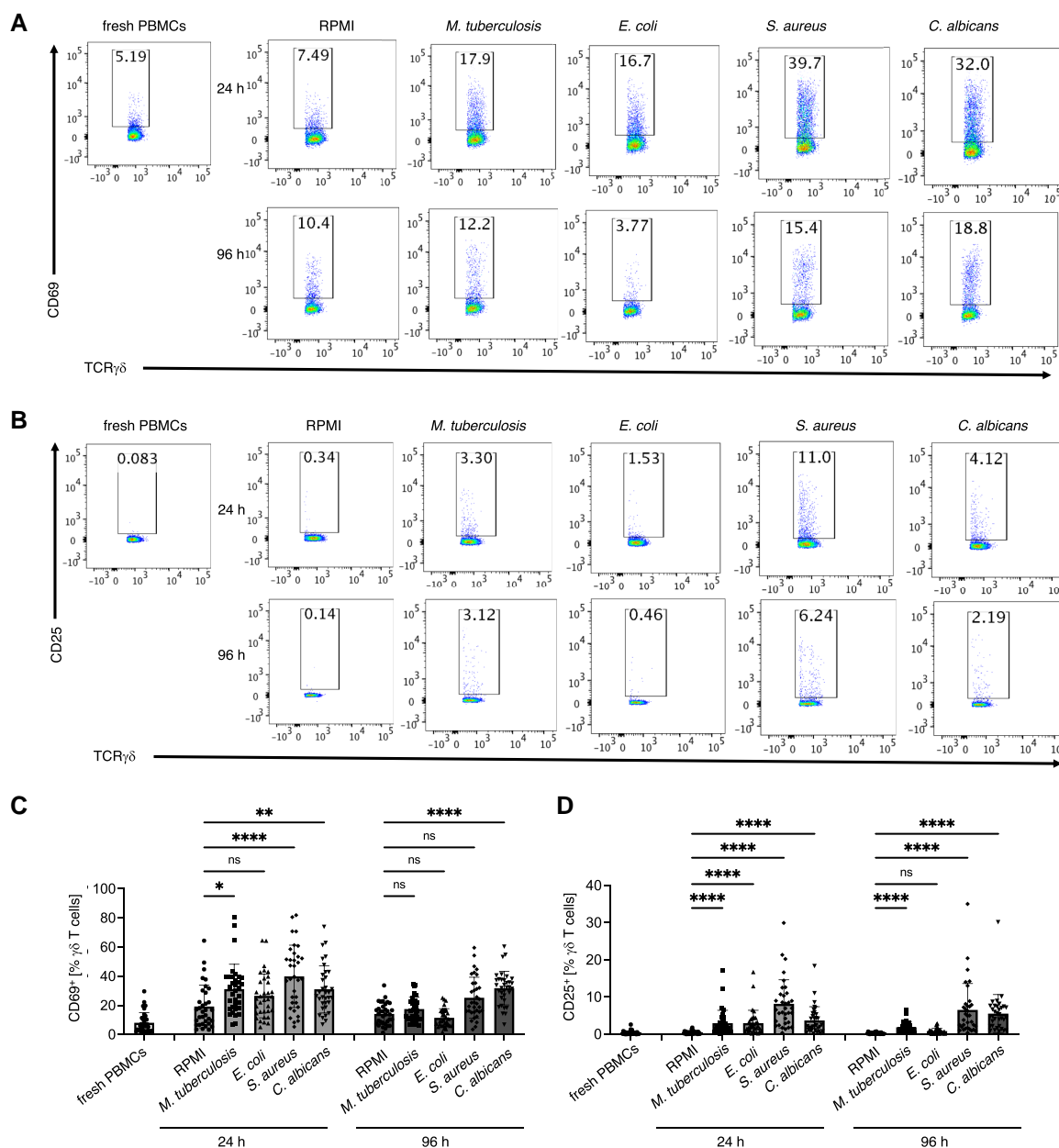
Fig. 1. Human peripheral blood γδ T cells upregulate activation markers: CD25 and CD69 in response to *M. tuberculosis*, *E. coli*, *S. aureus*, and *C. albicans* as early as 24 h after in vitro cultures. (A) Flow cytometry analysis of CD69 expression on γδ T cells in PBMC cultures stimulated in vitro with RPMI alone or with indicated heat-killed pathogens assessed before (0 h), 24 h, and 96 h after stimulation. PBMCs were gated for CD45⁺CD3⁺TCRγδ⁺ cells and representative dot plots are shown. (B) Cumulative data of percentage of CD69⁺ among γδ T cells assessed as in A. (C) Flow cytometry analysis of CD25 expression on γδ T cells assessed as in A. (D) Cumulative data of percentage of CD25⁺ among γδ T cells assessed as in C. Friedman test, *P* value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****); $n = 34$.

senders. Top 20 ligands were selected based on the Pearson correlation coefficients with TIGs. The genes expressed in at least 10% of the cells from the senders were used as the background genes.

2.10 Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software). Statistical significance of differences observed was calculated by the Friedman or Mann–Whitney test as indicated in the figure description. Differences were considered significant when *P* value was <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****).

3 Results

3.1 *S. aureus* and *C. albicans* are the most potent activators of human peripheral blood γδ T cells

In order to better understand the functional responses of human peripheral blood γδ T cells, we first assessed CD25 and CD69 activation markers on γδ T cells in response to in vitro stimulation with heat-killed pathogens using flow cytometry. In our experimental setup, PBMCs from nonvaccinated healthy individuals were cultured with *M. tuberculosis*, *E. coli*, *S. aureus*, and *C. albicans* up to 96 h. By gating on the TCR γδ T-cell population within PBMCs, we found that stimulation with all 4 inactivated pathogens caused upregulation of CD69 (Fig. 1A and C) and CD25
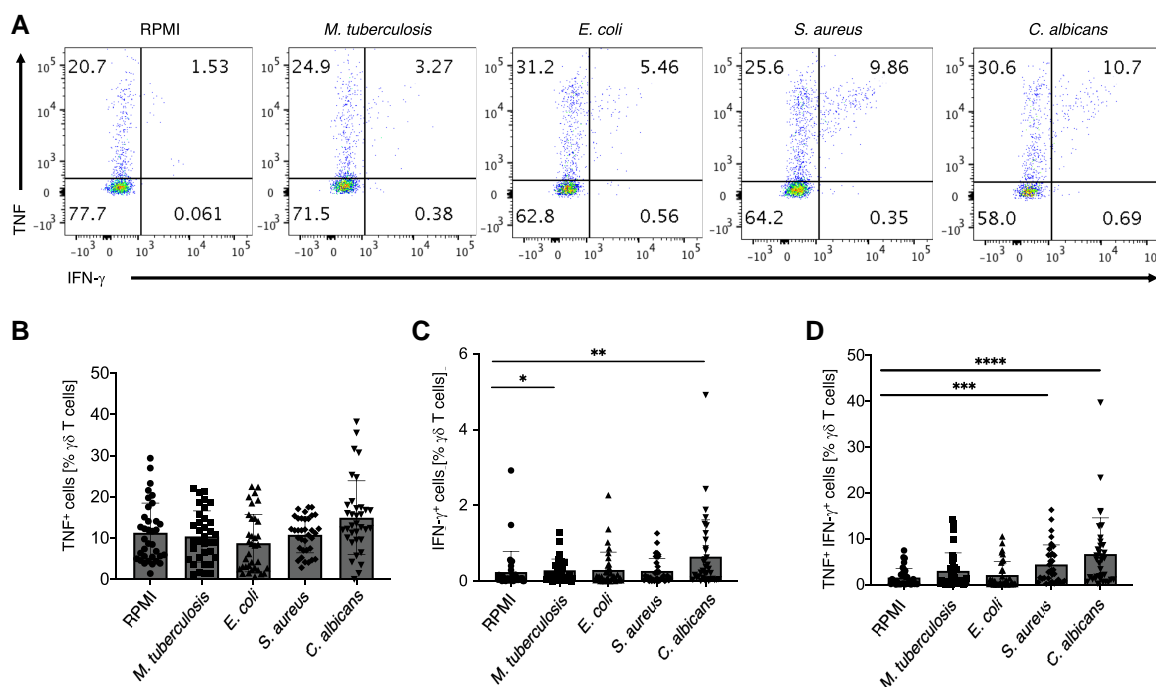
Fig. 2. *S. aureus* and *C. albicans* are the most potent inducers of cytokine production by peripheral blood γδ T cells. Human PBMCs were stimulated as in Fig. 1. TNF and IFN-γ cytokine production by γδ T cells was assessed by intracellular staining at day 4 of culture upon restimulation with anti-CD3/CD28 for 4 h. PBMCs were gated for CD45$^+$CD3$^+$TCRγδ$^+$ cells and representative dot plots are shown. (A) Representative dot plots of TNF and IFN-γ expression by γδ T cells in response to indicated stimuli. (B) Cumulative data of percentage of TNF$^+$ IFN-γ$^-$ γδ T cells assessed as in A. (C) Cumulative data of percentage of TNF$^-$ IFN-γ$^+$ γδ T cells assessed as in A. (D) Cumulative data of percentage of TNF$^+$ IFN-γ$^+$ γδ T cells assessed as in A. Friedman test, *P* value <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****); *n* = 34.

(Fig. 1B and D) activation markers on γδ T cells as early as 24 h after stimulation. As expected, *M. tuberculosis* induced significant CD25 expression on γδ T cells after 24 and 96 h, but a significant expression of CD69 was found on γδ T cells only after 24 h. *S. aureus* induced a significant expression of the early activation marker (CD69) on γδ T cells at 24 h (but not after 96 h), while the late activation marker (CD25) was significantly expressed on γδ T cells at both time points. Notably, *C. albicans* induced a statistically significant increase in both early and late activation markers on γδ T cells.

Additionally, we analyzed IFN-γ and TNF expression, which are effector molecules of γδ T cells. While we did not observe a significant increase in tumor necrosis factor (TNF) single-producing cells upon stimulation with selected pathogens (Fig. 2A and B), *M. tuberculosis* and *C. albicans* induced a significant increase in numbers of IFN-γ single-producing γδ T cells after 4 d of activation (Fig. 2A and C). IFN-γ and TNF double-producing T cells were highly induced by *S. aureus* and *C. albicans* (Fig. 2A and D). Thus, we found a distinct functional response of human γδ T cells toward the heat-killed pathogens.

3.2 BCG vaccination in adults enhances perforin production by γδ T cells

To determine the functional characteristics of γδ T cells, we investigated γδ T-cell responses to various stimuli in healthy adult volunteers who had been vaccinated with BCG. As represented in a schematic diagram, we assessed γδ T-cell subset distribution and activation status before and after BCG vaccination (Fig. 3A). BCG vaccination did not affect γδ T-cell percentages in the peripheral blood of vaccinated volunteers (Fig. 3B and C). Similarly, BCG did not influence the activation status of γδ T cells 2 and 12 wk after

vaccination, as characterized by the expression of CD69 (Supplementary Fig. 1A and B) and CD25 (Supplementary Fig. 1C and D). The assessment of the perforin production in PBMC cultures treated 24 h with RPMI alone revealed that perforin amounts were significantly increased in γδ T cells 3 mo after BCG vaccination (Fig. 3D and E). Therefore, BCG vaccination has no effect on activation status of human γδ T cells in a steady state but likely can boost their effector function by enhancing cytotoxic molecule production.

3.3 BCG vaccination induces transcriptional changes in γδ T cells

To further determine transcriptional changes induced in γδ T cells by the BCG vaccine, sRNA-seq was performed on PBMCs collected from 39 healthy volunteers. These cells were collected before and 3 mo after receiving the BCG vaccination, both with and without in vitro LPS stimulation. In total, we profiled ~200,000 PBMCs after filtering out doublets, followed by quality control. To identify γδ T cells from the scRNA-seq data set, we first selected CD4 T cells, CD8 T cells, and NK cells and visualized those cell types in UMAP (Fig. 4a). Based on manually curated markers, cluster 7 was annotated as γδ T cells with a high expression level of *TRDC*, *TRGC1*, *TRGC2*, *GZMB*, and *GZMK* (Fig. 4B and 4C). In total, 6,338 γδ T cells were obtained with 1,664, 1,754, 1,525, and 1,395 cells from four conditions: before BCG vaccination without/with LPS (T0_RPMI, T0_LPS) and 3 mo after BCG vaccination without/with LPS stimulation (T3m_RPMI, T3m_LPS), respectively.

We next used an unsupervised clustering method and identified 5 distinct subpopulations of trained γδ T cells (Fig. 5A). Samples collected before BCG and 3 mo after BCG vaccination with LPS stimulation were used for downstream analysis. Interestingly, *IFNG* was highly expressed in cluster 2 as one of
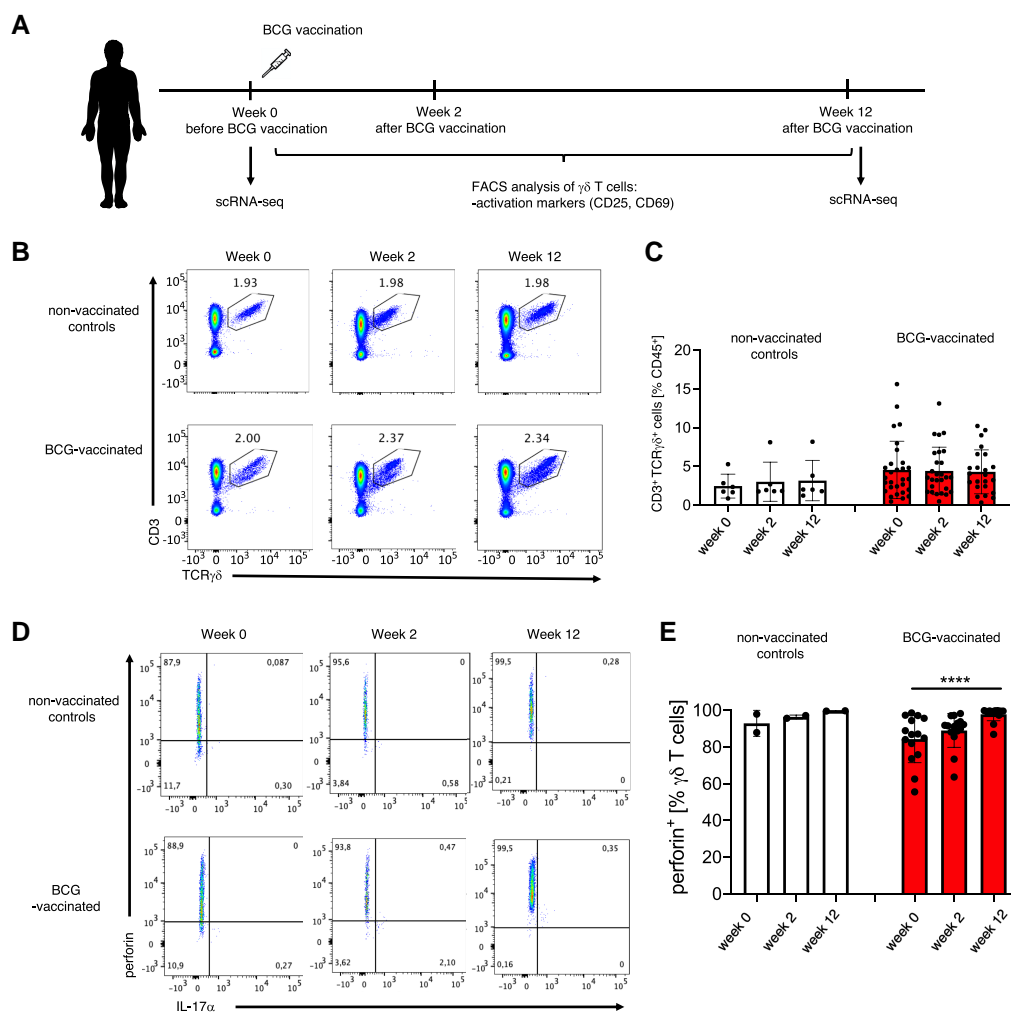
Fig. 3. BCG vaccination in adults enhances perforin production by γδ T cells. (A) Schematic representation of the experimental setup for γδ T-cell characterization in BCG-vaccinated volunteers. (B) Flow cytometry analysis of CD3$^+$TCRγδ$^+$ T cells among CD45$^+$ cells in peripheral blood of unvaccinated controls and BCG-vaccinated volunteers before (week 0) vaccination and 2 wk (week 2) and 3 mo (week 12) after vaccination; gated CD45$^+$ cells. (C) Cumulative percentages of CD3$^+$TCRγδ$^+$ T cells assessed as in B. (D) Representative flow cytometry dot plots. (E) cumulative percentages of perforin-producing γδ T cells (nonvaccinated controls, $n = 2$; BCG vaccinated, $n = 15$).

the main markers (Fig. 5B). In order to explore the trained immunity features of identified γδ T-cell subpopulations, we calculated TIGs by comparing gene expression levels between T3m_LPS and T0_LPS (Fig. 5c). Cluster 1 showed the greatest number of TIGs, with 80% of them being downregulated. Although cluster 0 had the highest number of cells, it had the least number of identified TIGs compared to the other clusters, which may indicate that the majority (67%) of γδ T cells were not strongly trained (Fig. 5A and C).

To further investigate the functions of the identified TIGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed based on the up- and downregulated TIGs in each cluster (Fig. 5D and E and Supplementary Fig. 2). GO analyses of the upregulated genes in cluster 1 and cluster 2 identified GO terms related to "response to IFN-γ" and "IFN-γ mediated signaling" (Fig. 5D). The KEGG pathway analyses showed that upregulated TIGs in cluster 1 and cluster 2 were enriched in the "antigen processing and presentation" pathway (Supplementary Fig. 2A). In contrast, the downregulated TIGs in γδ T-cell cluster 1 were enriched in the "NOD-like receptor signaling" pathway (Supplementary Fig. 2B).

Next, we tested if γδ T cells interact with monocytes during the training process through cell–cell communication analyses. Monocytes are well known to undergo metabolic and epigenetic reprogramming by BCG, resulting in a trained immunity phenotype[8,17,42] as well as being potent activators of γδ T cells,[43–45] raising the possibility that the interaction between the 2 cell types is important for the formation of trained immunity. Therefore, we explored the interaction pairs where monocytes express the ligand and γδ T-cell subpopulations express the receptor (Fig. 6A–D). This analysis predicted that the *CXCL* family from monocytes were highly ranked ligands, which likely affected the TIGs in γδ T cluster 1 (Fig. 6A). Meanwhile, *IL* family from monocytes were potential ligands to regulate receptors (TIGs) in γδ T cluster 2 (Fig. 6b). *IFNG*, as one of the receivers, was involved in the cross-talk between monocytes and γδ T-cell cluster 2. Combined with the GO analyses (Fig. 5D), the upregulated TIGs were enriched in IFN-γ–related biological processes, suggesting that *IFNG* played an important role in γδ T-cell impact on monocytes during induction of trained immunity. Additionally, we focused on the interaction in which γδ T-cell subpopulations express the ligand, while monocytes express the receptor (Fig. 6C and D). We observed that *IFNG* was highly expressed in cluster 2 and interacted
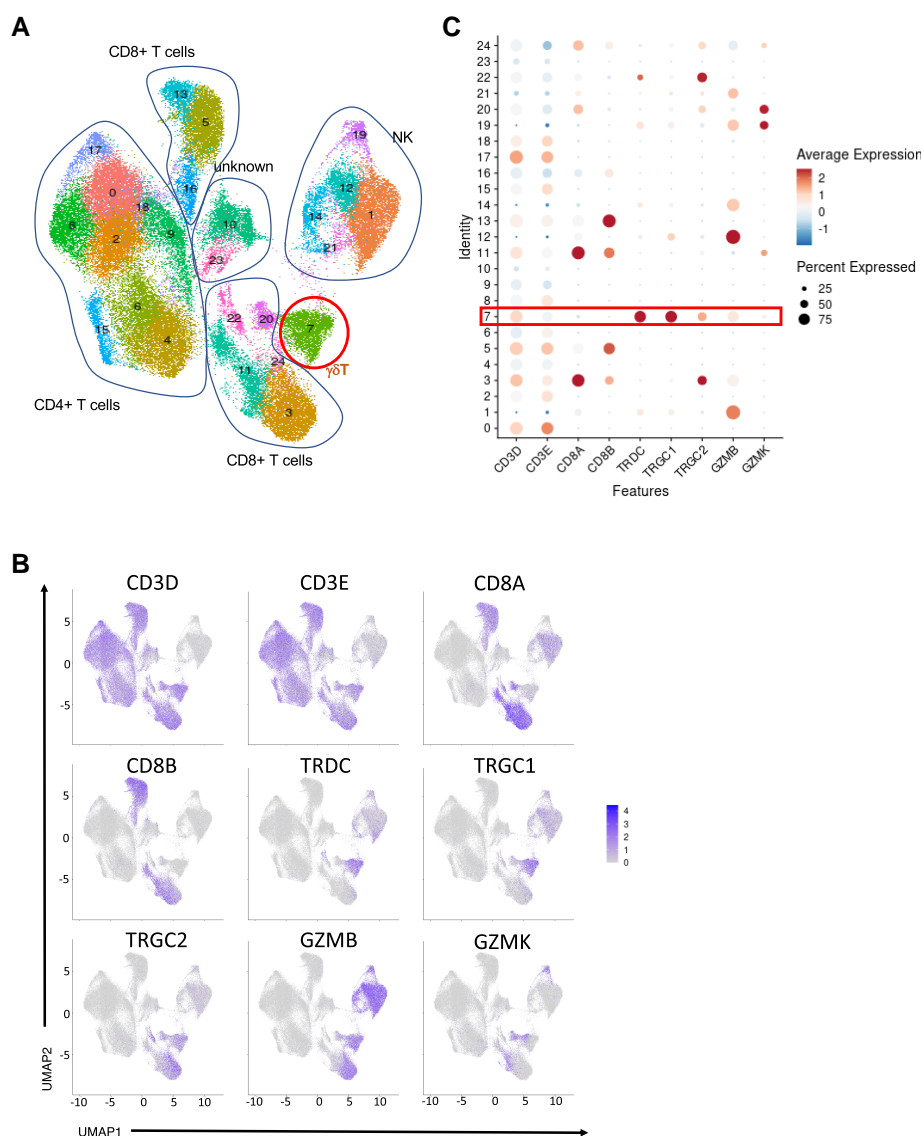
Fig. 4. Overview of γδ T cells in the 300BCG data set at day 0 before BCG vaccination and 3 mo postvaccination. (A) UMAP of CD4T, CD8T, and NK cells from 300BCG. (B) FeaturePlot of γδ T marker (CD3D, CD3E, TRDC, TRGC1, TRGC2, GZMB, GZMK) expression distribution. (C) γδ T marker expression level in each cluster.

with all the identified TIGs in monocytes. These analyses strongly suggest that after BCG vaccination, a subset of γδ T cells (but not all) displays an increase in responses to heterologous (nonmycobacterial) stimuli suggestive of induction of trained immunity, and 2 of the γδ T-cell subpopulations that possess features of trained immunity were related to IFN-γ–related pathways.

3.4 BCG vaccine enhances human γδ T-cell responses to heterologous stimuli

We next compared the γδ T-cell responses to in vitro stimulation in subjects before and after BCG vaccination (Fig. 7). We assessed the expression of activation markers CD25 and CD69 (data not shown) on γδ T cells, as well as cytokine production in response to the in vitro stimuli of PBMCs isolated from adults before (week 0) and 2 wk (week 2) and 12 wk (week 12) after BCG vaccination and compared to nonvaccinated controls (Fig. 7A). The fold change of CD69+ and CD25+ γδ T cells in response to all stimuli before and after vaccination was not different in BCG-vaccinated vs nonvaccinated

controls (Supplementary Fig. 3). In contrast, the fold change of IFN-γ–producing γδ T cells in response to *M. tuberculosis* in BCG-vaccinated volunteers 2 wk after vaccination was significantly increased compared to nonvaccinated controls, indicating the generation of specific immune memory by γδ T cells (Fig. 7B).

The heterologous responses of γδ T cells to nonmycobacterial stimuli followed a pattern previously described in BCG vaccination studies, in which only approximately half of the volunteers respond with a potent trained immunity phenotype.[46,47] The TNF and IFN-γ production capacity of γδ T cells after stimulation with *E. coli* and *S. aureus* was increased in 30% to 50% of the BCG-vaccinated individuals compared to controls (Fig. 7B and E). BCG vaccination significantly increased the TNF and IFN-γ production stimulated by *C. albicans* (Fig. 7C and D).

