

Influence of Vitamin C on the differentiation and functional plasticity of human gamma delta T cells

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Vorgelegt von

Léonce Kouakanou

aus Cotonou, Bénin

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1. Gutachter: Prof. Dr. rer. nat. Thomas Bosch
2. Gutachter: Prof. Dr. med. Dieter Kabelitz

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“There are things we know that we know. There are known unknowns. But there are also unknown unknowns”

Donald Rumsfeld

SUMMARY

$\gamma\delta$ T cells are a numerically small subset of CD3⁺ T cells in human peripheral blood. They have recently attracted substantial interest as effector cells for cellular immunotherapy, due to their MHC-independent recognition and lysis of many solid tumor and leukemia/lymphoma cells. Depending on the micro-environmental cues, $\gamma\delta$ T cells also produce Th1/Th2/Th9/Th17-type cytokines, process and present antigen to conventional T cells, and can acquire regulatory activity. Potential clinical applications of *in vitro* expanded human $\gamma\delta$ T cells include the adoptive transfer into cancer patients. $\gamma\delta$ T cells can use different mechanisms to exert their effector functions. However, a better understanding of how their functional plasticity can be modulated is required to improve their translational efficacy. In this regard, it is important to develop novel strategies to optimize the culture conditions enabling maximal proliferative and functional activity. Vitamin C (L-ascorbic acid) is an essential vitamin that has to be supplied through appropriate nutrition or dietary sources. Vitamin C plays important roles in many different biological processes, spanning from stem cell differentiation to cancer cell biology. Moreover, Vitamin C has multiple effects on immune cells, acts as cofactor for several enzymes, has antioxidant activity and is known as facilitator of DNA hydroxymethylation through its capacity to enhance the capacity of Ten-eleven-translocation enzymes. The goal of the present thesis was to analyze the influence of the more stable derivative of Vitamin C, L-ascorbic 2-phosphate (pVC) on the differentiation and effector functions of human $\gamma\delta$ T cells. Moreover, the mechanisms sustaining pVC-mediated $\gamma\delta$ T-cell differentiation were also investigated.

The first part of this thesis studied the effect of pVC on the proliferation, differentiation and cytotoxic effector function. The results demonstrated that supplementation of pVC to the *in vitro* expanded human $\gamma\delta$ T cells significantly enhances their proliferative expansion. Further investigations demonstrated that pVC does not prevent activation-induced death of (phospho)antigen-re-stimulated $\gamma\delta$ T cells but rather enhances cell cycle progression and thereby cellular expansion. Moreover, pVC sustained the polarization of expanded $\gamma\delta$ T cells towards a mixed Th1+2-like phenotype along with the expression of Th1 and Th2 signature cytokines. Finally, we observed that the cytotoxicity of the *in vitro* expanded $\gamma\delta$ T cells against pancreatic cancer cell lines was enhanced in the presence of pVC and was associated with an increased release of IFN- γ . In the second part, this thesis investigated the effect of pVC on the TGF- β -induced expression of the regulatory T-cell-specific transcription factor Foxp3 and the resulting regulatory activity of purified human V γ 9V δ 2 T cells. pVC induced a significant increase of TGF- β -induced Foxp3 expression (both at protein and transcriptional levels) and stability as well as an increase in suppressive activity *in vitro*. Methylation

analysis of the Treg-specific demethylated region (TSDR) revealed that the TGF- β -expanded $\gamma\delta$ T cells treated with pVC showed a more pronounced demethylation of *FOXP3*. The third pillar of this thesis addressed the influence of pVC on the genome-wide gene expression and DNA methylation of human $\gamma\delta$ T cells activated for eight days *in vitro*. At this time point, pVC had only minor effects on the transcriptome and genome-wide DNA methylation of the expanded $\gamma\delta$ T cells.

Taken together, the results of this thesis demonstrated that Vitamin C has a significant influence on the functional activity of human $\gamma\delta$ T cells and thus appears to be suitable for enhancing the effector function of $\gamma\delta$ T cells for adoptive cell transfer in cancer patients. In addition, Vitamin C may also enhance the regulatory activity of $\gamma\delta$ T cells (in the presence of TGF- β), which may be of interest in the context of autoimmune diseases.

ZUSAMMENFASSUNG

$\gamma\delta$ T-Zellen stellen eine kleine Untergruppe von CD3-positiven T-Zellen im menschlichen peripheren Blut dar. Aufgrund ihrer HLA-unabhängigen Erkennung und Abtötung vieler Tumorzellen (sowohl solide Tumoren als auch Leukämien und Lymphome) haben $\gamma\delta$ T-Zellen großes Interesse für die potenzielle Anwendung in der Immuntherapie erlangt. Abhängig vom umgebenden Mikromilieu können $\gamma\delta$ T-Zellen auch Th1/Th2/Th9/Th17 Zytokine produzieren, Antigen prozessieren und präsentieren, sowie regulatorische Aktivität erwerben. Potenzielle klinische Anwendungen umfassen auch den adoptiven Zelltransfer in Krebspatienten. $\gamma\delta$ T-Zellen können verschiedene Mechanismen benutzen, um ihre Funktion auszuüben. Ein besseres Verständnis ihrer funktionellen Plastizität ist jedoch erforderlich, um ihre translationale Anwendung zu verstärken. Es müssen daher neue Strategien zur Optimierung ihrer proliferativen und funktionellen Aktivität entwickelt werden. Vitamin C (L-Ascorbinsäure) ist ein essentielles Vitamin, das