4 Discussion

Understanding the biological mechanisms and immune cell subsets involved in the induction of immunologic memory is essential
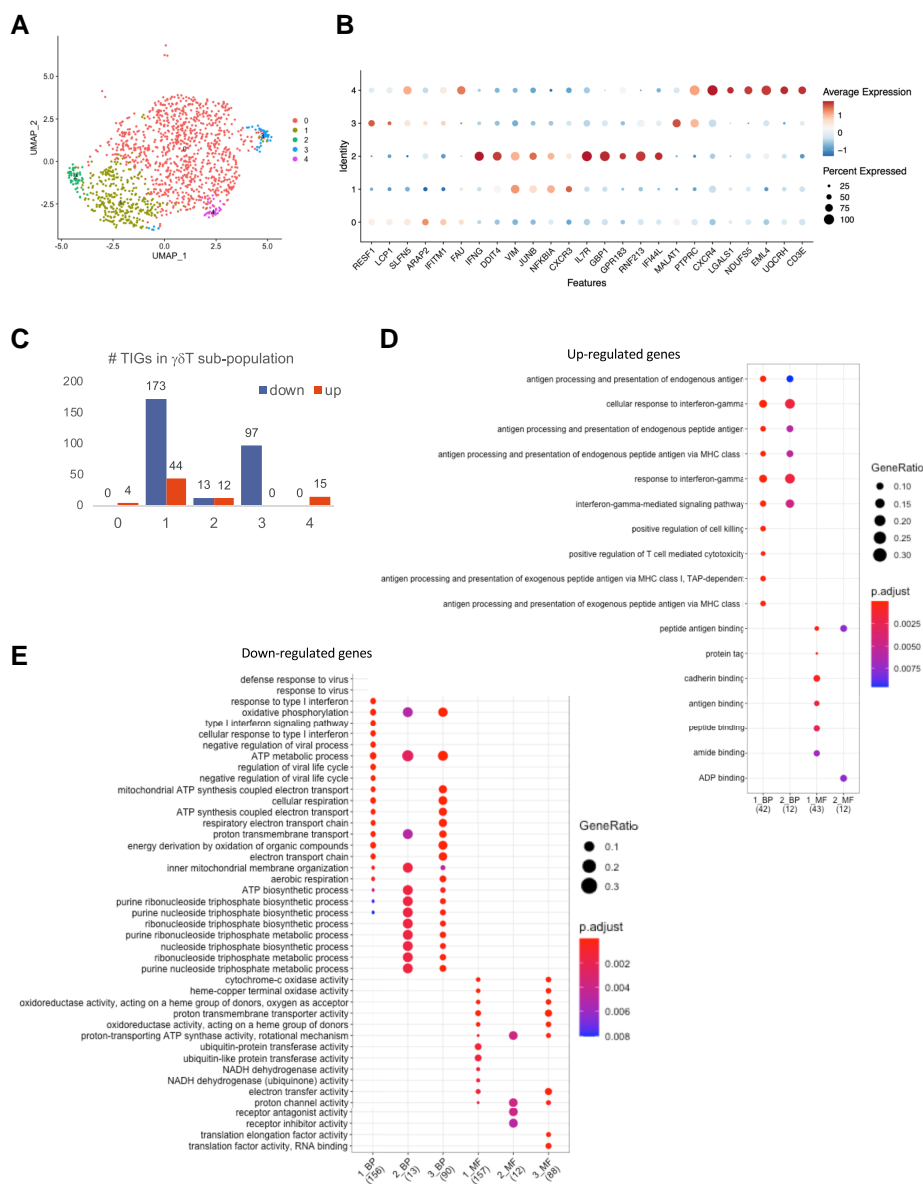
Fig. 5. Trained immunity characteristics of γδ T cells. (A) UMAP γδ T-cell subpopulation after being trained by BCG assessed 3 mo postvaccination. (B) Markers of identified subpopulations. (C) The number of TIGs in each subpopulation. (D) GO enrichment of upregulated TIGs. (E) GO enrichment of downregulated TIGs.

for the design and development of effective vaccines. For decades, immunologic memory was solely attributed to adaptive immune cells such as conventional T and B cells. Subsequently, these cells became the main target for vaccination strategies. The recent discovery that other cells of the immune system such as myeloid cells (monocytes, macrophages, neutrophils), NK cells, and innate-lymphoid cells can mount an enhanced response upon secondary stimulation[48] has opened a new avenue for the design of preventive measures against infections. Human γδ T cells have been long known to develop memory characteristics.[21,26,33] Despite the fact that they possess innate immune features (rapid and MHC-unrestricted activation, recognition of nonpeptide antigens, and expression of innate-like receptors), γδ T-cell memory has been mainly described in the pathogen-specific context,[26] with a very limited understanding about the nonspecific character of memory γδ T cells.[49] In this study, we address for the first time whether human peripheral blood γδ T cells can also display

memory responses to heterologous stimuli (trained immunity characteristics) after BCG vaccination.

BCG vaccination did not induce changes in γδ T-cell proportion 2 and 12 wk after vaccination. While various reports showed that γδ T cells expand in peripheral blood upon various types of infections[50] such as tularemia,[51,52] listeriosis,[53] ehrlichiosis,[54] toxoplasmosis,[55] malaria,[56,57] leishmaniasis,[58] and *Salmonella* infections,[59] there is no consistency between studies on *M. tuberculosis* infections. Some reports suggest an increased number of peripheral blood γδ T cells during the disease,[60,61] while other studies were not able to reproduce these findings.[62–65] Studies in macaques show that Vδ2 T cells indeed expand in peripheral blood upon BCG inoculation, with a sharp peak of expansion around days 14 to 30 and a decrease to steady-state cell numbers by day 40.[26,66] Intradermal inoculation of BCG in nonhuman primates slightly expands γδ T cells in peripheral blood only at high doses (100 times higher than used in our study).[66] It is therefore
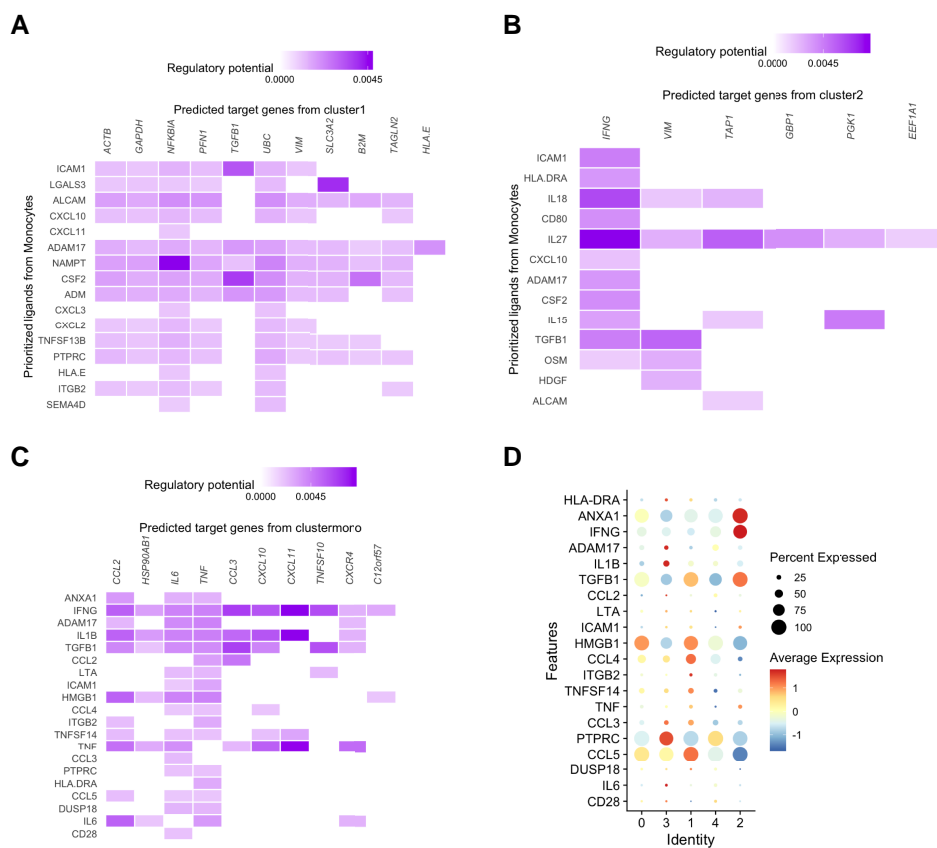
Fig. 6. Putative γδ T-cell interactions with monocytes assessed by scRNA-seq in PBMC cultures 3 mo after BCG vaccination. (A) Cell–cell interaction between monocytes (senders) and γδ T cluster 1 (receiver). (B) Cell–cell interaction between monocytes (senders) and γδ T cluster 2 (receiver). (C) Cell–cell interaction between γδ T subpopulation (senders) and monocytes. (D) The expression level of potential ligands in each γδ T-cell subpopulation.

not unexpected that regular intradermal BCG vaccination as performed in our study did not induce human γδ T-cell proliferation. This is similar to tularemia in which *Francisella tularensis* drives γδ T-cell expansion, while the tularemia vaccination does not.[52]

In contrast to the lack of an effect on the total number of γδ T cells, BCG vaccination resulted in increased production of the cytotoxic molecule perforin by γδ T cells, suggesting a functional reprogramming of these cells. This is in line with earlier studies that have shown that BCG can increase perforin production in in vitro PBMC cultures, bladder tissue in patients with cancer undergoing BCG therapy,[67] and peripheral immune cells of vaccinated infants.[68] However, to our knowledge, this is the first report showing the effect of BCG vaccination on perforin expression in human γδ T cells. Moreover, scRNA-seq of circulating immune cells stimulated before and 12 wk after BCG vaccination clearly shows strong changes in the transcriptional programs of γδ T cells. Interestingly, we identified 5 distinct γδ T-cell subpopulations when assessed based on the trained immunity responses. While cluster 0 contained the highest number of γδ T cells, this was also the cluster that showed the least changes after vaccination: this indicates that most (approximately two-thirds) of the γδ T cells are not trained. This is different from other innate immune cell types that are more potently trained after BCG vaccination (e.g. approximately three-quarters of monocytes undergo different trained immunity programs).[69] In contrast, trained immunity is induced in a smaller proportion of γδ T cells. While cluster 1 showed the greatest number of TIGs, 80% of them were downregulated after BCG vaccination. A very interesting pattern has been subsequently seen in cluster 2 of γδ T cells, in which *IFNG* was

highly expressed after BCG vaccination, as one of the main markers. In line with this, GO analyses of the upregulated genes in cluster 1 and cluster 2 identified GO terms related to "response to IFN-γ" and "IFN-γ mediated signaling." Consistently, a recent scRNA-seq analysis of in vitro BCG-treated PBMC cultures revealed an upregulated IFN-γ signature within γδ T cells.[70] KEGG pathway analyses showed that upregulated TIGs in cluster 1 and cluster 2 were enriched in the "antigen processing and presentation" pathway. These clusters might represent previously described γδ T cells with antigen-presenting properties.[66,67,71,72] The Vδ2 T cells pulsed with *M. tuberculosis* purified protein derivative acquired antigen-processing molecules and were able to induce proliferation of conventional αβ T cells in vitro.[66,67] Monocyte–γδ T-cell interaction analysis also predicted that *IFNG* played an important role in γδ T-cell impact on monocytes during induction of trained immunity. Previous studies have indicated that mycobacteria-infected human monocytes are potent in activating γδ T cells by an efficient antigen presentation.[65,66] Upon *M. tuberculosis* stimulation, antigen presentation and costimulation exerted by monocytes are also essential for triggering IFN-γ production by γδ T cells.[73] Here, our data confirmed the importance of this interaction not only for γδ T-cell activation but also for the induction of γδ T-cell trained immunity. In addition, we report for the first time a list of specific ligands that may induce such interaction. Because of the presence of various immune cells in the PBMC cultures, we do not exclude the possibility that interactions with other cells are important for the induction of trained immunity in γδ T cells. The identification of the molecular mechanism of trained immunity induction by these
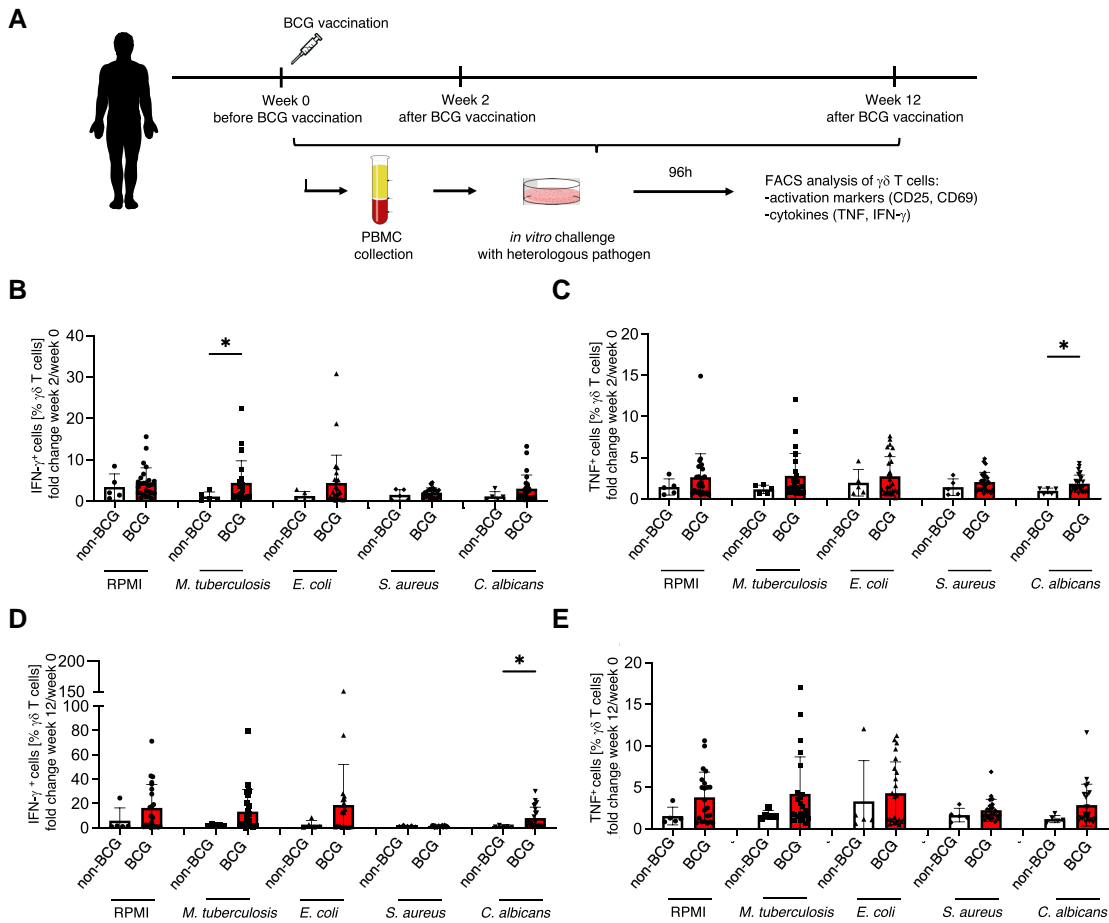
Fig. 7. BCG vaccination displays a tendency to induce innate immune memory in peripheral blood γδ T cells. (A) Experimental layout of the BCG vaccination trial. (B) Fold change of TNF-producing γδ T cells 2 wk after BCG vaccination over week 0 before vaccination. (C) Fold change of IFN-γ–producing γδ T cells 2 wk after BCG vaccination over week 0 before vaccination. TNF⁺ and IFN-γ⁺ γδ T cells were assessed by flow cytometry 96 h after in vitro culture with indicated pathogens or RPMI medium alone. (D) Fold change of IFN-γ–producing γδ T cells 12 wk after BCG vaccination over week 0 before vaccination. (E) Fold change of TNF-producing γδ T cells 12 wk after BCG vaccination over week 0 before vaccination. IFN-γ⁺ and TNF⁺ γδ T cells were assessed by flow cytometry 96 h after in vitro culture with indicated pathogens or RPMI medium alone. Mann–Whitney test, *$P < 0.05$. Nonvaccinated volunteers, $n = 6$; BCG-vaccinated volunteers, $n = 26$.

interactions requires further investigation. Altogether, the scRNA-seq analysis strongly suggested induction of a trained immunity program in γδ T cells, with the IFN-γ pathway likely to play an important role in their enhanced function.

The functional assessment of γδ T-cell responses supports this observation. Stimulation of PBMC cultures with heterologous heat-killed pathogens showed significantly increased numbers of IFN-γ–producing γδ T cells 2 wk after BCG vaccination, supporting the induction of a trained immunity phenotype of γδ T cells. BCG-expanded γδ T cells may be also effective in lysing cancer cells, as suggested by the recent report on in vitro BCG-treated PBMC cultures, indicating a secondary responses to heterologous stimuli.[70] It has to be noted, however, that an effective induction of trained immunity after bacterial stimulation was observed only in 30% to 50% of the individuals, similar to earlier observations during BCG vaccination in experimental models of malaria[74] or yellow fever vaccination.[47] In contrast, significant increases in TNF- and IFN-γ–producing γδ T cells have been observed in response to *C. albicans* stimulation. The smaller effect of BCG vaccination on γδ T cells in this study might be due to the adult age of the trial participants, as it is debated whether the BCG vaccine has a weaker effect in adults than in children.[75,76] Consistent with that hypothesis, in vitro BCG stimulation of PBMCs from

BCG-vaccinated children leads to a robust expansion of γδ T cells.[77] Furthermore, comparative studies show that γδ T-cell memory responses are more significant in individuals vaccinated at a neonatal age than in adulthood.[34] It is therefore of great importance to address the memory formation potential of γδ T cells in newborns in order to understand the heterogeneity of the efficacy of the BCG vaccine.

One additional important aspect to be considered is also the use of different strains of the BCG vaccine, with considerable differences reported between the immunomodulatory effects of various BCG strains.[78] The BCG-Bulgaria strain used in our study, which is similar to BCG-Russia used in 70% of the countries worldwide, showed a weaker effect on cytokine production in whole blood cell cultures than BCG-Denmark,[79] and this should be considered in the interpretation of our results. Indeed, scRNA-seq analysis of PBMC cultures treated in vitro with 2 different strains of BCG—namely, iBCG (BCG-Moreau) and BCG-Tice (OncoTICE)—revealed distinct effects on NK and γδ T-cell populations.[70] While iBCG was potent in activating and expending γδ T cells, BCG had a boosting effect on NK cells. Future studies are warranted to assess the impact of other BCG strains on γδ T cells.

It needs to be noted that we addressed γδ T-cell responses in terms of cytokine production, while most of the reports address

proliferative responses. As both are an indication of effector function, our data suggest that the 2 processes are uncoupled. Because of sample limitation, we focused our analysis on cytokine production as we believe it is of bigger relevance for the effector function. An additional limitation of the study is that our experimental set-up did not include the discrimination between Vδ1 and Vδ2 γδ T cells. However, because most γδ T cells in human peripheral blood are Vδ2 T cells,[29] which are also the subpopulation responding to BCG, we believe that the observed effect of vaccination on the γδ T-cell population is mainly attributed to Vδ2 T cells.

Interestingly, we found that *S. aureus* and *C. albicans* are potent activators of human peripheral blood γδ T cells based on CD69 and CD25 upregulation, as well as TNF and IFN-γ expression. While mouse γδ T cells are known to be involved in the defense against *S. aureus*[80–87] and *C. albicans* infections,[83,84,88,89] there are few studies addressing human γδ T-cell activation by these pathogens.[32,85–87] These studies reveal a proliferative response of human γδ T cells to *S. aureus* stimulation[32] and, in line with our findings, IFN-γ production, mainly by Vδ2 T cells.[90] However, this study reveals a strong proliferative response and a strong upregulation of IFN-γ and IL-17 of Vδ1 but not Vδ2 T cells to *C. albicans* in vitro.[91,92] Whether the cytokine-producing γδ T cells are Vδ1 or Vδ2 in our cultures has not been addressed and remains to be determined. Future studies should also investigate the molecules (HMBPP, β-glucans, TLR ligands, etc.), receptors, and signaling pathways involved in γδ T-cell stimulation by these microorganisms. As increasing evidence is pointing to the role of γδ T cells in the immune response against *S. aureus* and *C. albicans* infection in humans, others' and our observations pave the way for investigation of the function of these cells in candidiasis and *Staphylococcus* infections and the development of new therapeutic approaches.

In conclusion, we report induction of a trained immunity phenotype in γδ T cells in vivo in adults, although large interindividual variations are present. Because newborns contain truly naive γδ T cells, conducting a similar assessment in cord blood or blood from young children will provide the answer to whether human γδ T cells can develop stronger innate immune memory in childhood. Furthermore, our human study is limited in the capacity to provide causality proof due to inherent ethical limitations and can only characterize γδ T-cell trained immunity responses. However, based on the molecular mechanisms known to drive trained immunity phenotype in myeloid cells,[42] one can speculate that metabolites such as HMBPP produced by BCG and known to activate human peripheral γδ T cells[93,94] or other BCG components induce metabolic and epigenetic reprograming of these cells. As a result of metabolic and epigenetic changes induced by a primary trigger, the cells are in a more poised state to respond. Therefore, upon the secondary stimulation by unrelated pathogens/their components, γδ T cells produce elevated levels of effector molecules. These could be driven by the γδ TCR signaling but also by innate-like receptors such as TLRs present on the surface of γδ T cells.[95] Importantly, we did find that *S. aureus* and *C. albicans* are very potent stimulators of human peripheral blood γδ T cells. This observation might be of clinical relevance as candidemia is a major cause of morbidity and mortality in health care settings,[96] while staphylococcal infections are an increasing threat due to the emergence of multidrug-resistant strains.[97] Whether γδ T cells can be a new therapeutic or diagnostic tool to fight these infectious needs further investigation.

Authorship

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

Funding

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MMR vaccination induces a trained immunity program characterized by functional and metabolic reprogramming of γδ T cells

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Abstract

The measles, mumps and rubella (MMR) vaccine protects against all-cause mortality in children, but the immunological mechanisms mediating these effects are poorly known. We systematically investigated whether MMR can induce long-term functional changes in innate immune cells, a process termed trained immunity, that could at least partially mediate this heterologous protection. In a randomized placebo-controlled trial, 39 healthy adults received either the MMR vaccine or a placebo. By using single-cell RNA-sequencing, we found that MMR caused transcriptomic changes in CD14-positive monocytes and NK cells, but most profoundly in γδ T cells. Surprisingly, monocyte function was not altered by MMR vaccination. In contrast, the function of γδ T cells was significantly enhanced by MMR vaccination, with higher production of TNF and IFNγ, as well as upregulation of cellular metabolic pathways. In conclusion, we describe a new trained immunity program characterized by modulation of γδ T cell function induced by MMR vaccination.

One-sentence summary: 125 characters including spaces

MMR vaccination induces cellular and metabolic reprogramming in γδ T cells towards a more active phenotype.

INTRODUCTION

60   Vaccines are developed to target specific pathogens. However, an accumulating body of evidence suggests that certain live-attenuated vaccines provide a broad spectrum of protection against heterologous infections as well (non-specific beneficial effects; NSEs)(*1, 2*). Vaccine-induced NSEs are accompanied by epigenetic and metabolic changes in innate immune cells, also known as trained immunity (*3-6*). Recent studies suggest that induction of trained immunity is an attractive strategy to

65   boost broad protection against infections and as anti-cancer therapy (*7*). Most of the studies aiming to study trained immunity induced by vaccines in humans have used the tuberculosis vaccine Bacille Calmette-Guérin (BCG) (*8-10*), while very little is known about the effects of other vaccines on innate immune cells. Although there is ample epidemiological evidence that other live attenuated vaccines also have NSEs (*2*), their potential effects on innate immune cells have not been studied.

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Measles containing vaccines (MCVs) are one such group of vaccines associated with beneficial heterologous effects (*11*). Measles vaccines are routinely used in childhood immunization programs worldwide and were recently re-confirmed to be safe and effective (*12*). MMR vaccine is composed of a live-attenuated negative stranded measles virus, combined with mumps and rubella, and provides

75   life-long immunity against measles after two doses. Several studies have confirmed higher child survival and lower morbidity after measles immunization, independent of measles-attributable disease (*13, 14*). This suggests that MMR induces trained immunity that can provide broad heterologous protection (*15, 16*). Here, we assess the potential of MMR vaccination to induce trained immunity against SARS-CoV-2 and a range of other microbial stimuli. We used single-cell multi-'omics approaches

80   to compare cellular heterogeneity in a randomized placebo-controlled trial of MMR re-vaccination in Dutch adults. Surprisingly, we found that MMR vaccination caused transcriptomic and functional changes in γδ T cells, rather than monocytes. This suggests that γδ T cells might have a key role in the mechanisms underlying MMR-induced trained immunity.

RESULTS

Study design and baseline characteristics

To investigate the potential non-specific effects of MMR re-vaccination in adults, we conducted an exploratory randomized controlled trial (**Fig. 1A**; see methods for more details). Briefly, thirty-nine healthy adults (19 female and 20 male) were randomly assigned to receive either the MMR vaccine or a placebo. All participants were between 18 and 50 years of age and there were no significant differences in sex, age, or BMI between the vaccination arms (**Fig. S1**; **Table S1**). Peripheral venous blood was collected to conduct immunological analyses was collected immediately before vaccination and one month later. No infections occurred between visits or at least two weeks prior to the baseline measurement.

Circulating biomarkers of inflammation after MMR vaccination

Previous studies have shown that trained immunity induced by BCG increases the responsiveness of innate immune cells upon rechallenge, but reduces systemic inflammation during homeostasis(*8*). We sought to investigate if this is also the case after MMR vaccination. To that end, we used Proximity Extension Assay technology (Olink) to assess targeted proteomics biomarkers (which have previously been related to inflammation, oncology, neurology, or cardiometabolic function) before and after MMR vaccination (**Fig. 1B**). There were no major changes in plasma proteome composition between the baseline measurement and 1 month after vaccination. Of all analyzed parameters (1289 after quality control), only 4 met our selected cutoffs for log2 fold change > 0.5 and unadjusted p-value < 0.05. These were PPY (a pancreatic protein associated with counter-regulation of gastric emptying(*17*)), S100A12 (a calcium-binding alarmin protein(*18*)), TMPRSS15 (a peptidase known to activate trypsin(*19*)), and CALCA (a vasodilating peptide hormone involved in calcium regulation and thought to also function as a neurotransmitter(*20*)).

We subsequently assessed the proteins that met the statistical significance threshold, independently of the fold change (**Fig. 1C-F**). Of the protein subcategories, the inflammation-related proteins showed the highest number of changes, with a trend towards upregulation after vaccination (**Fig. 1C**).

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PNLIPRP2, the top significant hit from this sub-panel, is a pancreas-associated protein involved in lipid metabolism. Among the other enriched proteins were factors related to NK cell and T cell activities (IL-12B, IL-15, IFNγ, CCL20, GZMA). Of the cardiometabolic-related proteins (**Fig. 1D**), most suggestive hits were also related to immunological processes either directly (GP2, CST6, NCAM1, MARCO) or indirectly (GH1). The proteins significantly changes in the oncology panel (Fig. 1E) were similarly enriched for immunologically relevant proteins (S100A12, PQBP1, CCL8, FCGR2B), with a trend towards upregulation after vaccination. Finally, in the neurology panel (**Fig. 1F**) there was also an upregulation of immunology-related proteins such as CCL2, VSTM1, and PLA2G10. These results indicate that inflammation-related proteins tend to be upregulated in the circulation after MMR vaccination, although the effect size is relatively limited. However, in terms of the effects of MMR vaccination on the systemic inflammatory status, this differs significantly from the inhibitory effects exerted by BCG vaccination.

We then considered the effects of MMR vaccination on circulating leukocyte counts (**Fig. 1G**). MMR vaccination, but not placebo treatment, significantly increased (p = 0.04) the number of circulating leukocytes after one month, although there was a large inter-individual variation. This change appeared to be driven mainly by an increase in myeloid cells (neutrophils and monocytes), although the comparison did not reach statistical significance for the individual cell types. Together, our results show that MMR-vaccinated individuals present with slightly increased systemic inflammation one month after vaccination.

Transcriptome effects in monocytes and γδ T cells after MMR vaccination

We decided to further investigate the effects of MMR vaccination on the composition and cellular states of circulating leukocytes. To that end, we performed single-cell (sc)ATAC-seq and scRNA-seq on peripheral blood mononuclear cells (PBMCs) of a subset of participants. For scATAC-seq, we selected 12 participants from both the placebo and MMR-treated groups. We then selected half of those

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individuals to also perform scRNA-seq, taking care to balance male and female participants from both the MMR and placebo groups.

An integrated analysis of both scRNA-seq and scATAC-seq data (**Fig. 2A, Fig. S2A-B**) based on marker genes enabled us to identify 13 cell types within the PBMC fraction (**Fig. S2C**). Since the white blood cell differential (**Fig. 1G**) suggested there might be changes in the PBMC composition, we leveraged the single-cell datasets to investigate this in more detail. Indeed, we observed intra-individual shifts in PBMC composition between timepoints (**Fig. , Fig. S3A**). However, we did not observe any consistent changes in either the MMR or placebo group (**Fig. 2B, S3A-B**). Although the low number of participants hindered statistical comparisons, our data suggest that it is unlikely that MMR vaccination has a major influence on PBMC composition one month after vaccination.

Previous studies have shown that vaccination with other live-attenuated vaccines such as BCG induces transcriptional and functional changes (trained immunity) in innate immune cells such as monocytes (*21, 22*). We therefore hypothesized that although the PBMC composition was not altered by MMR vaccination, changes in monocyte transcriptional and functional programs could account for its known NSEs, and possibly also for our observation that MMR increases systemic inflammation. We thus assessed the transcriptional effects of MMR and placebo between timepoints, across different cell types. In every cell type except CD4$^+$ T cells, MMR vaccination had a more pronounced transcriptional effect than placebo administration (**Fig. 2C**; top panel). Indeed, CD14$^+$ monocytes and NK cells, classical executors of BCG-induced trained immunity, were prominently influenced by MMR. Surprisingly however, γδ T cells were the most strongly affected cells at transcriptional level among all assessed cell types. Cell-type specific analysis of differentially accessible genes revealed the chromatin to be impacted by MMR vaccination only in CD14$^+$ monocytes (**Fig. 2C**; bottom panel).

MMR has minor effects on the transcriptome and epigenome of monocyte subpopulations

Exploring further the monocyte sequencing data, we found different subpopulations defined by their transcriptional or open-chromatin signatures. Among them, we found a subpopulation highly expressing HLA genes and another one upregulating alarmins, in both data layers (**Fig. 3A**). We did not

identify significant changes in the subpopulations in either data layer (**Fig. S3A**). Investigating the transcriptional changes induced by MMR, we identified more than 20 differentially expressed genes

165 (adjusted p-value < 0.05) and 7 genes with changed chromatin accessibility (**Fig. S4C**). Upregulated genes were enriched in pathways associated with response to mechanical stimulation or exposure to metals such as calcium (*FOS*, *FOSB*, *JUN*, *JUNB* and *NFKBIA*). Downregulated genes were enriched in cell-cell adhesion programs (*B2M*, *FGL2*, *CD46*, *PRKAR1A*) (**Fig. 3B,C**). Thus, while monocytes were moderately affected at the transcriptional level by MMR vaccination, these cells were not explicitly

170 more pro-inflammatory during homeostasis.

Cytokine production capacity of PBMCs after MMR vaccination

Innate immune memory is defined as the long-term functional reprogramming of innate immune cells by a first stimulation, leading to an altered response towards re-stimulation. To investigate functional immune responses after MMR vaccination or placebo, we stimulated PBMCs for 24 hours with a variety

175 of bacterial (LPS, heat-killed *Staphylococcus aureus*), fungal (heat-killed *Candida albicans*), and viral (poly(I:C), R848, Influenza A (H1N1), SARS-CoV-2) stimuli, and measured monocyte-associated cytokine responses by ELISA. We measured TNF, IL-6, and IL-1RA for all stimuli. Additionally, we measured IP10 and IFNα specifically for the viral stimulations. We calculated log2-transformed fold changes corrected for age, sex, and BMI between one month after vaccination and baseline measurements, and

180 statistically compared placebo treatment to MMR vaccination (**Fig. 3B-H**). We observed large intra- and inter-individual variations in cytokine responses between baseline and one month after treatment. However, there were no significant differences between placebo and MMR for any measured cytokine across all stimuli. Thus, we found no differences in PBMC cytokine production capacity after MMR vaccination. As monocytes are the main producers of the measured cytokines in PBMCs during 24-

185 hour stimulations, this suggests that monocyte cytokine secretion capacity is not changed by MMR vaccination.

Transcriptional and functional reprogramming of Vδ2 T cells after MMR vaccination

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Because the most prominent changes in response to MMR were seen in γδ T cells according to the scRNA-seq data (**Fig.2**), in the next set of experiments we assessed whether vaccination changed the transcriptional and functional programs of these cells. A closer look into the γδ T cell subpopulations revealed differences in their transcriptional and open-chromatin dynamics. Transcriptionally, two distinct subpopulations were found, one characterized by transcription of granzyme genes, and the other by upregulation of IL7R (**Fig. 4A**; left). Integrating open-chromatin landscape with their transcriptional profiles from the same individuals led to differentiating Vδ1 (higher in GZMB and GZMH) and Vδ2 (higher in GZMK) T cell populations (**Fig. 4A**; right). Notably, the vaccination did not affect the proportion of the subpopulations identified (**Fig. S5A-B**). Interestingly however, MMR induced a metabolic shift in γδ T cells at the transcriptional level, with downregulation of genes involved in cellular respiration and ATP synthesis (**Fig. 4B-C**), exemplified by *NDUFA3*, *ATP5F1E*, *ATP5MD*, and *ATP5MG*. We subsequently investigated the functional consequences for Vδ2 T cells (the most abundant γδ-T cell population in the blood) by flow cytometry.

There was a small but significant decrease in Vδ2 T cells present in the PBMC fraction of MMR-vaccinated individuals (p = 0.0425); a pattern that was not present in the placebo controls (**Fig. 5A**). On the other hand, we did not find any significant differences in relative abundance of subpopulations expressing CD27 and/or CD45RA, indicating MMR did not have strong effects on classical memory-Vδ2 T cell formation (**Fig. S6A**). There were also no changes in the expressions of CTLA4, PD1, LAG3, and TIM3, markers that are commonly associated with T cell dysfunction or exhaustion. Notably, following stimulation of the γδ T cell receptor using anti-CD3/anti-CD28 beads, the percentage (but not the mean fluorescence intensity [MFI]) of Vδ2 T cells positive for TNF or IFNγ increased (**Fig. 5B, Fig. S6B**). This indicates that the γδ T cell population has become more responsive towards secondary stimulation, a feature resembling the classical monocyte trained immunity. Unstimulated Vδ2 T cells showed a trend towards lower expression of the degranulation marker CD107a after MMR vaccination (**Fig. S6C**); this effect was not present in CD3/CD28-stimulated Vδ2 T cells, which showed no difference between timepoints in the percentage of cells staining positive for CD107a, granzyme B, or perforin (**Fig. 5C**).

215   These data suggest that granule release by Vδ2 cells is more tightly regulated at baseline following

MMR vaccination, but that this effector function is not weaker following stimulation.

The single-cell analyses revealed that unstimulated γδ T cells modulate genes associated with oxidative

phosphorylation (**Fig. 4B-C**). Therefore, we determined the functional metabolism profile of Vδ2 T cells

before and after MMR vaccination using SCENITH (*23*)). This flow cytometry-based technique uses

220   puromycin incorporation as a proxy for protein synthesis activity, which in itself reflects a significant

portion of total ATP used by the cell. Briefly, we measured protein synthesis levels (puromycin MFI;

Fig. 5D) upon treatment with metabolic inhibitors. This allowed us to calculate fatty acid/amino acid

oxidation capacity (**Fig. 5E)**, glycolytic capacity (**Fig. 5F**), mitochondrial dependence (**Fig. 5G**), and

glucose dependence (**Fig. 5H**) of Vδ2 T cells. These experiments revealed a trend towards higher

225   protein synthesis activity ($p = 0.203$) in unstimulated Vδ2 T cells following MMR vaccination. The same

pattern was visible following stimulation with isopentenyl pyrophosphate (IPP, a common antigen

specifically for Vδ2 T cells; $p = 0.129$), and was statistically significant following CD3/CD28 stimulation

($p = 0.00391$). The metabolic processes fueling this change remained unclear however, as only

CD3/CD28-stimulated Vδ2 T cells had changed metabolic parameters: a decrease in glycolytic capacity

230   and thus a concomitant increased dependence on mitochondrial energy metabolism. In conclusion,

these data show, for the first time, transcriptional and functional changes consistent with induction of

trained immunity in γδ T cells following MMR vaccination.

DISCUSSION

235   Trained immunity entails the process of boosting innate immune function following vaccination or

infection (*4*), and this process has been proposed to mediate at least in part the heterologous

protective effects of live attenuated vaccines such as BCG or MMR. While extensive studies have

documented induction of trained immunity by BCG (including but not limited to (*3, 5, 6, 8-10, 22, 24,

25*)), nothing is known regarding the capacity of MMR vaccination to induce trained immunity. We

240   performed a randomized, placebo-controlled trial investigating the potential of the MMR vaccine to

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induce innate immune memory. Using single-cell multi-omics transcriptional and epigenetic analysis combined with functional immunological and metabolic assays, we show that MMR vaccination induces a trained immunity phenotype in γδ T cells, while it has limited effects on monocyte function.

245 Most studies on the heterologous protection induced by certain vaccines such as BCG or influenza have focused on the induction of trained immunity in myeloid cells (*3, 26*). MMR vaccination has beneficial heterologous effects on overall mortality in children, therefore, we hypothesized that it also induces trained immunity. MMR induced some modest changes in the chromatin accessibility and transcriptional programs of monocytes, related to cellular responses to metal ions (specifically calcium; upregulated) and cellular adhesion (downregulated). However, these transcriptional effects did not result in significant effects on monocyte-derived cytokine production, despite the well-known role of calcium-dependent signaling in the function of immune cells (*27-29*). Future studies should be conducted to analyze in more depth the role of these pathways in monocytes after MMR vaccination, and if these changes are functionally relevant.

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Instead, we discovered that MMR induced much stronger transcriptomic and functional changes in the innate lymphoid population of γδ T cells. In this respect, MMR vaccination modulated the expression of genes involved in aerobic energy metabolism. We sought to functionally validate these findings and therefore closely examined Vδ2 T cells, the most abundant γδ T cell subpopulation in human peripheral blood. MMR vaccination preceded a significant increase in the proportion of Vδ2 T cells producing TNF and IFNγ, and these cells were more metabolically active, especially after CD3/CD28 stimulation. Our findings indicate that γδ T cells have a more activated phenotype in MMR-vaccinated individuals, providing a plausible mechanistic explanation for the NSEs conferred by MMR.

265 Because of their non-canonical antigen-recognition mechanisms, the exact receptors and pathways that trigger the effects of MMR vaccination on γδ T cell transcriptome and function remain to be

investigated by future studies. Previous gene expression and functional analyses demonstrate that γδ T cells have hybrid innate- and adaptive immune functions; the single-cell transcriptome of these cells has similarities to both CD8+ T cells and NK cells (*30*). Their innate-like features encompass the ability

270   to mediate antibody-dependent cellular cytotoxicity, phagocytose pathogens, and direct rapid non-specific responses against threats (*31*). On the other hand, classic adaptive features include somatic recombination of their functional T cell receptor, memory cell formation, and professional antigen-presenting capabilities (*32*). Unlike classical αβ T-cell receptor signaling antigen-recognition by γδ T cells is not MHC-restricted (*33*). Vδ2 T cells, for example, predominantly recognize phosphoantigens

275   such as isopentenyl pyrophosphate, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) in the context of butyrophilins 2A1 and 3A1 ((*34*)), and tetanus toxoid (*35, 36*). In cancer immunology, γδ T cells are known to exert strong anti-tumor effects by the release of pro-inflammatory cytokines, granzymes, perforin and via activation of apoptosis-triggering receptors (*36*), although pro-tumor effects have also been described ((*37*)). Interestingly, previous studies have suggested that IFNγ-

280   producing γδ T cells are more dependent on glycolysis than on oxidative metabolism (*38*), whereas our analyses show increased reliance on mitochondrial metabolism and simultaneously an increase in IFNγ-producing Vδ2 T cells. This contrast could potentially be explained by the differences in used models: our study uses human samples rather than mice, and the previous work was done in a cancer model. Future studies need to confirm our results and assess the full array of pathways and functional

285   consequences induced by MMR on γδ T cells.

Our data have several practical and theoretical implications. On the one hand, the differences between the BCG-induced (myeloid-dependent) and MMR-induced (lymphoid-dependent) trained immunity programs demonstrate that different vaccines can induce different types of innate immune memory.

290   Remarkably, even when considering effects on lymphoid cells with innate properties, a major difference emerges between MMR-induced effects on γδ T cells rather than BCG effects on NK cells. On the other hand, demonstrating that MMR is also able to induce trained immunity could lead to the

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hypothesis that it may have beneficial heterologous effects in other groups of individuals with increased susceptibility to infections. Indeed, MMR has been proposed as a potential approach to

295   prevent COVID19 in the period before the SARS-CoV-2 specific vaccines were available (*39, 40*). In a case-control study during a recent measles outbreak, a reduced COVID19 incidence was detected in men vaccinated with MMR (*41*).

Not only induction of trained immunity has been proposed as a mechanism explaining the

300   heterologous effects of MMR on COVID-19. An inverse correlation between COVID-19 disease severity and MMR-specific IgG-titers was found in adults in the United States (*42*). A potential explanation for this observation could lie in cross-reactivity against structurally similar components of SARS-CoV-2 and MMR epitopes, which was described by Marakosova et al (*43, 44*). However, this cannot account for the entire breadth of protection offered by MMR vaccines and these studies did not investigate the

305   potential impact of MMR on innate immune cells.

Our study also has some limitations. First, the sample size was limited due to the exploratory nature of this investigation, which barred us from investigating the host and environmental factors that impact these effects of MMR vaccination. Future research should encompass an increased number of

310   participants and a broader range of study parameters such as microbiome constituents, epigenetic histone modifications, and more follow-up timepoints, similar to the large-scale vaccination studies performed with BCG (*8, 25*). Second, while NK cell activation related inflammatory proteins were increased in the serum of MMR-vaccinated individuals, we could not substantiate this finding with functional NK cell experiments due to limitations in the cell numbers available. Third, it is unknown

315   which MMR components triggered γδ T cell activation, or if they reacted for example upon interaction with other activated cells. Moreover, in this study we in fact re-vaccinated adults who had previously received the MMR vaccine as part of the Dutch national vaccination program. Future studies should investigate if primary MMR vaccination has similar effects.

320   In conclusion, MMR is the first described vaccine that induces a program of trained immunity based on long-term transcriptional and functional changes of γδ T cells. The immunological and metabolic cellular responses to MMR reveal γδ T cells as a novel population of innate-like cells that mediate trained immunity. Our findings warrant further research to investigate the possibility that γδ T cells activation may be a component of trained immunity programs of other vaccines as well, and to assess

325   the potential to improve vaccine efficacy by inducing these effects.

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RJR, PAD, and JBB contributed equally to this work, and each has the right to list themselves first in author order on their CVs.

CRediT statement:

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Formal analysis: JBB, RJR, PAD, OB, GK, VACMK
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Supervision: YL, MGN
Verification: RJR, JBB, TKS, KP
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Writing – review & editing: RJR, PAD, JBB, MGN

Competing interests

MGN is a scientific founder and member of the scientific advisory board of Trained Therapeutix Discovery.

Data and materials availability

The sequencing data used in this manuscript will be made accessible in the EGA archive (EGAS00001006787). Olink data will be added as a supplementary file. Other data are available upon request to the corresponding author.

Supplementary materials

Materials and methods
Figs. S1 – S6
Tables S1 – S3
References: 45-55

18
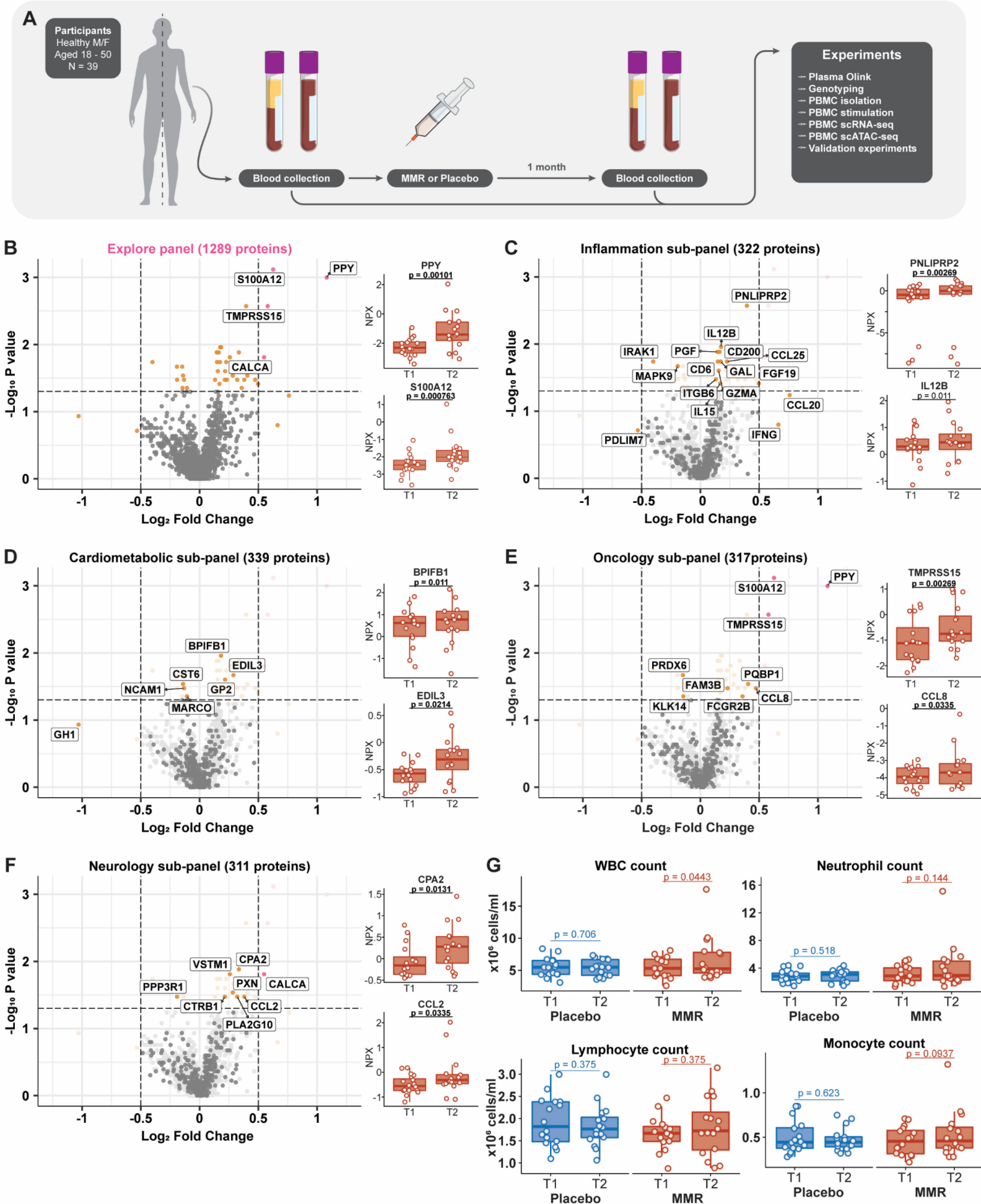
Fig. 1. Study setup, plasma proteomics analysis after MMR vaccination, and white blood cell counts.
(A) Setup of the present randomized, placebo-controlled trial of MMR vaccination. (B) Volcano plot of
Olink targeted proteomics (1289 analyzed proteins in total) in plasma, after MMR vaccination (n = 16).
(C-F) Volcano plots of sub-categories of the plasma proteome measured by proximity extension assay
technology (Olink). In volcano plots for sub-panels, the full panel is depicted in light gray as the
background. The side plots of B-F show the relative expression values (NPX) of selected proteins in
each (sub)panel. (G) Total- and differentiated white blood cell counts (WBC), before and after
vaccination in placebo and MMR-vaccinated groups. The p-value cutoff for the volcano plots is
unadjusted $p < 0.05$. WBC, white blood cell; T1, baseline; T2, one month after treatment.

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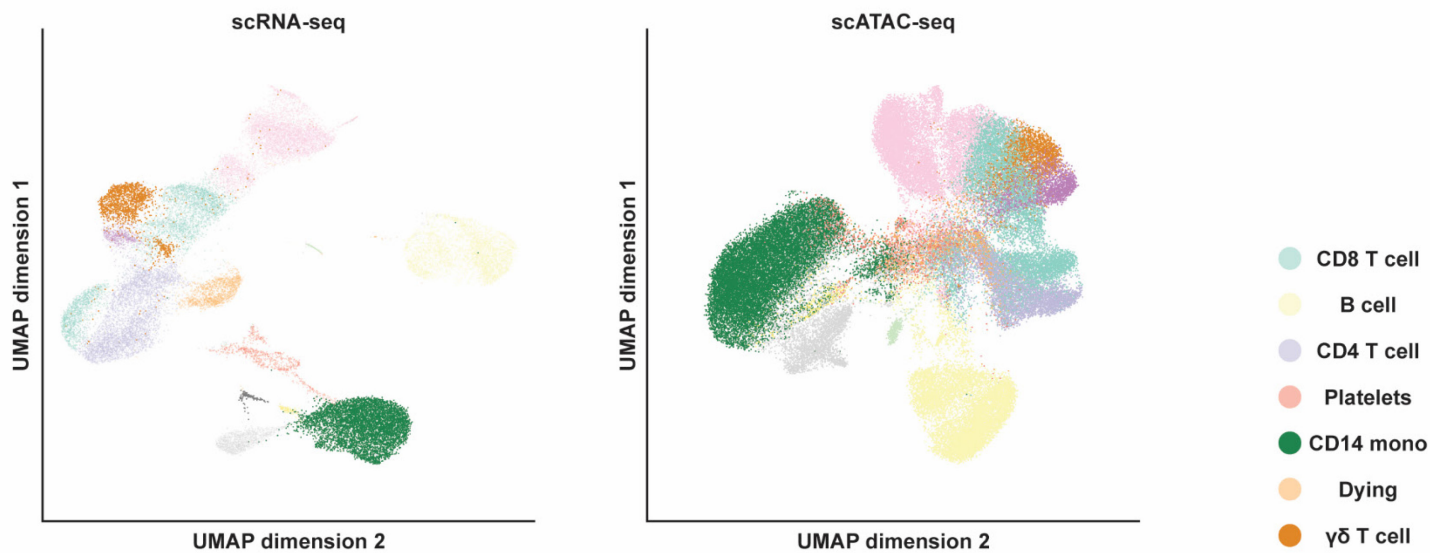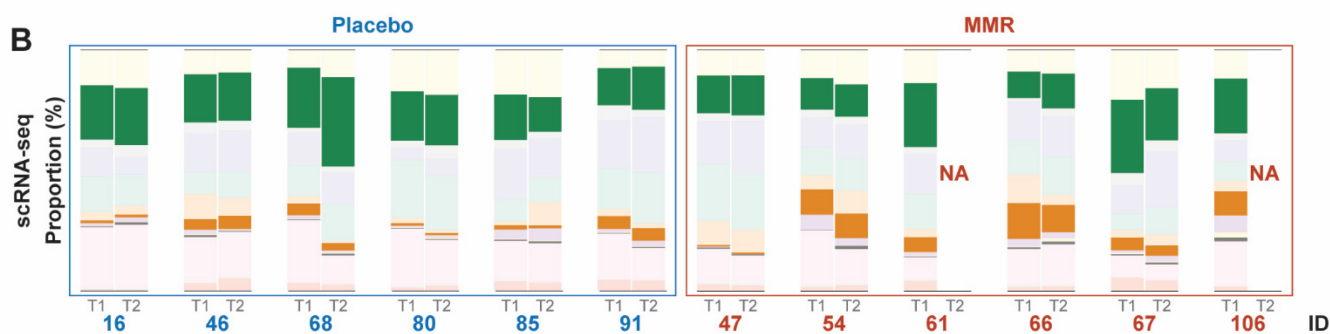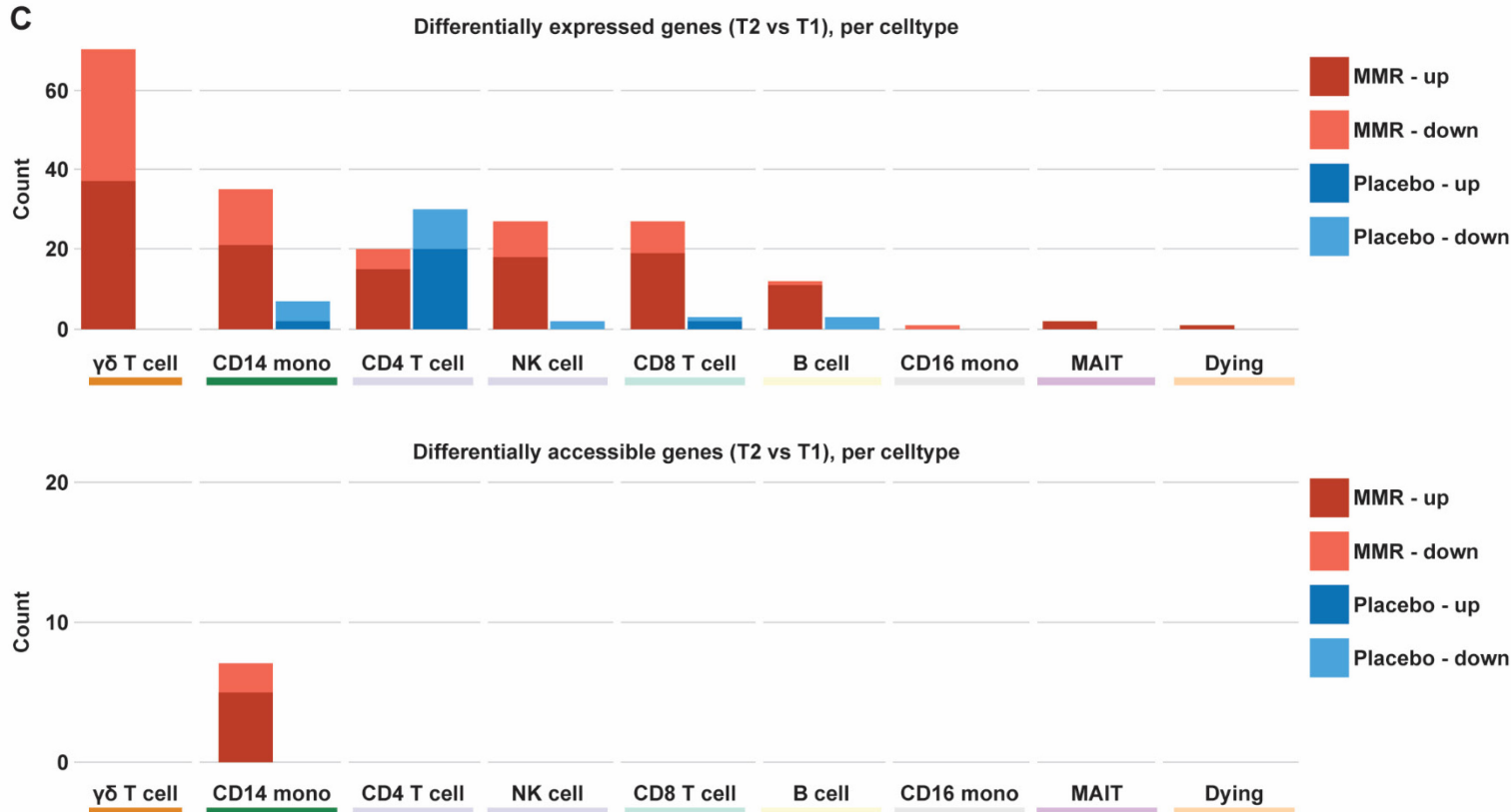
Fig. 2. Single-cell analysis of PBMCs following MMR vaccination. (A) UMAP analysis of single-cell RNA-seq (left) and single-cell ATAC-seq of PBMCs (right). (B) Proportions of cell types annotated according to the scRNA seq data in placebo and MMR samples. (C) Differentially expressed genes (scRNA seq) per cell type, between baseline (T1) and one month after placebo or MMR (T2). (D) Differentially accessible genes (scATAC-seq) per cell type, between baseline (T1) and one month after placebo or MMR (T2).

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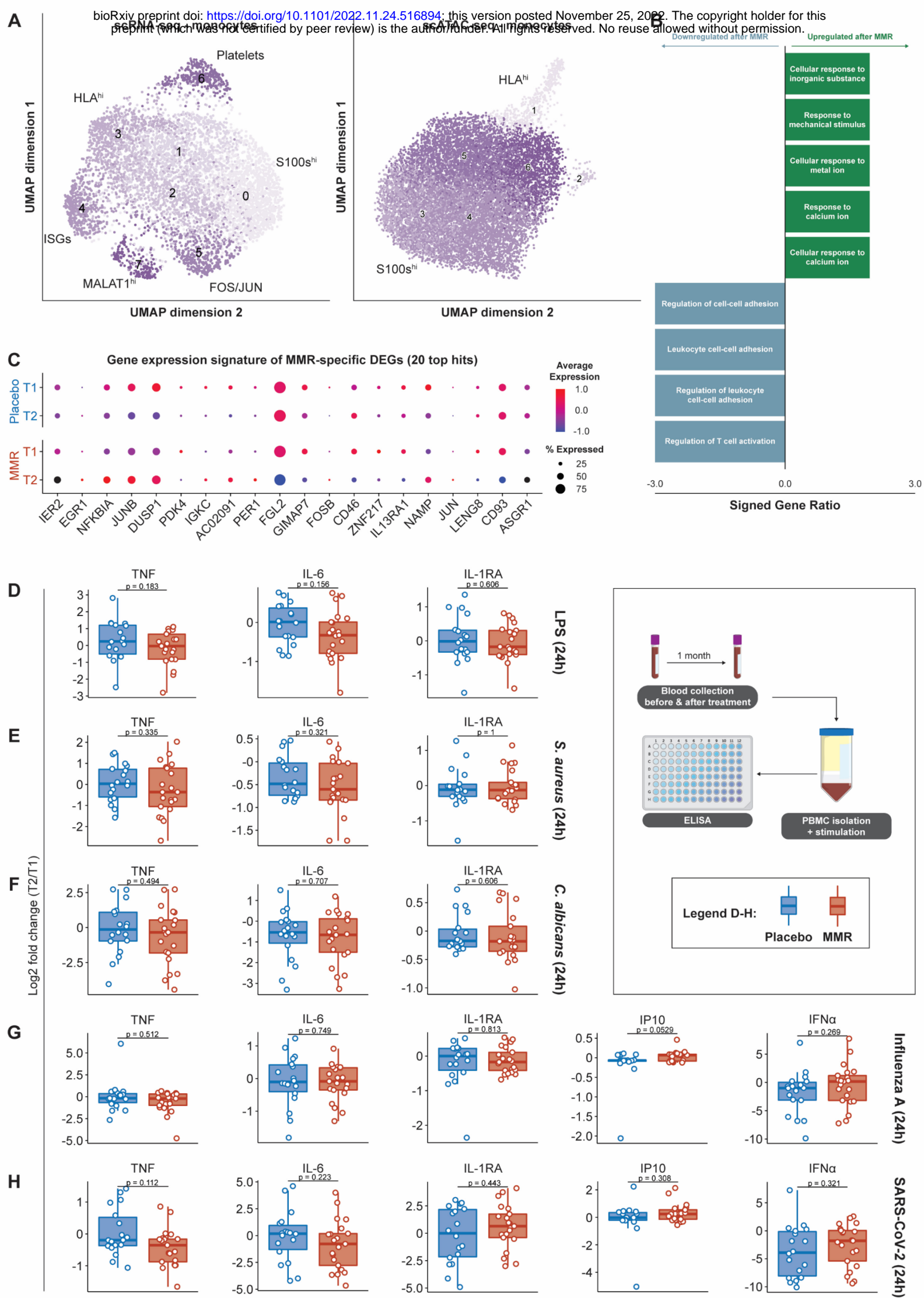
Fig. 3. Single-cell analysis of monocyte subpopulations, and monocyte-associated cytokine production by PBMCs. (A) UMAP analysis and sub-population identification of single-cell RNA-seq (left) and single-cell ATAC-seq (right), specifically in monocytes. (B) Pathway enrichment of genes that are differentially expressed in monocytes after MMR vaccination. (C) Top 20 differentially expressed genes in monocytes following MMR vaccination, by timepoint and treatment group. (D-H) Monocyte-associated cytokines produced by PBMCs following diverse stimulations; the data are expressed as log2 fold-changes between baseline and one month after treatment.

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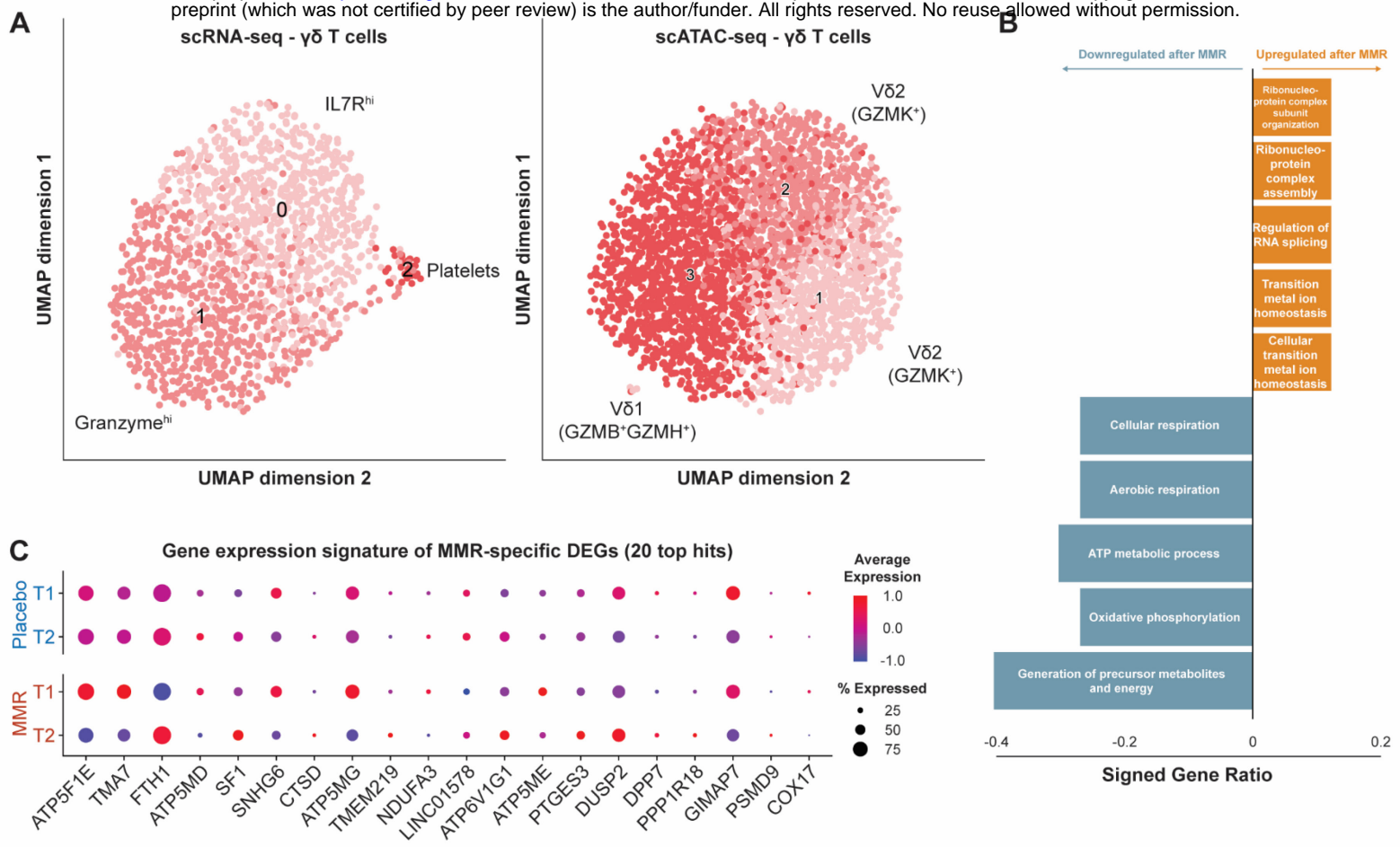
Fig. 4. Single-cell analysis of γδ T cell populations. (A) UMAP analysis and sub-population identification of single-cell RNA-seq (left) and single-cell ATAC-seq (right), specifically in γδ T cells. (B) Pathway enrichment analysis of genes that are differentially expressed in γδ T cells after MMR vaccination. (C) Top 20 differentially expressed genes in γδ T cells following MMR vaccination, by timepoint and treatment group.

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A Circulating Vδ2 T cells

B Cytokine-producing CD3/CD28-stimulated Vδ2 T cells

C Cytotoxic granulation markers - CD3/CD28 stimulated Vδ2 T cells

SCENITH metabolic profiling of Vδ2 T cells

D Unstimulated Vδ2 T cells | CD3/CD28 stimulated Vδ2 T cells | IPP stimulated Vδ2 T cells

Legend: Placebo   MMR
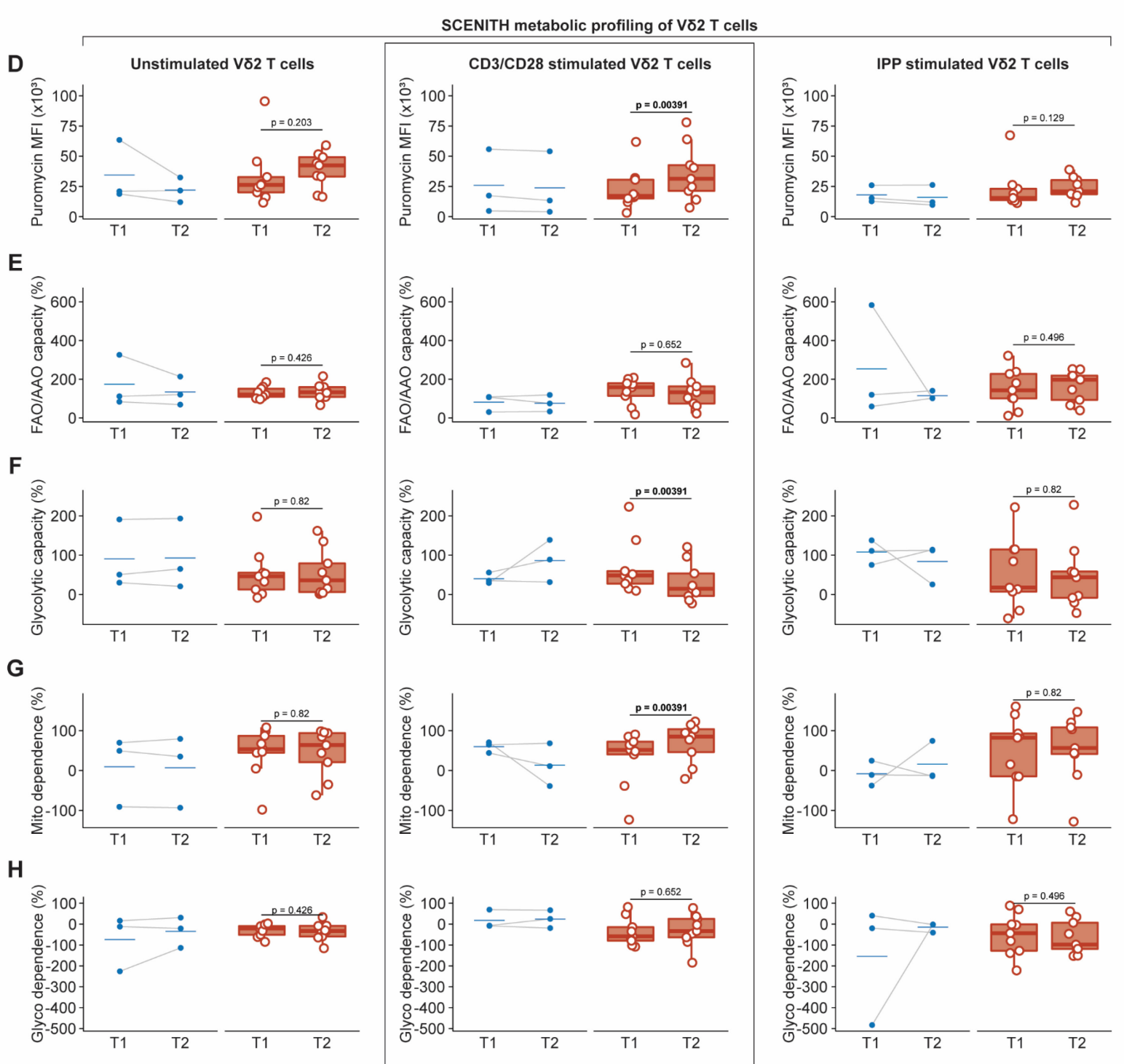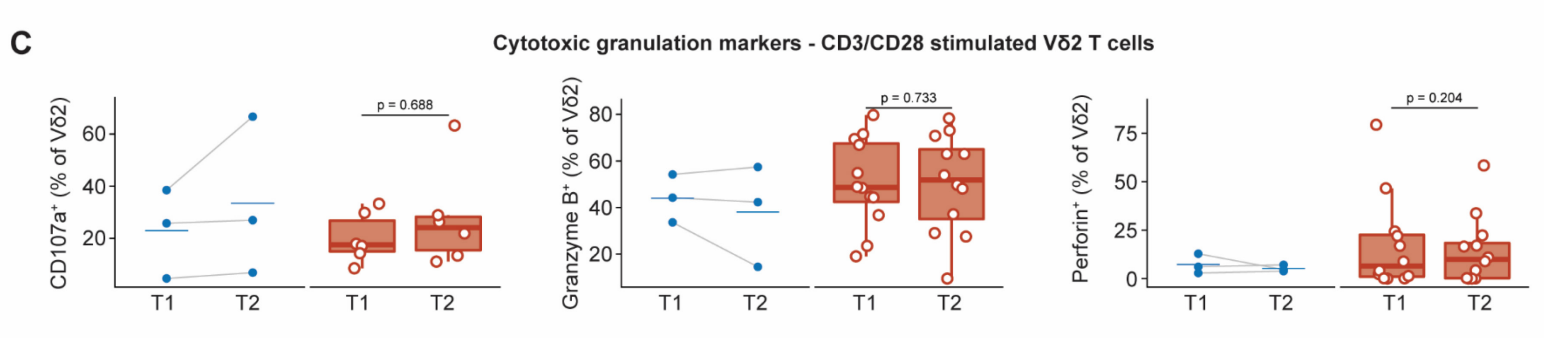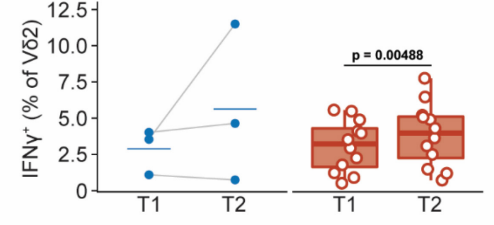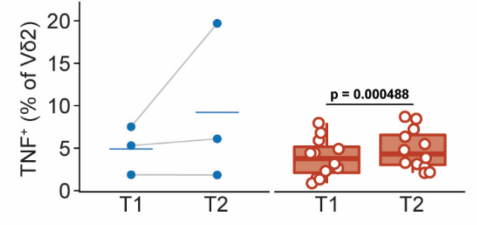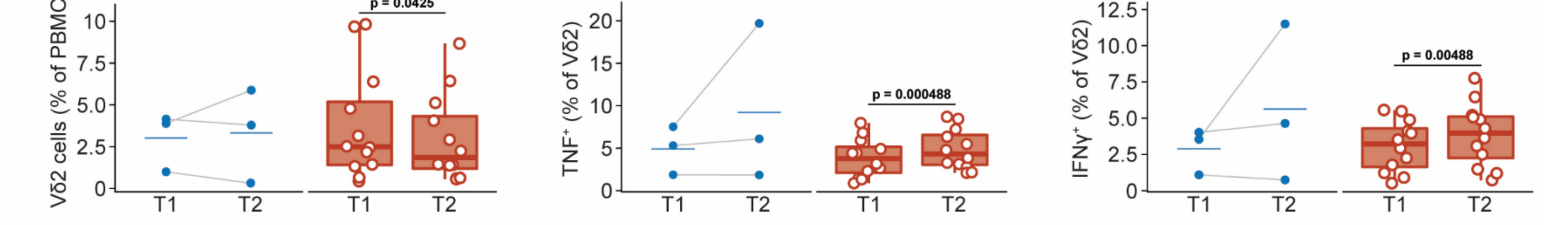
665 **Fig. 5. Functional and metabolic characterization of Vδ2 cells following MMR vaccination. .** (A) The percentage of Vδ2 T cells in isolated PBMCs. (B) The percentage of Vδ2 T cells that produce TNF or IFNγ following CD3/CD28 stimulation. (C) The percentage of Vδ2 T cells expressing markers of cytotoxic granule release (CD107a, left) or production (Granzyme B and perforin, middle and right). metabolic parameters by modified SCENITH^TM (https://www.scenith.com) calculated as in Argüello *et al.* (*23*): (D)
670 puromycin incorporation, (E) FAO/AAO capacity, (F) Glycolytic capacity, (G) Mitochondrial dependence, (H) Glycolysis dependence. All parameters were measured by flow cytometry.

Supplementary materials for:

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MMR vaccination induces a trained immunity program characterized by functional and metabolic

reprogramming of γδ T cells

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This PDF file includes:

Materials and Methods
695 Figs. S1 to S6
Tables S1 to S3

Other Supplementary Materials for this manuscript include the following:
Data S1 (Olink before and after MMR)
700

MATERIALS AND METHODS

Study design

705   This randomized placebo-controlled trial, depicted in Fig. 1A, was designed to research the ability of MMR vaccination to establish trained immunity. Therefore, participants were 1:1 allocated to receive either a placebo vaccination (0.1 ml of 0.9% saline solution), or an MMR vaccination (SD, 0.5 ml, live attenuated mumps virus [strain 'Jeryl Lynn', at least 12.5 * 10^3 CCID50]; live attenuated measles virus [strain 'Enders' Edmonston', at least 1 * 10^3 CCID50]; live attenuated rubella virus [strain 'Wistar RA

710   27/3', at least 1 * 10^3 CCID50]). Vaccination was performed intramuscularly in the right upper arm and. Blood was drawn at baseline ("T1") and one month after vaccination ("T2"). The trial protocol registered under NL74082.091.20 in the Dutch trial registry, was approved in 2020 by the Arnhem-Nijmegen Ethics Committee. All experiments were conducted in accordance with the Declaration of Helsinki and no adverse events were recorded.

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Study subjects

Thirty-nine healthy volunteers (**Table S1**) within the age of 18 and 50 years, were recruited between June and September 2020. Subjects with a medical history associated with immunodeficiency or a solid or non-solid malignancy within the two preceding years were excluded. Vaccination three months prior

720   to the start of the study or plans to receive other vaccinations during the study period was not allowed. Acute illness within two weeks before study initiation or the use of drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) less than four weeks before the start of the trial, with the exception of oral contraceptives, also resulted in exclusion. Pregnant subjects were not eligible. All participants gave written informed consent.

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Blood collection and sample processing

EDTA whole blood (8 x 10 ml) was collected via venipuncture. Two of the EDTA tubes were centrifuged immediately after collection at 2970 x g for 10 minutes at room temperature (RT) and plasma was

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stored at -80 °C until later analysis. Hematological parameters such as white blood cell count and

730 differential were measured on a Sysmex XN-450 apparatus. Additionally, 1 ml of whole blood was

stored at -80 °C for genotyping analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation over

Ficoll-Paque. Briefly: EDTA blood was diluted in calcium/magnesium-free PBS and layered on Ficoll-

Paque solution. After centrifugation for 30 minutes at 615 x g (no brakes; RT), the PBMC layer was

735 collected and washed at least 3 times with cold calcium/magnesium-free PBS. The cells were

resuspended in RPMI-1640 with Dutch modifications (Invitrogen) supplemented with 50 mg/ml

gentamicin (Centrafarm), 2 mM GlutaMAX (Gibco) and 1 mM pyruvate (Gibco) and counted by Sysmex.

For cytokine production assessments, PBMCs were seeded in round-bottom 96 well plates at $0.5 * 10^6$

cells/well. The cells were stimulated for 24 hours using the stimuli described in **Table S3** (all in the

740 presence of 10% human pooled serum, at 37 °C and 5% CO_2). Supernatants were collected and stored

at -20 °C until further analysis.

PBMC freezing and thawing

Leftover PBMCs were resuspended in ice-cold, heat-inactivated fetal bovine serum (FBS) prior to

745 cryopreservation. Ice-cold 20% DMSO in FBS was added dropwise to the cells until a final concentration

of 10% DMSO was reached. The cells were stored for up to 24 hours in CoolCell alcohol-free freezing

containers (Corning) at -80 °C, after which they were transferred to a -150 °C freezer for long-term

storage.

For subsequent experiments, PBMCs were thawed following a protocol modified from Hønge et al (*45*).

750 The PBMCs were retrieved from the -150 °C storage and kept on dry ice until the moment of thawing.

The cells were rapidly warmed in a water bath of 37 °C until only a small clump of ice was present in

the vial. The contents were immediately transferred into a 10x volume of pre-warmed thawing

medium (RPMI supplemented as described above, further supplemented with 20% FBS and 12.5 µg/ml

DNase-I). The cells were centrifuged at 500 x g for 10 minutes at RT and resuspended in thawing

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755   medium without DNase-I. The cells were again centrifuged, resuspended in cold PBS and counted with

trypan blue to assess recovery and viability.

ELISA cytokine measurements and data analysis

The cytokines TNF (commonly referred to as TNF-α), IL-6, IL-1Ra, and IP-10 were measured using

760   DuoSet ELISA kits from R&D systems, and IFNα with a kit from PBL Assay Science, using the

manufacturer's protocol. To account for plate-to-plate variation, the participants were randomized

over different plates (timepoints were kept together on the same plate). Cytokine concentrations were

calculated relative to the standard curve in Gen5 software (BioTek). Log2-fold changes were calculated

between T2 and T1, and corrected for sex, age, and BMI using a linear regression approach. The MMR

765   and placebo groups were compared using Mann-Whitney U tests.

Targeted proteomics analysis by proximity extension assay

Plasma samples from 16 MMR-vaccinated individuals were sent to Olink (Sweden) for targeted

proteomics analysis using proximity extension assay technology. In total, 1472 proteins were

770   measured, of which 183 were removed from the analysis due to them being poorly detectable in >25%

of samples (30 cardiometabolic proteins, 46 inflammatory proteins, 56 neurology proteins, 51

oncology proteins). Unadjusted p-values were calculated using the Wilcoxon signed rank test.

DNA isolation and genotyping

775   Whole blood samples were shipped on dry ice to the Human Genomics Facility of the Genetic

Laboratory of the Department of Internal Medicine at Erasmus MC, Rotterdam, The Netherlands.

There, DNA isolation was performed and samples were genotyped using Illumina GSA Arrays "Infinium

iSelect 24x1 HTS Custom Beadchip Kit".

780   **Single cell library preparation and sequencing**

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Cryopreserved PBMCs were thawed as described above and washed an additional time with ice-cold PBS. Single cell gene expression libraries were generated on the 10x Genomics Chromium platform using the Chromium Next GEM Single Cell 3' Library & Gel Bead Kit v3.1 and Chromium Next GEM Chip G Single Cell Kit (10x Genomics) according to the manufacturer's protocol. Single-cell ATAC-seq libraries were generated on the 10x Genomics Chromium platform using the Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit v1.1 and Chromium Next GEM Chip H Single Cell Kit (10x Genomics) according to the manufacturer's protocol. Gene expression and ATAC-seq libraries were sequenced on a NovaSeq 6000 S4 flow cell using v1.5 chemistry (Illumina).

Single-cell sequencing data analysis

Pre-processing and demultiplexing scRNA-seq and snATAC-seq data

The proprietary 10x Genomics CellRanger pipeline (v4.0.0) was used with default parameters. CellRanger count or CellRanger-atac count was used to align read data to the reference genome provided by 10x Genomics. refdata-cellranger-arc-GRCh38-2020-A-2.0.0 was used for the snATAC-seq experiments and refdata-gex-GRCh38-2020-A for the scRNA-seq. In scRNA-seq, a digital gene expression matrix was generated to record the number of UMIs for each gene in each cell. In snATAC-seq, fragment files were created.

Each library was further demultiplexed by assigning cell barcodes to their donor. Souporcell (v1.3 gb) (*46*) was used for genotype-free demultiplexing by calling candidate variants on the pre-mapped bam files. Cells were clustered by their allelic information and each cluster was matched to a donor with a known genotype.

scRNA-seq data analysis

The expression matrix from each library was loaded to R/Seurat package (v3.2.2) (*47*) for downstream analysis. To control the data quality, we first excluded cells with ambiguous assignments from Souporcell demultiplex. Next, we further excluded low-quality cells with > 25% mitochondrial reads, < 100 or > 3,000 expressed genes, or > 5000 UMI counts.

After QC, we applied LogNormalization (Seurat function) and scaled the data, regressing for total UMI

810   counts, number of features, percentage of mitochondrial genes and percentage of ribosomal genes.

We then performed principal component analysis (PCA) based on the 2,000 more highly variable

features identified using the vst method implemented in Seurat. As batches showed a good integration

of the data, no integration algorithm was applied. Cells were then clustered using the Louvain

algorithm with a resolution of 0.75 based on neighbors calculated the first 30 principal components.

815   For visualization, we applied UMAP based on the first 30 principal components.

Annotation of scRNA-seq clusters

Clusters were annotated by manually checking the expression of known marker genes. Cluster 5

showed a higher expression of gamma and delta chain genes (*TRGC1*, *TRDC*), along with T cell markers

820   (*CD3E*). Performing dimensionality reduction and clustering on this subset revealed two mixed

populations: MAIT cells overexpressing *KLRB1* and γδ T cells over expressing *TRDC/TRGC*, these cells

were then annotated accordingly.

Differential gene expression and gene-set enrichment

825   For paired comparison between timepoints in both MMR and placebo, differential expression (DE)

tests were performed using the FindMarkers functions in Seurat with MAST (*48*). Patient ids were

regressed out, in order to perform a paired analysis. Genes with a Bonferroni-corrected P-value < 0.05

were regarded as differentially expressed.

Gene-set enrichment was performed using the enrichGO function from the R package clusterProfiler.

830   Gene-sets enriched with a Benjamin-Hochberg corrected p-value below 0.05 and more than 4 genes

were considered significant.

snATAC-seq data analysis

835   ArchR (*49*) was used for the downstream analyses on snATAC-seq data, reading the fragment files created in by CellRanger-atac. Cells with fewer than 1,000 unique fragments, a transcription start site enrichment below 4, identified as doublets by ArchR, or ambiguously labelled by souporcell were removed.

        After QC, we used the ArchR function 'addIterativeLSI' to process iterative latent semantic indexing using the top 25,000 variable features and top 30 dimensions. For visualization, we applied UMAP with

840   nNeighbors = 30 and minDist = 0.5.

        Gene scores were calculated for each cell based on accessibility. In order to aid the analysis of gamma-delta T cells, a modified reference was used. Adding the gtf gene reference used by CellRanger, gene scores could be calculated for *TRDC*, *TRGC1* and *TRGC2*.

845   *snATAC-seq annotation and integration with scRNA-seq data*

        ArchR function 'addGeneIntegrationMatrix' was used to compare the calculated snATAC-seq gene score matrix and the measured gene expression in scRNA-seqdata. This resulted in a matched scRNA profile and predicted cell type per sequenced cell in the snATAC-seq data. Cell types were therefore assigned to the snATAC-seq data based on the predicted cell type of the integration. UMAPs of the

850   integrated blocks were inspected in order to examine the quality of the integration (**Fig. S2A-B**).

        Per cell type analysis of snATAC-seq data

        A common approach was followed to inspect the open-chromatin changes in each cell type. The same method as described before for the whole cell pool was used for visualization and clustering separately

855   in each cell type. After, open-chromatin peaks were calculated by running 'addReproduciblePeakSet' using Macs2 algorithm (*50*). Transcription factor motif deviations were calculated based on the 'CIS-BP' database annotation (*51*) using the 'addDeviationsMatrix' function. The effects of MMR vaccination and placebo were assessed by running 'getMarkerFeatures' comparing both timepoints

for all data types: open-chromatin peaks, TF motifs and gene score. FDR < 0.05 was indicative of

860   significant changes.

Flow cytometry measurements of γδ T cell parameters
The flow cytometry staining was performed as follows: 5×10^5 thawed PBMCs were stained for surface

markers using the antibodies described in **Table S3**, for 30 minutes in the dark at 4 °C, in FACS buffer

865   (PBS, 5% FBS, 2 mM EDTA Intracellular proteins were analyzed after fixation and permeabilisation in

Cytofix permeabilization/fixation reagent (BD biosciences) for 30 mins. Following two washes with

Cytofix permeabilization/washing buffer (BD biosciences), the cells were stained with the antibodies

against intracellular markers detailed in **Table S3**, for 30 minutes in the dark at 4 °C. After completion

of the staining procedure, the cells were washed with PBS and stored in CellFIX reagent (BD

870   biosciences) until acquisition on a LSR II cytometer (BD biosciences).

The flow cytometry data was analyzed in FlowJo (vX.07). The gating strategy was as follows: events

corresponding to lymphocyte size were selected based on FSC-A/SSC-A, followed by selection of single-

cell events in subsequent FSC-H/FSC-A and FSC-W/FSC-A gates. Viable cells were selected by gating on

875   viability-dye-negative cells. The analyses were performed on $CD45^+CD3^+V\delta2^+$ cells.

For measurement of surface markers on unstimulated Vδ2 T cells, the thawed PBMC were stained as

described above immediately after thawing. For measurements of cytokine expression or surface

markers after stimulation, the PBMCs were first treated with soluble anti-CD3/anti-C28 (BD bioscience)

880   for 4 hours in the presence of a Golgi plug (Brefeldin A; BD bioscience), under standard cell culture

conditions.

SCENITH

We modified the original SCENITH[TM] technique (https://www.scenith.com) to analyze energy

885   metabolism of γδ T cells. Briefly, PBMCs were plated at 0.3×10^6 cells/well in 96-well plates. The cells

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were cultured in RPMI alone or stimulated with soluble anti-CD3/anti-CD28, or IPP for 4 hours under standard cell culture conditions. Cells were then untreated (control) or treated with 2-DG (final concentration 100 mM), Oligomycin (O, final concentration 10 µM) and combination of 2-DG and Oligomycin (DGO, final concentration 100 mM and 10 µM) for 30 min under standard cell culture

890   conditions. Following addition of puromycin (final concentration 10 µg/ml), the cells were incubated for an additional 45 minutes. The cells were subsequently harvested and washed in cold FACS buffer before being stained as described above.

Statistical analysis and software

895   All data was analyzed in R as described in each relevant section of the methods. Unless otherwise indicated, two-tailed p-values of <0.05 were considered statistically significant. If correction for multiple testing was applied, the method is described in the relevant methods section. In cases where the p-value is not provided, an asterisk (*) indicates statistical significance.

The following R packages were used for the present work: the Tidyverse core packages 1.3.2 (*52*),

900   Seurat 4.1.1, ArchR 1.0.2, SeuratObject 4.1.0, GenomicRanges 1.48.0, data.table 1.14.2, ggplot2 3.3.6, colortools 0.1.6, clusterProfiler 4.4.4. magrittr 2.0.3, ggprism 1.0.3, ggsci 2.9, rstatix 0.7.0, pzfx 0.3.0, janitor 2.1.0, readr 2.1.3, openxlsx 4.2.5, psych 2.2.9. The figures were compiled in Adobe Illustrator.

905 **Supplementary figures**

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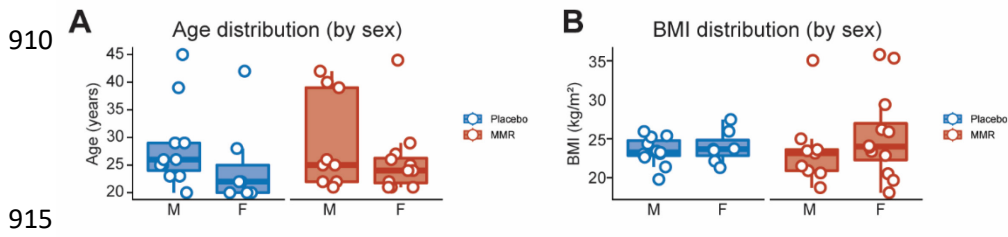
920 **Figure S1: Participant characteristics.** (**A**) Participant age, stratified by sex and treatment group. (**B**) Participant BMI, stratified by sex and treatment group.

A

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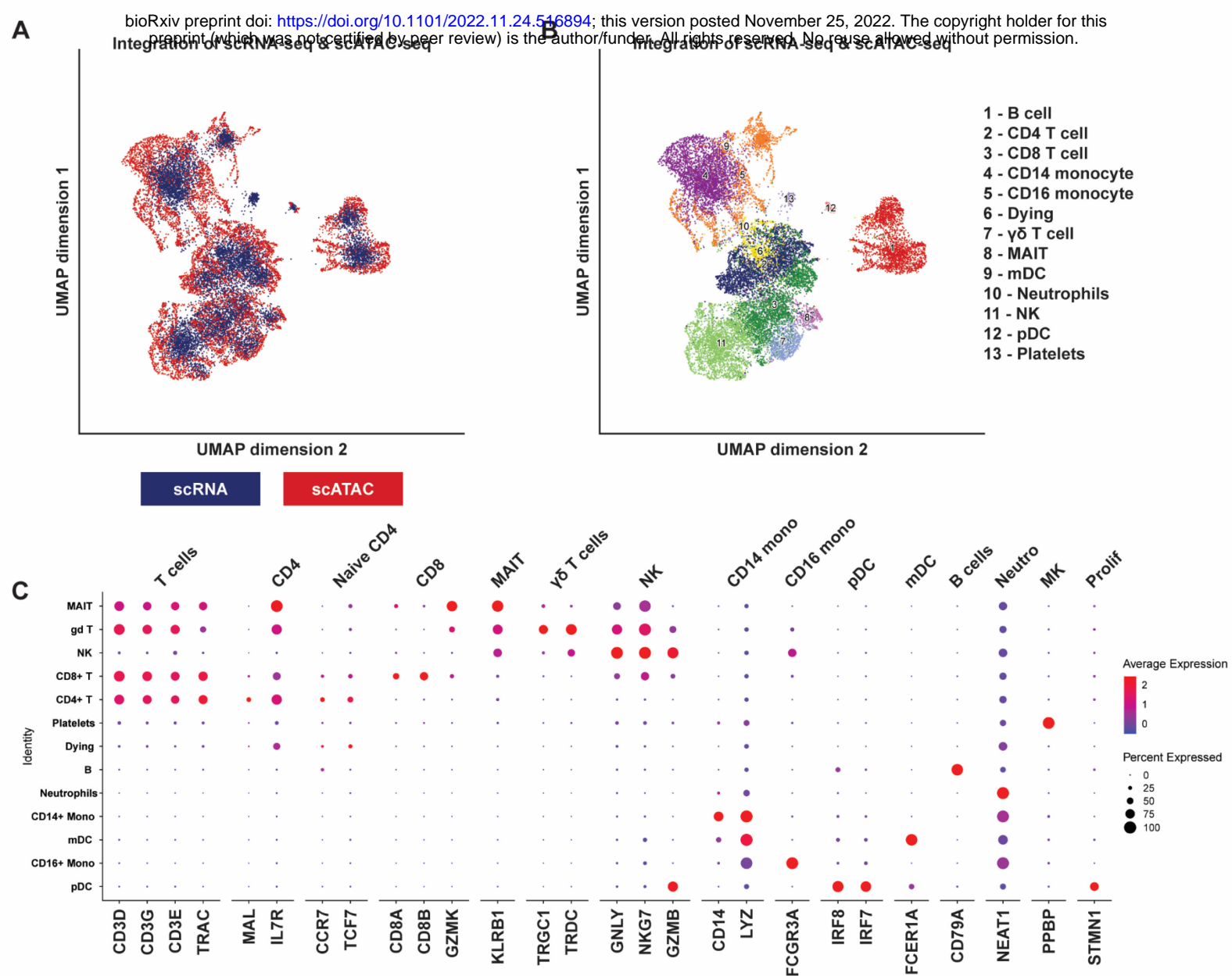
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Figure S2: Integration of scRNA and scATAC-sequencing data and cell-type annotation. (**A**) UMAP of scRNA-seq and scATACseq integrated data. Integration was performed using canonical correlation analysis between gene expression values and gene score values calculated based on gene accessibility. ( ) Same as before, colored by the different celltypes present. (**C**) Dotplot of markers used for celltype annotation.

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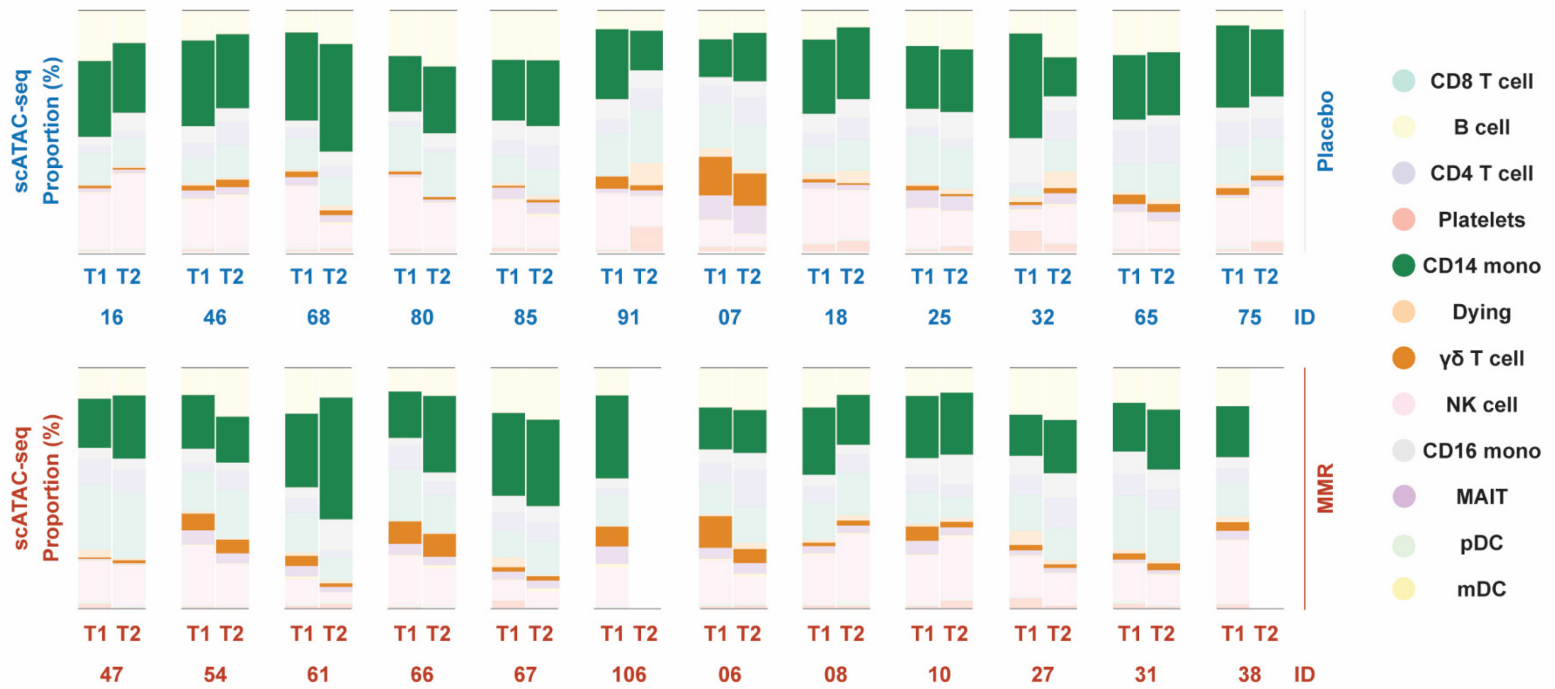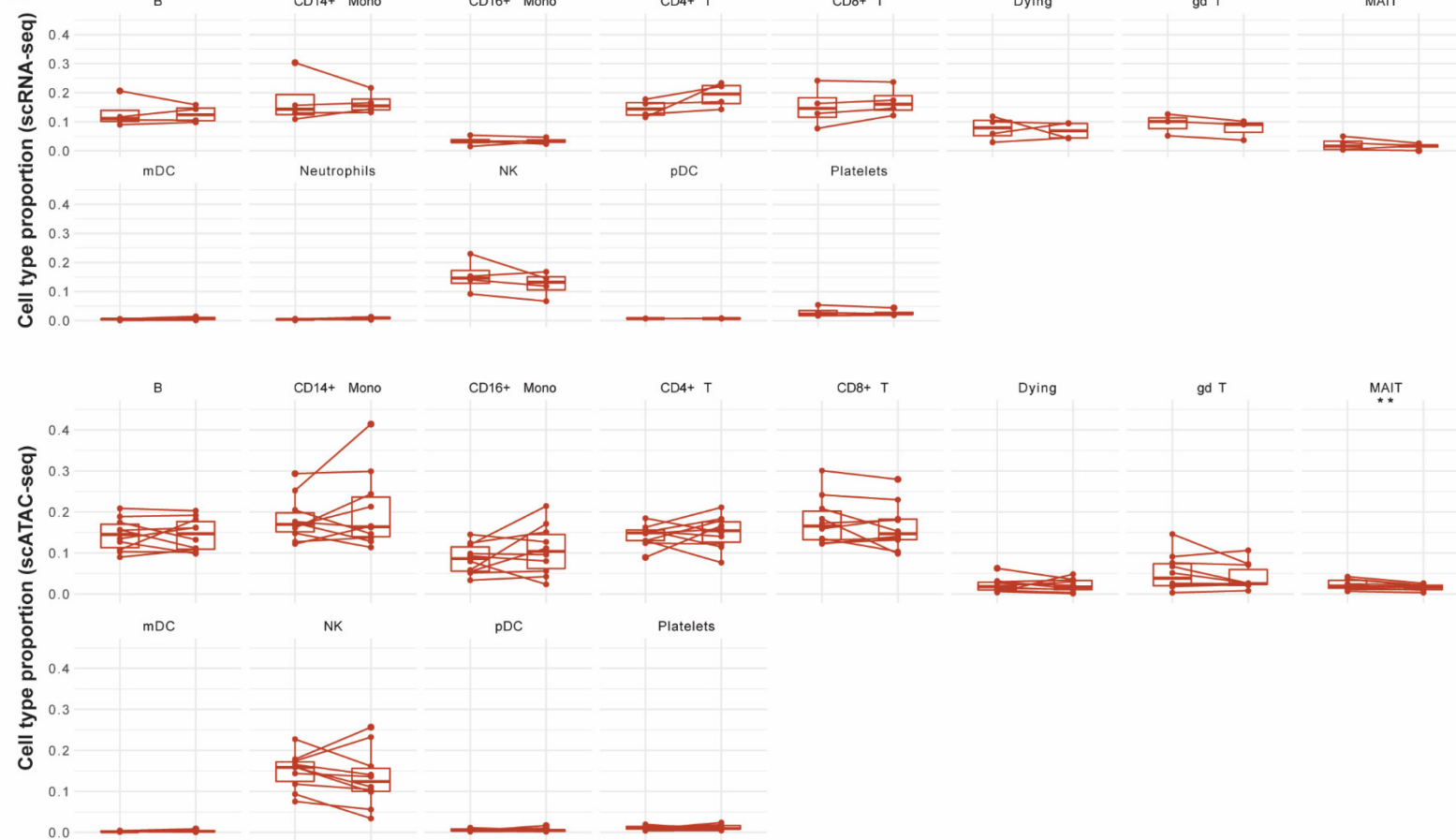
Figure S3: Cell-type proportions before and after treatment. (A) Proportions of cell types annotated according to the scATAC-seq data in placebo and MMR samples. (B) The same data, only displayed as boxplots per cell type to enable paired comparisons.
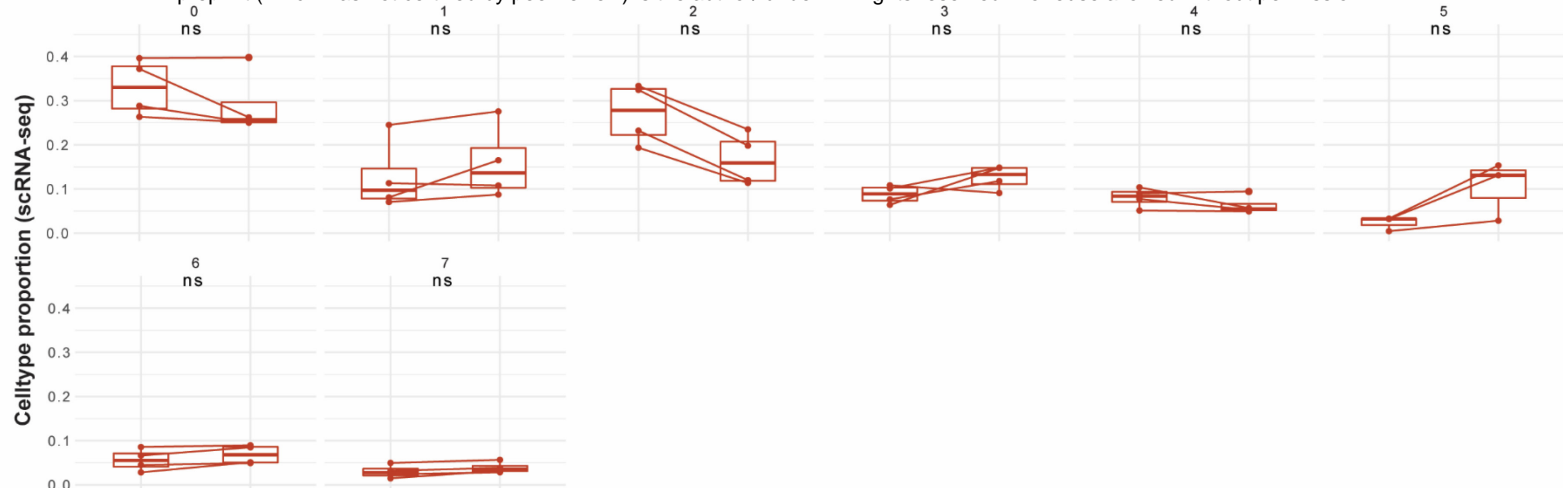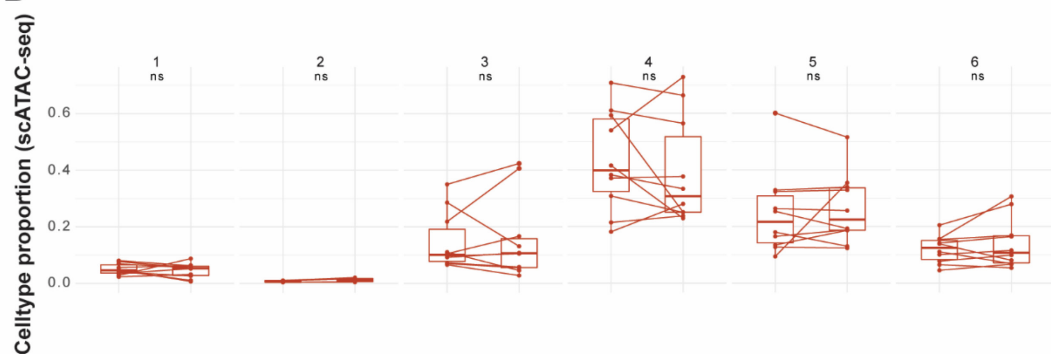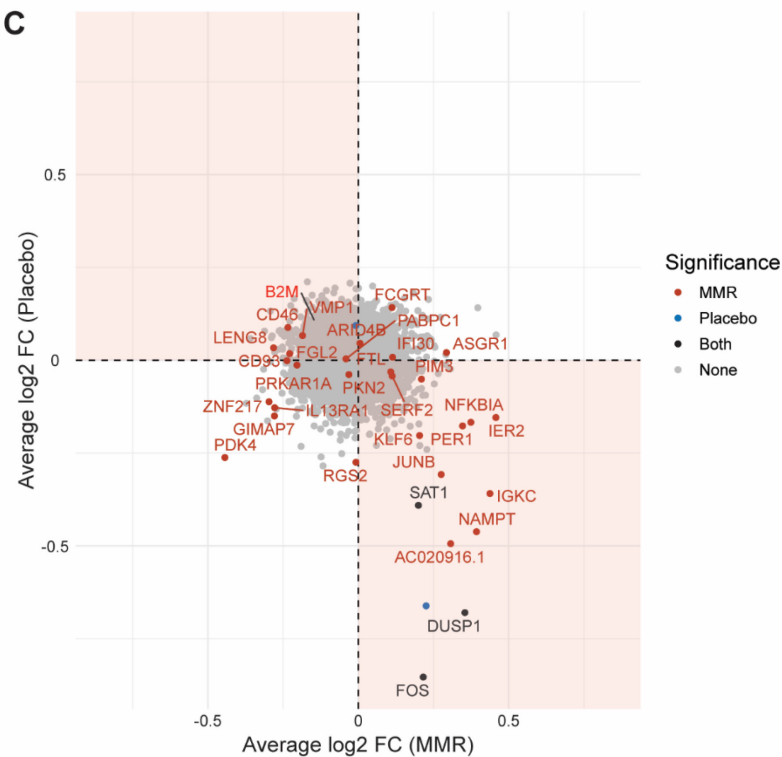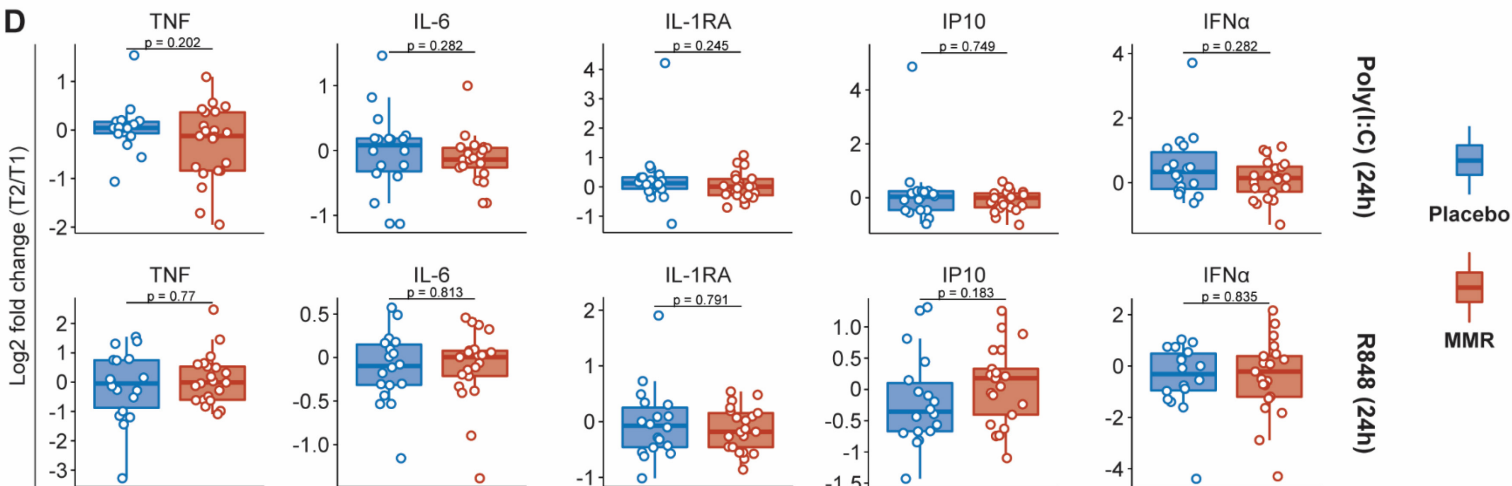
1015 **Figure S4: Single-cell analysis of monocyte subpopulations and monocyte-associated cytokine production by PBMCs.** (**A**) Proportions of monocyte sub-populations (scRNA-seq) before and after MMR vaccination. (**B**) Proportions of monocyte sub-populations (scATAC-seq) before and after MMR vaccination. (**C**) Combined 'volcano plot' showing average log2 fold changes of monocyte gene expression between timepoints for both placebo and MMR. Bonferroni adjusted p-value < 0.05,

1020 paired test using MAST. (**D**) Monocyte-associated cytokines produced by PBMCs following diverse stimulations; the data are expressed as log2 fold-changes between baseline and one month after treatment.
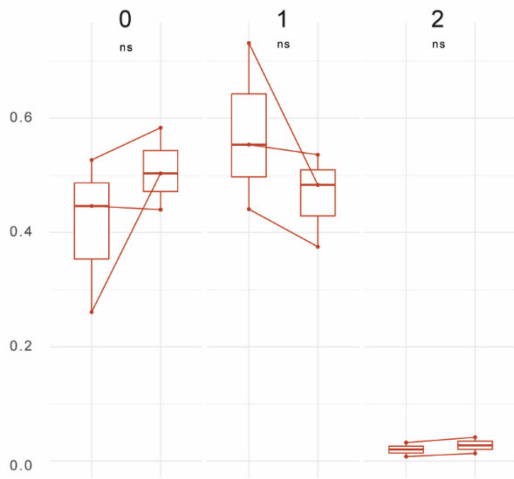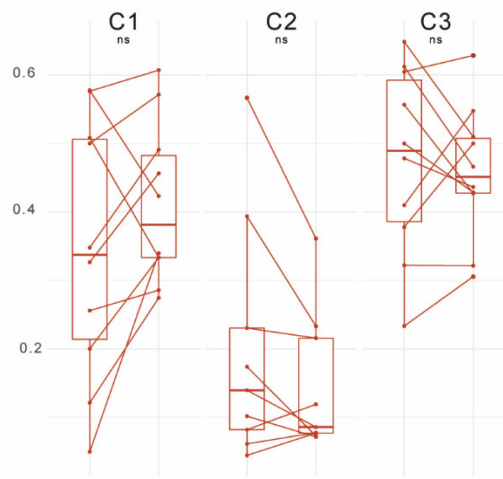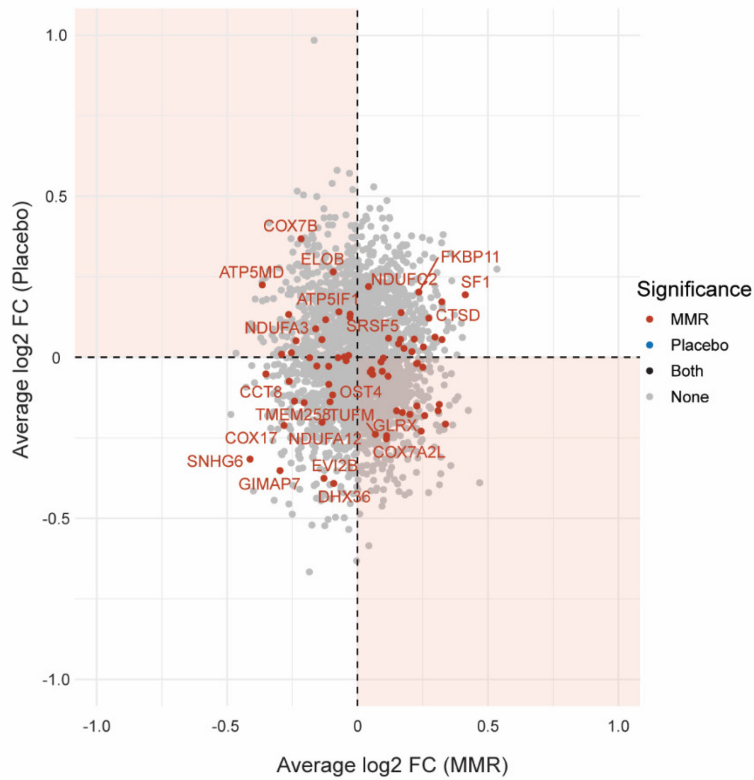
Figure S5: Single-cell analysis of γδ T cell populations. (A) Proportions of γδ T cell sub-populations (scRNA-seq) before and after MMR vaccination. **(B)** Proportions of γδ T cell sub-populations (scATAC-seq) before and after MMR vaccination. **(C)** Combined 'volcano plot' showing average log2 fold changes of γδ T cell gene expression between timepoints for both placebo and MMR. Bonferroni adjusted p-value < 0.05, paired test using MAST.
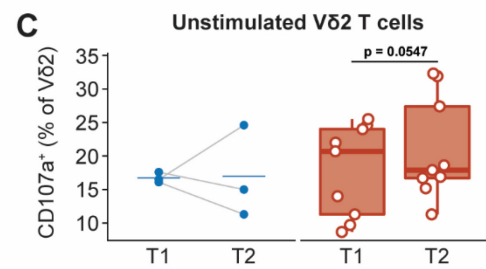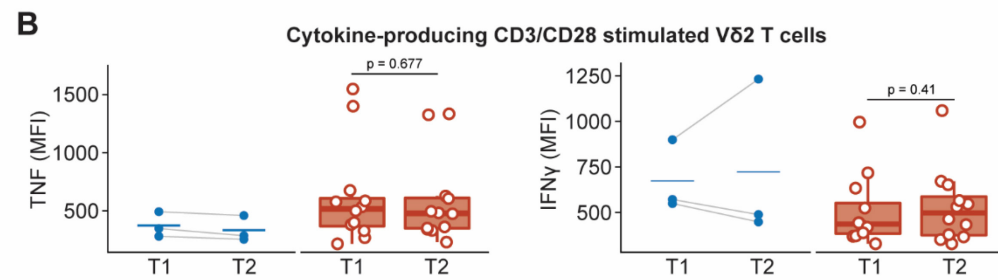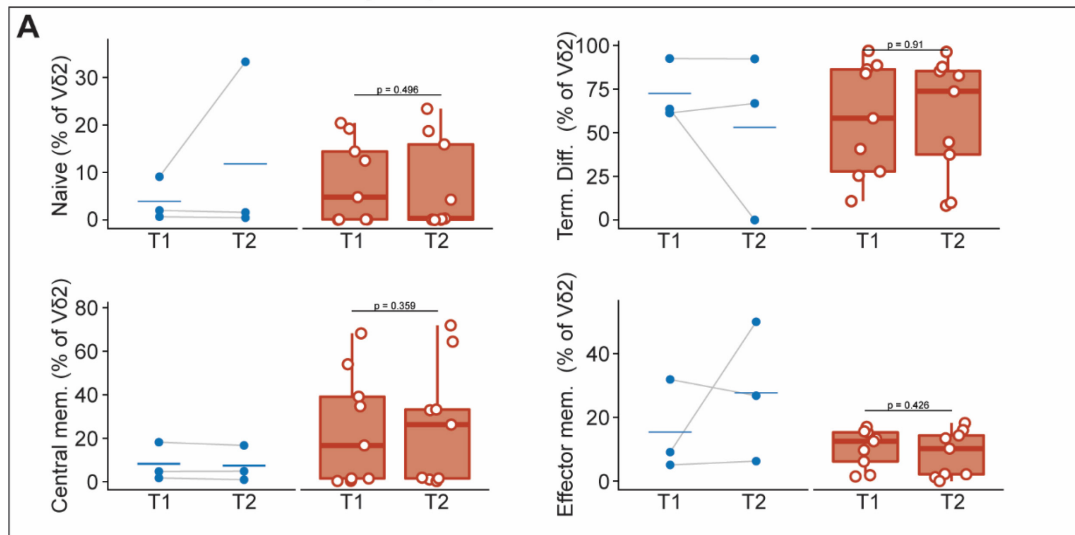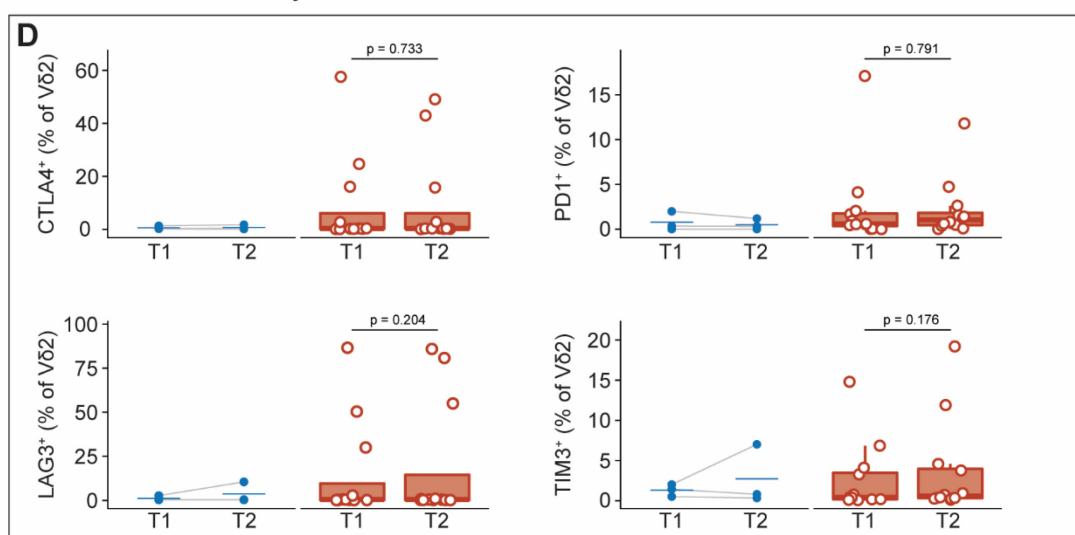
Figure S6: characterization of Vδ2 cells following MMR vaccination. (**A**) Proportions of memory compartments characterized by expression of CD27 and CD45RA. (**B**) Mean fluorescence intensities (MFI) of TNF and IFNγ in stimulated Vδ2 T cells. (**C**) Percentage of unstimulated Vδ2 T cells that stain positive for CD107a, a marker of cytotoxic degranulation. (**D**) Proportions of Vδ2 T cells that stain positive for markers commonly associated with T cell dysfunction (CTLA4, PD1, TIM3, LAG3).

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Supplementary tables

Characteristic*	Placebo n = 18	MMR n = 21	p-value†
Sex, female	7 (38.89)	12 (57.14)	0.3406
Sex, male	11 (61.11)	9 (42.86)	
Age, years	25 ± 7.63	25 ± 7.38	0.876
BMI, kg/m^2	23.47 ± 1.89	23.45 ± 5.22	0.945
* Median +/- SD for continuous variables and n (%) for categorical variables			
† Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables			

Table S1: characteristics of the study population.

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Stimulus	Concentration in experiment	Cat. No.	Manufacturer
E. coli LPS serotype O55:B5, further purified as in (*53*)	10 ng/ml	-	Prepared in-house by Heidi Lemmers, Raboudumc
Heat-killed *S. aureus* ATCC 25923	$1 * 10^6$ CFU/ml	-	Cultured and heat-killed in-house by Jelle Gerretsen, Radboudumc
Heat-killed *C. albicans* yeast, UC820 (ATCC MYA-3573), prepared as described in (*54*)	$1 * 10^6$ CFU/ml	-	Prepared in-house by Diletta Rosati, Radboudumc
Poly(I:C)	10 µg/ml	tlrl-pic	Invivogen
R848	3 µg/ml	tlrl-r848	Invivogen
Influenza A H1N1, prepared according to the methods in (*55*)	$3.3 * 10^5$ /ml	-	Kindly prepared by KLG, NR, PNO, LM, HS, OA, AB
SARS-CoV-2, prepared according to the methods in (*55*)	$1.4 * 10^3$ /ml	-	

Table S2: Stimuli used to assess PBMC cytokine production capacity.

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Target	Fluorophore	Clone	Cat. No.	Manufacturer	Panel
Viability	Live-or-Dye Fixable Viability Stain	-	32006	Biotium	1
CD3	Pacific blue	UCHT1	300431	Biolegend	1
CD4	PE-Cy7	OKT4	317414	Biolegend	1
CD8	APC-Cy7	SK1	344746	Biolegend	1
CD45	BV605	HI30	304042	Biolegend	1
Vδ2 TCR	FITC	REA771	130-111-009	Miltenyi	1
Vδ1 TCR	PE	REA173	130-120-440	Miltenyi	1
TNF	APC	MAb11	502912	Biolegend	1
IFNγ	PerCP-Cy5.5	B27	560704	BD Pharmingen	1
Target	**Fluorophore**	**Clone**	**Cat. No.**	**Manufacturer**	**Panel**
Viability	Live/dead Fixable Aqua dead cell stain	-	L34957	Invitrogen	2
CD3	Pacific blue	UCHT1	300431	Biolegend	2
Vδ2 TCR	FITC	REA771	130-111-009	Miltenyi	2
Vδ1 TCR	PE	REA173	130-120-440	Miltenyi	2
CD4	BV605	OKT4	317438	Biolegend	2
CD8	APC-Cy7	SK1	344746	Biolegend	2
PD1	PerCP-Cy5.5	A17188B	621613	BD Bioscience	2
Perforin	Pe-Cy7	dG9	308126	Biolegend	2
Granzyme B	AF647	GB11	515406	Biolegend	2
CD45	PE/Dazzle594	HI30	304052	Biolegend	2
Target	**Fluorophore**	**Clone**	**Cat. No.**	**Manufacturer**	**Panel**
Viability	Live/dead Fixable Aqua dead cell stain	-	L34957	Invitrogen	3
CD3	APC	UCHT1	300458	Biolegend	3
CD4	PE-Cy7	OKT4	317414	Biolegend	3
CD8	APC-Cy7	SK1	344746	Biolegend	3
Vδ1 TCR	Pacific blue	REA173	130-100-555	Miltenyi	3
Vδ2 TCR	FITC	REA771	130-111-009	Miltenyi	3
CD107a	PE	H4A3	328607	Biolegend	3
CTLA4	BV605	BNI3	369610	Biolegend	3
Lag3	PE/Dazzle594	11C3C65	369331	Biolegend	3
Target	**Fluorophore**	**Clone**	**Cat. No.**	**Manufacturer**	**Panel**
Viability	Live/dead Fixable Aqua dead cell stain	-	L34957	Invitrogen	4
CD45	PE/Dazzle594	HI30	304052	Biolegend	4
CD45RA	APC	HI100	304111	Biolegend	4
CD3	Pacific blue	UCHT1	300458	Biolegend	4
CD4	PE-Cy7	OKT4	317414	Biolegend	4
CD8	APC-Cy7	SK1	344746	Biolegend	4
Vδ1 TCR	PE	REA173	130-120-440	Miltenyi	4
Vδ2 TCR	FITC	REA771	130-111-009	Miltenyi	4
CD27	PerCP-Cy5.5	M-T271	560612	BD Bioscience	4
NKG2D	BV605	1D11	320832	Biolegend	4

Target	Fluorophore	Clone	Cat. No.	Manufacturer	Panel
Viability	Live-or-Dye Fixable Viability Stain	-	32006	Biotium	5
CD3	Pacific blue	UCHT1	300431	Biolegend	5
CD4	PE-Cy7	OKT4	317414	Biolegend	5
CD8	APC-Cy7	SK1	344746	Biolegend	5
CD45	BV605	HI30	304042	Biolegend	5
Vδ2 TCR	APC	REA771	130-111-009	Miltenyi	5
Vδ1 TCR	PE	REA173	130-120-440	Miltenyi	5
Puromycin	AF488	12D10	MABE343	Merck	5

Table S3: Antibodies used in the flow cytometric analyses of Vδ2 T cells.

ORIGINAL ARTICLE

Peripheral T Cell Populations are Differentially Affected in Familial Mediterranean Fever, Chronic Granulomatous Disease, and Gout

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Abstract

Both innate errors of immunity, such as familial Mediterranean fever (FMF) and chronic granulomatous disease (CGD), 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)-1ß 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. In order to widen our understanding of T cell involvement in CGD, FMF and gout pathology, we developed multicolour immunophenotyping panels for flow cytometry to characterize 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. Our study is the first deep immunophenotyping analysis of T cell populations in CGD, FMF, and gout patients. We found that CGD affects the frequencies and activation status of T cells, while gout impairs the cytokine production capacity of Vδ2 T cells. FMF was characterized by decreased percentages of regulatory T cells in circulation and attenuated IFN-γ production capacity by Vδ2 T cells. Autoinflammatory syndromes and congenital defects of phagocyte differentially affect T cell compartments. Future studies are warranted to assess whether these phenotypical changes are relevant for disease pathology.

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Introduction

Inborn errors of immunity (IEI) are caused by genetic variants which alter the function of individual genes and compromise innate and/or adaptive immunity [1]. To date, this large group of disorders encompasses 485 diseases [2]. Systemic autoinflammatory diseases (SAID), a subgroup of IEI, are described as distinct heritable disorders, mostly affecting the 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 [3, 4]. Several SAIDs, including familial Mediterranean fever (FMF), are mediated by excessive interleukin (IL)-1β production. 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, and stress).

Alterations in inflammasome activation [5] and in various metabolic pathways [6] can lead to dysregulation of IL-1β secretion and cause pathology. Gout, which is not an IEI, is another autoinflammatory disease with dysregulated IL-1β biology. Congenital defects of phagocyte are another group of IEI in which phagocytes are unable to function properly to kill the invading pathogens. CGD is one of the diseases in this subgroup with a particular defect of respiratory burst. While many of the SAIDs have been originally characterized by the absence of auto-antibodies or auto-reactive T cells, CGD-causing mutations result in the presence of lupus-like auto-antibodies and auto-reactive T cells [7–9]. Therefore, the lymphoid compartment might still be affected and contribute to the pathology of the diseases [10, 11]. Furthermore, dysregulated IL-1β production likely affects the T cell compartment in these diseases, as the effect of this cytokine on lymphocytes has been reported [12–16]. Indeed, many autoinflammatory conditions, including FMF and gout, display upregulation of T helper type 17 (Th17)-related cytokines [17–19]. Despite of this, a systematic analysis of the T cell compartment, including innate-like gamma delta (γδ) T cells, has not been performed in theis group of diseases.

Our study focuses on two diseases with known dysregulation of IL-1 cytokine production: FMF, the most prevalent monogenic autoinflammatory disease worldwide, and gout, which is the most common form of inflammatory arthritis, as well as chronic granulomatous disease (CGD) which is a rare inherited primary immunodeficiency disorder with hyperinflammatory characteristics [20–22]. FMF is recessively inherited and caused by gain-of-function mutations in the *MEFV* (Mediterranean FeVer) gene encoding the pyrin protein [23]. Pyrin is mainly expressed in innate immune cells [24] 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 [25]. Although the expression of *MEFV* gene has not been detected in T cells according to The Human Protein Atlas [26], 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 the T cell compartment during inflammatory attacks in FMF patients [10, 27]. Another inborn error of immunity, CGD, is caused by mutations in genes encoding components of the reduced nicotinamide dinucleotide phosphate (NADPH) oxidase complex: gp91^{phox}, p22^{phox}, p67^{phox}, p40^{phox}, or p47^{phox}, which generates reactive oxygen species (ROS) [28, 29]. 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 [28]. This leads to life-threatening bacterial and fungal infections. Interestingly, ROS defects can also lead to a defective regulation of IL-1β production

and granuloma formation [30]. In contrast, 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 [31]. Genetic factors, however, might play a role in the gout pathogenesis. Genome-wide association studies link high serum urate levels and gout to defective gene variants involved in the renal urate-transport system including: *ABCG2*, *SLC2A9*, *SLC17A1*, *SLC22A12*, *CGKR*, *PDZK1*, and others [32]. The pathology of gout is known to be driven mainly by innate immune cell responses, in which inflammasomes play a crucial role [33]. As such, innate immune cells produce an excess amount of IL-1β upon engulfing MSU crystals [34]. It has been shown that uric acid and MSU crystals have stimulatory effects also on T cells and can enhance T cell responses to secondary stimuli [35, 36]. Moreover infiltrated T cells were found in the tissues of gout patients [37]. 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 in patients with CGD, FMF and gout, with a focus on innate-like unconventional gammadelta (γδ) T cells as well as CD4 and CD8 conventional alphabeta (αβ) T cells.

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 min. The antibody mixes (Table S1) in staining buffer (BD Bioscience) were added, and cells were incubated for 30 min 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 mM sodium pyruvate (Gibco), 2 mM glutamax (Gibco), 100 U/ml Penicillin, and 100 ng/ml

Streptomycin (Pan Biotech)) for 4 h at 37 °C. Then, the 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 min at 4 °C in the dark. The cells were washed with the washing solution (BD Bioscience), and antibody mix in the washing solution was added. After 30 min of 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. The graphs were generated using the GraphPad Prism (version 8.4.3) software. Non-parametric Mann–Whitney 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 CGD, FMF, 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 without known ongoing infections 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, 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 CGD, FMF, and gout 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 (Tregs), and NKT-like cells (Fig. 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 [38]. 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 [39], they were the main focus among γδ T cells in this study. We found significantly increased percentages of regulatory T cells and decreased percentages of CD56$^+$ NK cells in CGD patients compared to healthy controls (Fig. 1f, h). We have also observed lower percentages of Vδ2 and CD4 T cells in CGD patients compared to healthy controls (Fig. 1b, c). However, statistical analysis did not reach significance possibly due to the small size of the cohort. The distribution of cell populations within PBMCs remained largely similar in FMF and gout patients compared to healthy controls, with the exception of almost significantly reduced percentages of regulatory T cells in FMF patients (Fig. 1f).

Naïve and Memory T Cell Compartments are Not Affected by CGD, FMF, and Gout

To further scrutinize T cell populations in CGD, FMF, 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) [40]. These distinct T cell subsets differ in their effector function such as, T_{Naive} cells (CD45RA$^+$CD27$^+$) do not mediate effector immune responses effectively, T_{CM} (CD45RA$^-$CD27$^+$) cells have a higher sensitivity to antigenic stimulation and proliferative potential, and T_{EM} (CD45RA$^-$CD27$^-$) cells exhibit rapid effector function and lack of proliferative capacity, while T_{EMRA} (CD45RA$^+$CD27$^-$) cells represent the most differentiated type of memory cells and express high levels of cytotoxic molecules [40]. 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, CGD, and FMF.

Vδ2 and CD8 T Cells Exhibit Increased Activation Status in CGD

CD38 and CD69 are induced on T cells upon activation and are therefore commonly used as markers of activated or differentiated cells [41, 42]. We observed elevated proportions of CD38- and CD69- expressing Vδ2 T cells and CD69-expressing CD8 T cells in CGD patients (Fig. 2 and S3). Overall, this data suggests that the activation status of these T cells is increased in CGD. We further assessed the expression profile of the death receptor CD95 (Figure S4a), which is known to induce apoptosis [43]. The
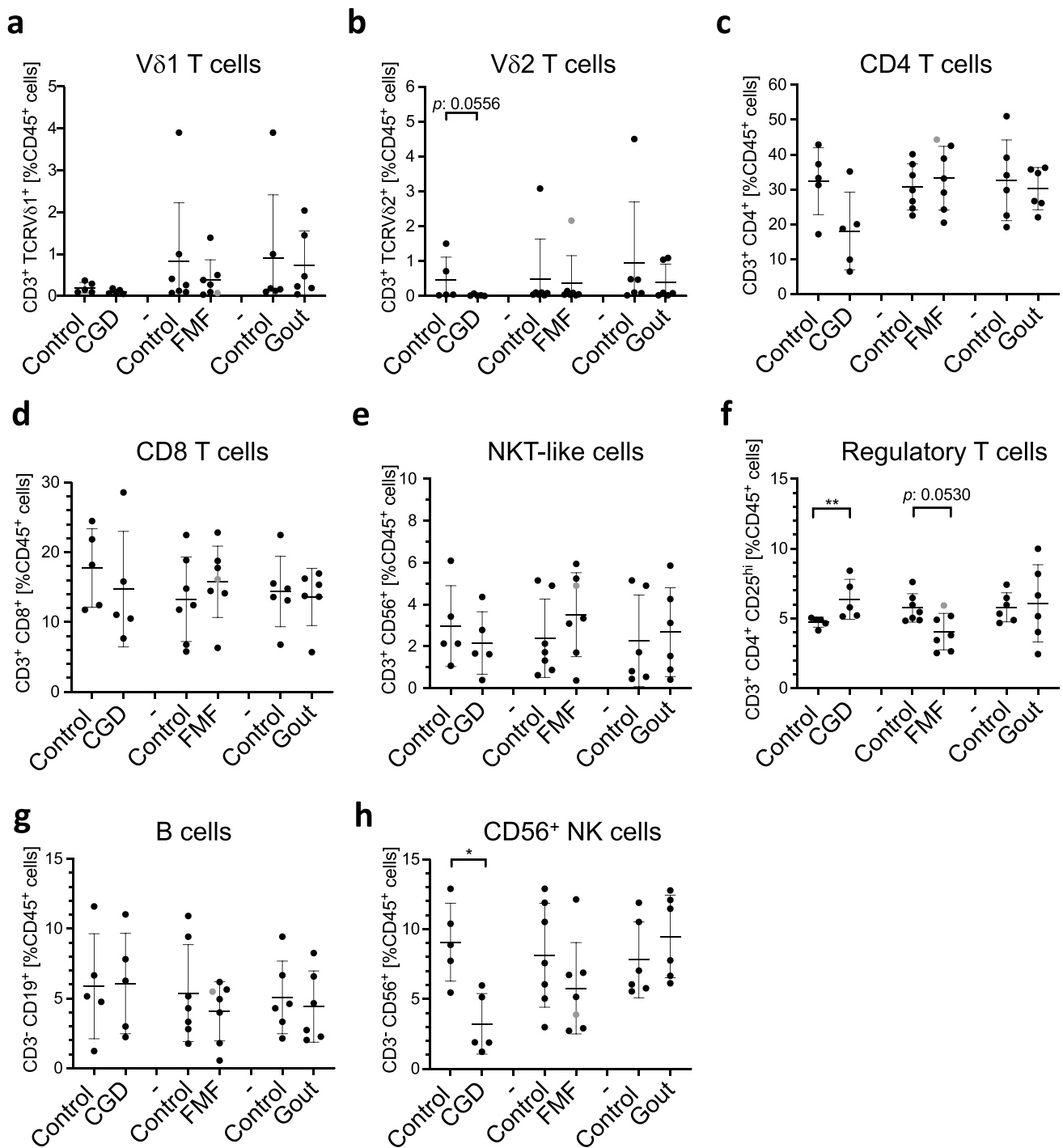
Fig. 1 The distribution of circulating lymphocyte populations is significantly affected in CGD patients. **a–h** Percentages of different cell populations: CD3^{+} TCRVδ1^{+} cells (**a**), CD3^{+} TCRVδ2^{+} cells (**b**), CD3^{+} CD4^{+} cells (**c**), CD3^{+} CD8^{+} cells (**d**), CD3^{+} CD56^{+} NKT-like cells (**e**), CD3^{+} CD4^{+} CD25^{hi} CD127^{−} regulatory T cells (**f**), CD3^{−} CD19^{+} B cells (**g**), and CD3^{−} CD56^{+} NK cells (**h**) in freshly isolated PMBCs from healthy controls and patients determined by flow cytometry. CGD chronic granulomatous disease ($n = 5$), FMF familial Mediterranean fever ($n = 7$), gout ($n = 6$). Gray circle: FMF patient with Behcet disease. Non-parametric Mann–Whitney test was applied. *$p < 0.05$, **$p < 0.01$

number of CD95-expressing Vδ2 T cells was elevated in CGD (Fig. 3a). The number of CD95-expressing CD4 and CD8 T cells was also increased in gout patients but did not reach statistical significance, possibly due to the small number of donors (Fig. 3c). Increased activation of T cells can lead to the exhaustion phenotype. Our results show that the
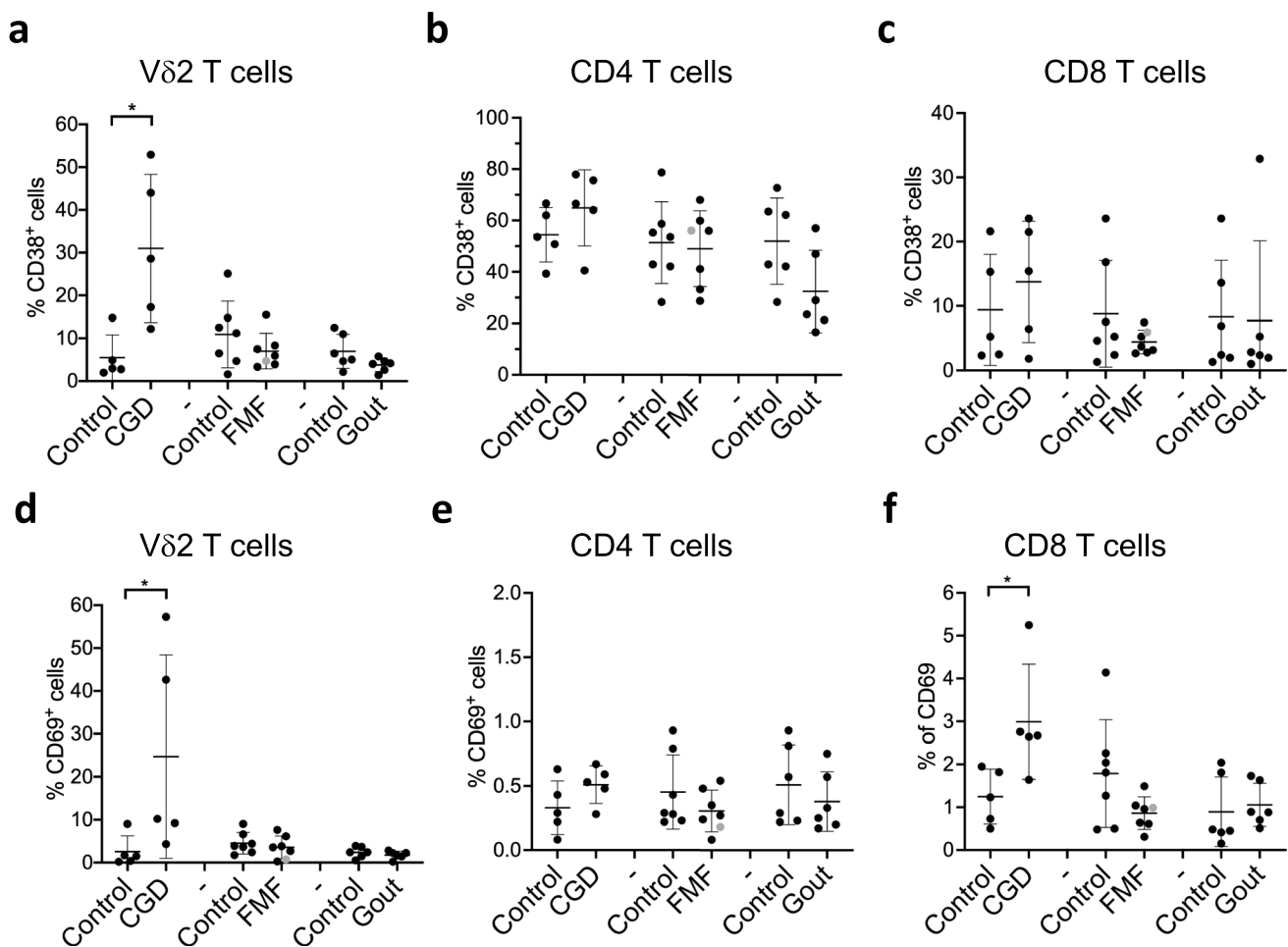
Fig. 2 CGD results in increased expression of activation markers on Vδ2 and CD8 T cells. **a–f** Percentages of Vδ2 (**a**, **d**), CD4 (**b**, **e**), and CD8 T cells (**c**, **f**) expressing CD38 (**a–c**) and CD69 (**d–f**) assessed by flow cytomet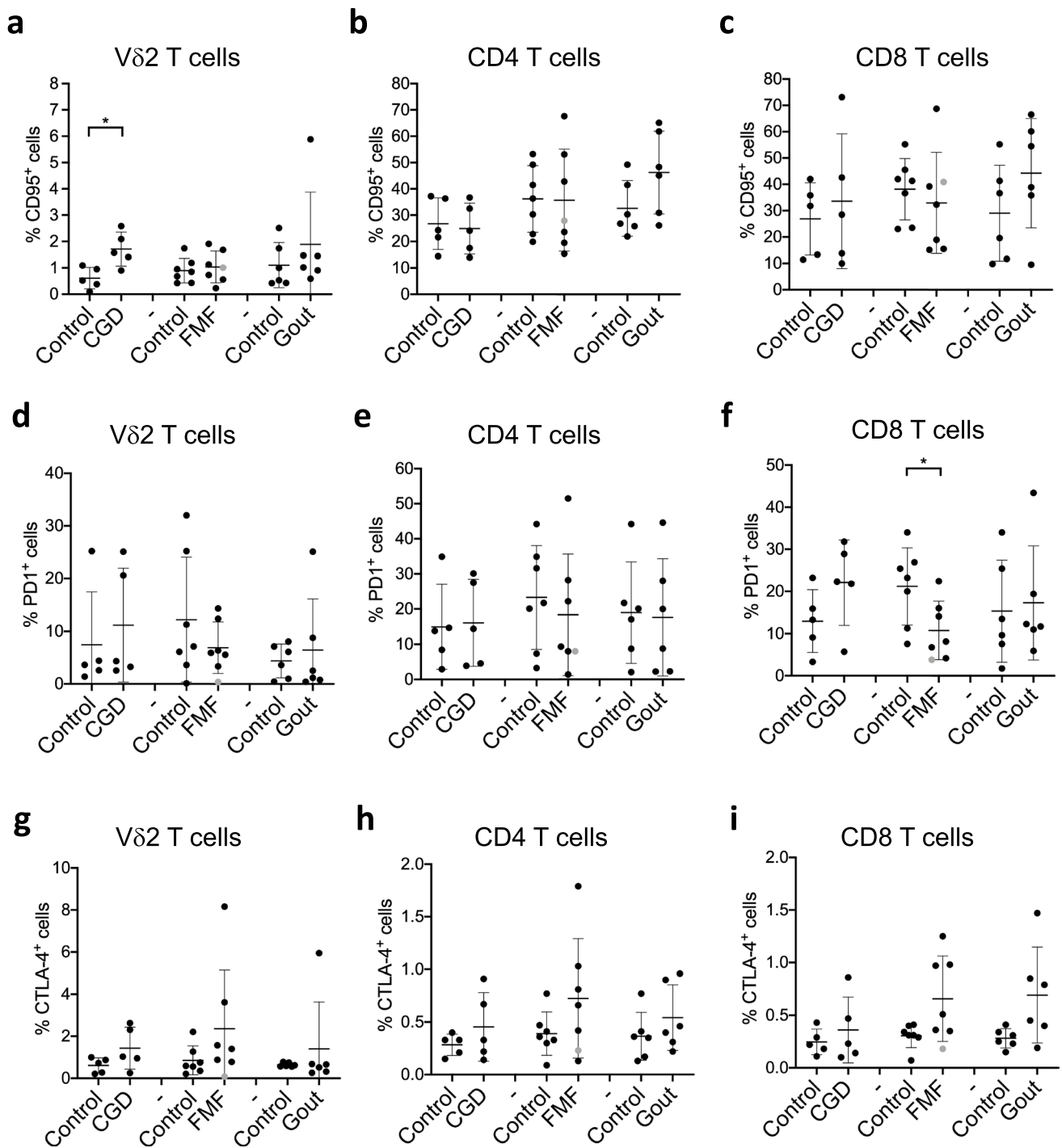ry on freshly isolated PMBCs from healthy controls and patients. CGD chronic granulomatous disease ($n = 5$), FMF familial Mediterranean fever ($n = 7$), gout ($n = 6$). Gray circle: FMF patient with Behcet disease. Non-parametric Mann–Whitney test was applied for statistical analysis. *$p < 0.05$, **$p < 0.01$

expression of the exhaustion marker PD-1 is significantly decreased in FMF patients in comparison to healthy controls (Fig. 3f), while numbers of CTLA-4-expressing CD8 T cells have tendency to increase in FMF and gout patients (Fig. 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 condition may affect the exhaustion status of CD8 T cells.

Cytokine Production by Vδ2 T Cells, but Not Conventional αβ T Cells, is Impaired in Gout and FMF Patients
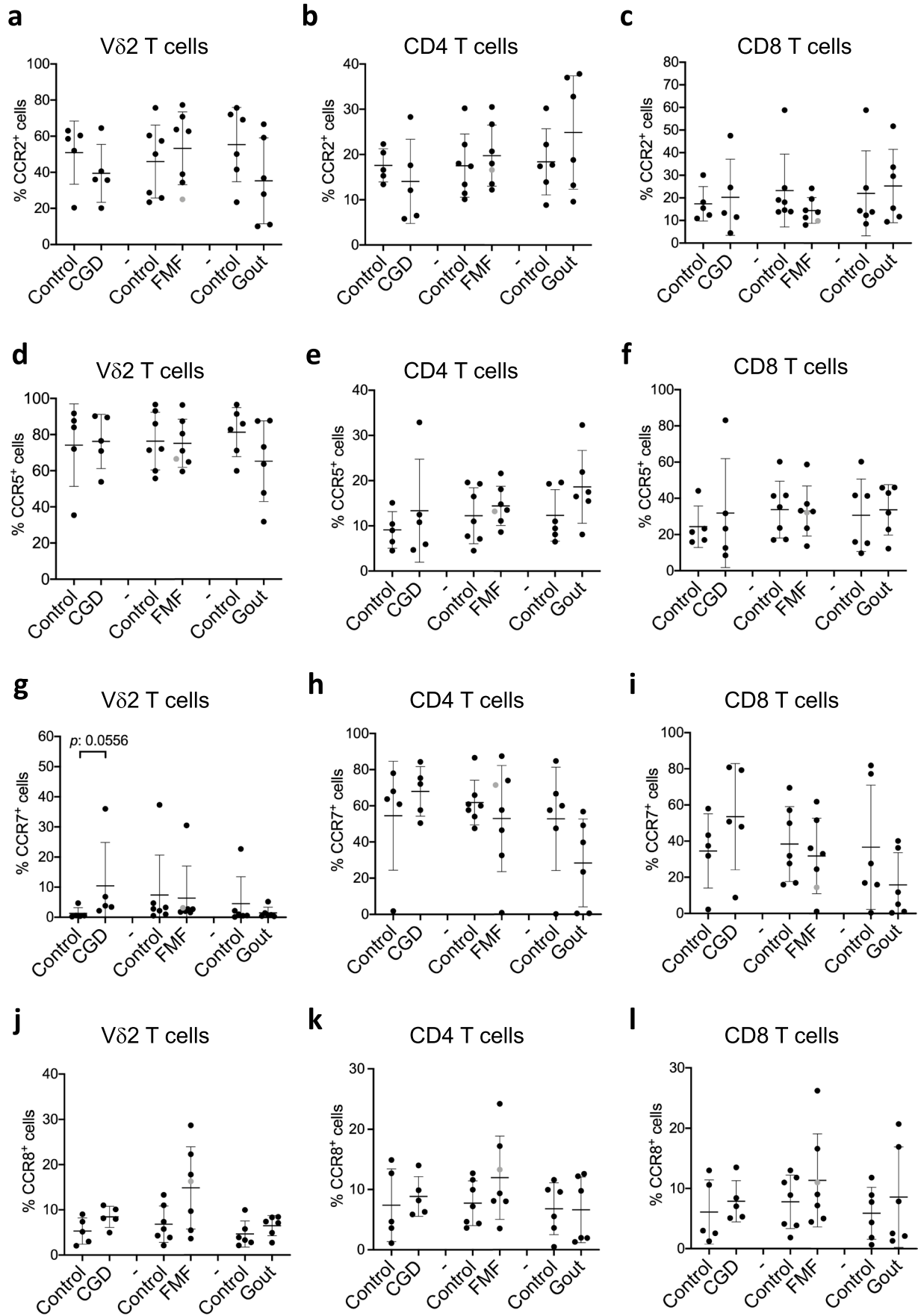
The effector function of T cells is largely driven by their cytokine production potential. Previous clinical studies demonstrated that cytokine production patterns are disrupted in patients with inborn errors of immunity, and these changes might help to distinguish different syndromes and their severity [44]. We assessed whether the capacity to produce cytokines by T cells is also affected in patients with CGD, FMF, and gout (Fig. 4). Our analysis revealed a significant reduction of IFN-γ- and TNF-α- producing Vδ2 T cells in gout (Fig. 4a, d, and S5). Furthermore, the percentages of IFN-γ-producing Vδ2 T cells were also significantly reduced in FMF patients (Fig. 4a). There were no significant changes in cytokine production among CD4 and CD8 T cells in all patients. We also analyzed the production of other cytokines, including the following: 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 in examined conditions.

Fig. 3 CGD and FMF affect PD-1 and CD95 expression on T cells. **a–i** Percentages of Vδ2 (**a**, **d**, **g**), CD4 (**b**, **e**, **h**), and CD8 T cells (**c**, **f**, **i**) expressing CD95 (**a–c**), PD-1 (**d–f**), and CTLA-4 (**g–i**) assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. CGD chronic granulomatous disease ($n = 5$), FMF familial Mediterranean fever ($n = 7$), gout ($n = 6$). Gray circle: FMF patient with Behcet disease. Non-parametric Mann–Whitney test was applied for statistical analysis. *$p < 0.05$
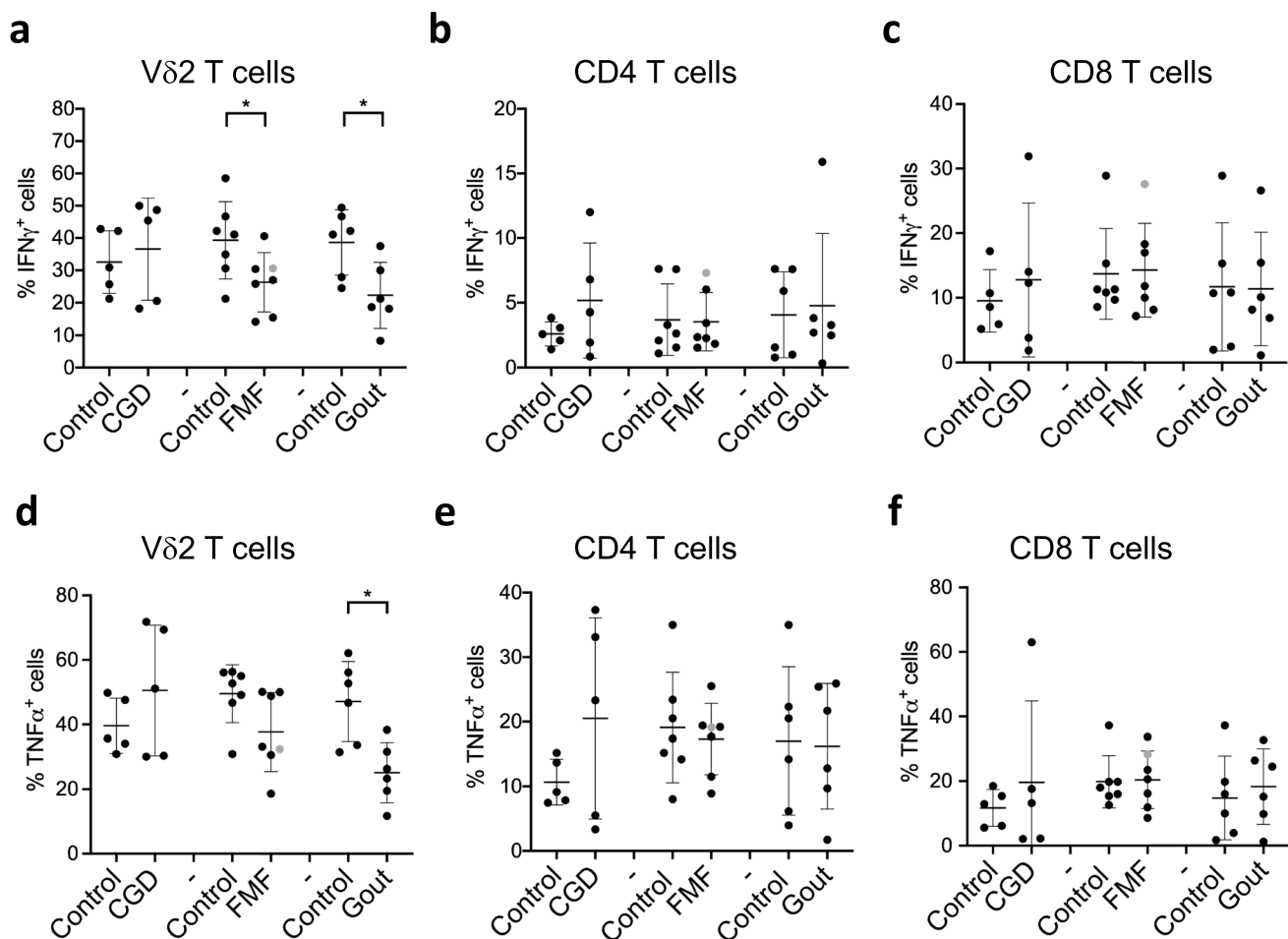
Fig. 4 FMF and gout patients show reduced numbers of cytokine-producing Vδ2 T cells. **a–f** Percentages of IFN-γ- (**a–c**) and TNF-α- (**d–f**) producing Vδ2 (**a**, **d**), CD4 (**b**, **e**), and CD8 T cells (**c**, **f**) assessed by flow cytometry in freshly isolated PMBCs from healthy controls and patients. CGD cChronic granulomatous disease ($n = 5$), FMF familial Mediterranean fever ($n = 7$), gout ($n = 6$). Gray circle: FMF patient with Behcet disease. Non-parametric Mann–Whitney test was applied for statistical analysis. *$p < 0.05$, **$p < 0.01$

CGD, FMF, and Gout Do Not Significantly Alter the Homing Receptor Expression on T Cells

Immune cell migration to inflamed tissues is an important component of 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 CGD, FMF, and gout (Fig. 5, S6, and S7). The expression of the following chemokine receptors was assessed by flow cytometry: CCR2 (Fig. 5a–c and S7a), which induces cell recruitment to sites of inflammation [45]; CCR5 (Fig. 5d–f and S7b), which regulates cell trafficking to the site of inflammation but also retention in tissues [46]; CCR7 (Fig. 5g–i and S7c), which mediates T cell migration from the blood to secondary lymphoid tissues [47]; CCR8 (Fig. 5j–i and S7d) and CCR4 (Figure S6a-c and S7e), which are skin homing receptors [48, 49] as well as CXCR3

(Figure S6d-f and S7f), which functions as a homing receptor to sites of infection and inflammation [50]. We observed increased numbers of CCR5-expressing CD4 T cells and reduced numbers of CCR7-expressing CD4 and CD8 T cells in gout patients (Fig. 5e, h, i). However, these numbers did not reach statistical significance, likely because of the sample size of the groups. Our results also show a trend for the increased number of CCR7+ Vδ2 T and CD8 T cells in CGD patients as well as the increased number of CCR8+ Vδ2 T, CD4 and CD8 T cells in FMF patients (Fig. 5g, i, j, k, l). Other homing receptors, CCR4 and CXCR3, did not show alterations in expression among T cell subpopulations in CGD, FMF, and gout patients compared to healthy controls (Figure S6). Altogether, no significant changes in the homing receptor expression pattern were detected in comparison to healthy controls, suggesting an unaltered migratory potential of T cells.

The Adhesion Potential of T Cells is Not Affected by CGD, FMF, 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 [51]. 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 LFA-1 and CD54 expression on T cells did not reveal any significant changes in all examined disorders (Fig. 6a–c, S8a-c, and S9a, d). Among T cells, CD8 and Vδ2 T cell can directly kill target cells [52, 53]. We examined whether this cytotoxic property is also affected in T cells by analyzing expression of CD56, NKG2D, and CD16 [54] (Fig. 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 [55, 56]. They have therefore been 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 (Fig. 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 differential alterations in the lymphoid compartment in terms of (*i*) distribution of circulating immune cell types, and (*ii*) expression of cell surface markers and cytokines in the examined disorders.

We found that the distribution of lymphoid cells was the most affected in CGD patients, in whom CD56⁺ NK cell percentages were significantly lower and regulatory T cell percentages were significantly higher than in healthy controls (Fig. 1). Furthermore, in our study, the percentages of Vδ2 and CD4 T cell populations tend to be lower in CGD patients compared to healthy controls (Fig. 1). Previous studies reported contradictory observations, where increased, reduced, or not affected T cell

numbers were shown [57–60]. The discrepancy between findings might be due to the differential treatment of patients at the time of analysis, the different distribution of patients' age, or causative mutations in p91^{phox} or p47^{phox} 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 [57]. 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 [59]; but whether this is 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 [61]. Deficiency in its activity in mice results in enhanced activation of MEK-Erk pathway and augmented Th1 and Th2 responses [62] as well as reduced differentiation and suppressive activity of regulatory T cells [62, 63]. 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 [61] and with human CGD biopsies from inflamed tissues [64]. 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 [65]. 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 the X-linked form of CGD, caused by p91^{phox} mutations, our cohort is mainly composed of patients with p47^{phox} mutations (Table S1). These two forms of disease might have distinct pathophysiology [66, 67]. While animal models suggest ROS-dependency of Tregs [63, 68], CGD patients with p47^{phox} mutations have been shown to have similar numbers of circulating Treg in comparison to healthy controls [68, 69]. These findings are not consistent with our observations of increased regulatory T cell numbers in CGD patients. The discrepancy in results might stem from the differences in age between the studies: our study focuses on adults (median age 36 years), while van de Geer et al. examined children (median age 13.6 years) (Table S1) [69]. A study with an increased number of inclusions will reveal if there is indeed a difference in T cell phenotype between these subgroups of patients.

Our analysis revealed an increased number of CD69-expressing CD8 and Vδ2 T cells in CGD patients (Fig. 2). CD69 is considered as an early activation and a tissue retention marker [70]. Increased expression on circulating T cells in CGD patients is less likely to be due to recent activation of these cells since the patients did not display any signs of
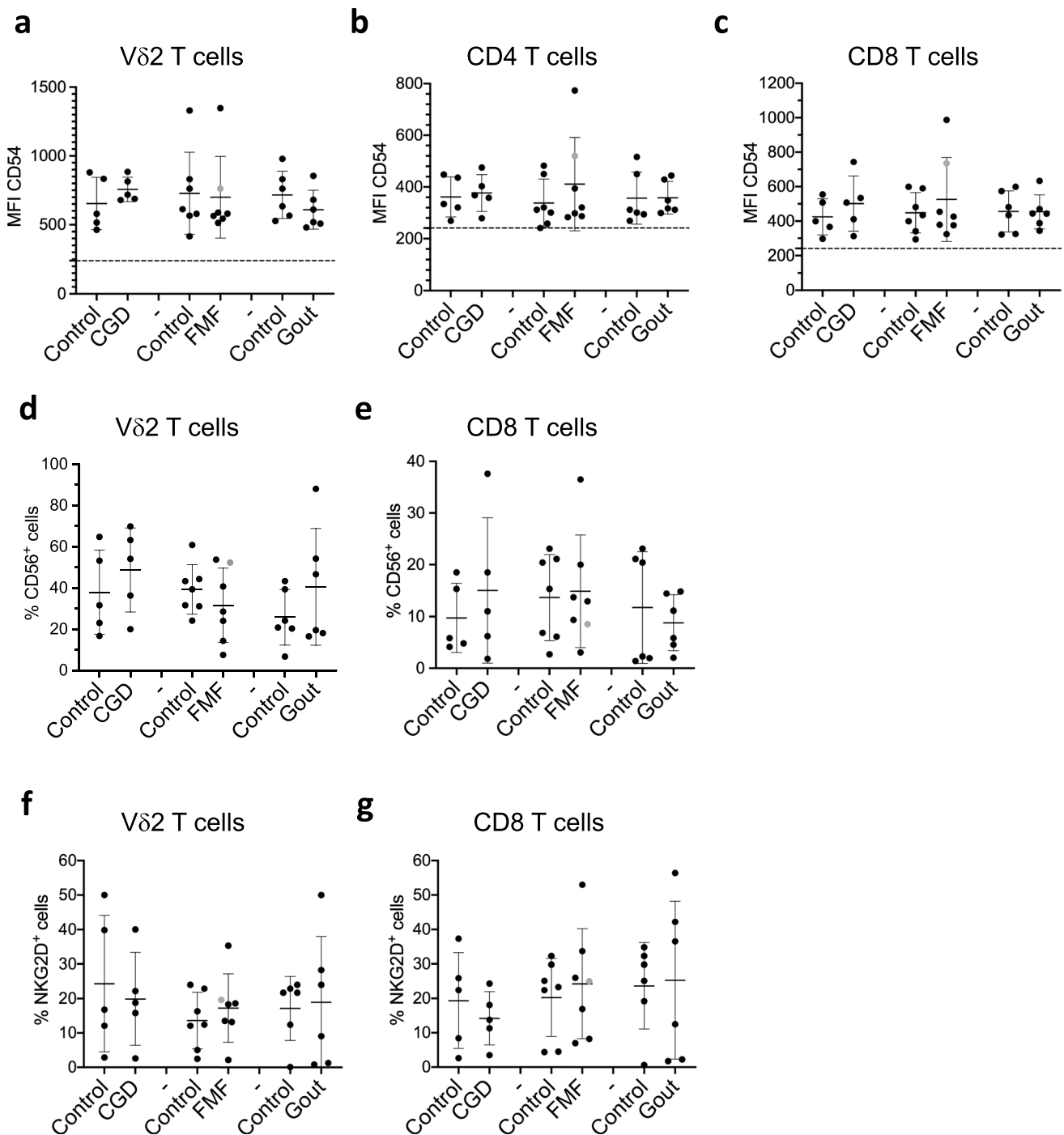
Fig. 6 T cells exhibit no change in expression of cytotoxicity markers in FMF, CGD, and gout. **a–g** Expression levels of CD54 (**a–c**), and CD56 (**d, e**) and NKG2D (**f, g**) on peripheral Vδ2 (**a, d, f**), CD4 (**b**), and CD8 T (**c, e, g**) assessed by flow cytometry on freshly isolated PMBCs from healthy controls and patients. Dashed lines in y-axis show the MFI of fluorescence minus one (FMO) control. CGD chronic granulomatous disease ($n = 5$), FMF familial Mediterranean fever ($n = 7$), gout ($n = 6$). Gray circle: FMF patient with Behcet disease. Non-parametric Mann–Whitney test was applied for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

acute attacks at the time of examination but could rather be 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 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. 3a) was significantly elevated on Vδ2 T cells. This suggests an increased activation status and susceptibility to undergo apoptosis, possibly explaining the reduced numbers of Vδ2 T cells in CGD (Fig. 1b). Our analysis has also revealed a reduced number of CD56$^+$ NK cells (Fig. 1h). Although early studies show a normal cytotoxic function of NK cells in CGD patients [71], a more recent cohort study revealed an association of NK cell lymphopenia with the more severe granulomas in patients with immunodeficiencies [72], indicating an involvement of these cells in granuloma formation. This suggests that development, rather than the functions of NK cells, is affected by NADPH complex deficiency. The analysis of a larger cohort is necessary to confirm these speculations.

FMF is a widely studied monogenic autoinflammatory disease, in which the T cell compartment has been best characterized so far. Early reports revealed an increased number of CD4 and CD8 T cells [10] as well as an increased number of IFN-γ-producing T cells in the asymptomatic phase and during acute inflammatory attacks [73]. 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 the attack-free or acute inflammation phases [73–75]. However, reduced, rather than increased, IFN-γ concentrations were found in PBMC cultures from FMF patients at different stages of the disease [76]. We did not observe any significant differences in CD4 and CD8 T cell frequencies (Fig. 1) between FMF patients and healthy volunteers, but we observed lower percentages of IFN-γ-producing Vδ2 cells (Fig. 4). The discrepancy in T cell frequencies between earlier studies and our findings might be due to differences in age: while our cohort included only adults (ages 35 to 55 years old) (Table S1), earlier studies investigated pediatric patients (ages 2 to 17 years old) [10, 73]. 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 the T cell compartment. We observed decreased percentages of regulatory T cells in our FMF cohort compared to healthy controls, suggesting that the reduced suppressive activity of Tregs might contribute to the severity of inflammatory attacks (Fig. 1). On the other hand, a small study of 6 FMF patients [77] reported unchanged Treg frequencies in FMF patients at different stages of febrile attacks compared to the healthy control group [77] despite the increased concentration of Treg-related cytokines such as IL-10 and TGF-ß in the circulation of FMF patients [75, 78]. The discrepancy between the findings might be due to the different medication history of the patients (Table S2) or different timing of the blood sampling.

Moreover, our results suggest a reduced exhaustion status of CD8 T cells in FMF patients based on a significant decrease in the expression of PD-1 (Fig. 3). These results suggest that CD8 T cells might be more active in FMF and contribute to the inflammatory flares. Indeed, increased percentages of the activation markers CD69 and CD25 expressed on CD8 T cells were found in FMF patients during the inflammatory attack [56]. The speculation on the role of CD8 T cells in the pathology of FMF warrants further functional validation. We also found a tendency toward increased expression levels of the CCR8 homing receptor by all examined T cell populations in FMF patients (Fig. 5). CCR8 expression points to changes in the migratory patterns of T cells. Yet, the frequencies of CCR8$^+$ cells in peripheral blood are very low, in accordance with other studies [79, 80] but enriched in the skin [79]. Examination of the immune cells in the skin of FMF patients will reveal whether T cells are involved in inflammatory reactions at the site of rashes, for example.

Of note, one of our FMF patients also has Behçet disease (Table S1). As this patient exhibits a huge increase in Vδ2 T cells, the observation also reported in previous studies [81] 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 also been found in the gouty tophi [37]. Furthermore, a recent discovery of the NLRP3 assemblage in T cells [82], which is known to be activated by MSU crystals in innate immune cells and to drive inflammatory flares in gout [33], suggests that adaptive immune cells can also be involved in gout pathology. Our analysis revealed some trends in the expression of homing receptors CCR5 and CCR7 as well as CD38 on CD4 T cells in gout patients (Figs. 5 and 2) indicating that the migratory pattern of T cells can be affected. While CCR7 regulates trafficking to lymph nodes and intestinal Peyer's patches [83], CCR5 mediates migration and effector function of T cells to sites of inflammation [46]. Increased frequency of CCR5$^+$ CD4 T cells and reduced numbers of CCR7$^+$ CD4 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) [84–87]. CCR7 signaling has been shown to influence the Th1/Th2 balance by skewing CD4 T cell differentiation towards Th1 fate [88–91]. The Th1 cells potentiate inflammatory responses to MSU crystals [19, 92]. However, we did not find increased numbers of circulating IFN-γ$^+$ T cells in gout condition (Fig. 4), consistent with recent reports [93, 94]. 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 [18, 19]. 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 [18]. 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.

As there is currently no cure for FMF, gout, and CGD current treatment strategies are aimed at reducing symptoms and preventing inflammatory attacks (in case of FMF and gout) or at preventing and managing bacterial and fungal infections and granulomas (in case of CGD). As such, the prescribed medications specifically target immunological dysfunctions, and therefore we cannot exclude the possibility that they have collateral effects on the peripheral T cell compartment. FMF and most gout patients from our cohort are on colchicine treatment which, by inhibiting the microtubule function, can affect various cell types including T cells (Table S2). Indeed, early studies reported that colchicine normalizes the CD4 to CD8 T cell ratio in FMF patients, while in healthy individuals, it reduces the total T cell numbers [95]. CGD patients, apart from receiving antibiotics (trimethoprim, sulfamethoxazole, flucloxacillin, metronidazole cream) and anti-fungals (ketoconazole, posaconazole), are also prescribed steroids (triamcinolonacetonide, prednisone, prednisolone, ciclesonide aerosole, Emovate) (Table S2). Consistent with the notion of a general immunosuppressive effect, steroids have been reported to exhibit numerous direct effects on T cells via increasing the expression of immunoregulatory proteins, inhibitory receptors, and apoptotic genes and decreasing the expression of pro-inflammatory cytokines, co-stimulatory molecules, and cell cycle mediators [96]. Other immunomodulatory medications that might influence the T cell compartment in FMF, gout, and CGD patients are IL-1β inhibitors (canakinumab or anakinra, used by two of the FMF patients) and TNF blockers (etanercept, used by one of the FMF patients), which by targeting these cytokines might modulate the polarization signals for T cells; non-steroidal anti-inflammatory drugs (NSAID) (diclofenac, ibuprofen; five FMF patients); statins (fluvastatin, simvastatin, taken by two gout patients); and allopurinol (four gout patients) (Table S2). The effect of these treatments on T cellshas either not been well investigated or has been reported contradictory. TNF blockers, for example, have been shown to either suppress cytokine production by circulating T cells in some inflammatory conditions [97–100] or to increase T cell responsiveness [101, 102]. Whereas in vitro treatment of PBMC cultures with allopurinol, a xanthine oxidase inhibitor which is used to reduce uric acid levels and treat gout, impairs cytokine production capacity by

T cells [103], yet the in vivo effect is not well defined. Apart from known immunomodulators, pain medications such as opioids (oxycodone, tramadol), proton pump inhibitors reducing the amounts of stomach acid (omeprazole, pantoprazole), or even vitamins (vitamin D, folic acid) might modulate the T cell compartment in our cohort (Table S2). Yet, we cannot exclude vitamin uptake by healthy volunteers. As there is a possible impact of ongoing medical interventions on T cell populations in studied patients, it is important to report the immune aberrations in patients undergoing treatments as a potential target for further improvement of the disease burden.

Overall, our findings indicate that IEI are complex diseases 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. However, we cannot exclude the possibility that the observed alterations in the lymphoid compartment are influenced by patient treatment, genetics, clinical history, or other factors. Performing a broader examination of the T cell compartment over the course of the 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 the 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 IEI and prompt a broader assessment of T cell involvement in gout, CGD and FMF.

Author Contribution B.A. designed, performed, and analyzed the experiments, wrote the manuscript; M.B., R.J.R., S.J.C.F.M.M., V.K., R.L., P.A.D., O.G., L.A.B.J. and F.vdV recruited patients; T.K.S. performed and analyzed the experiments; 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.

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

Declarations

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.

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

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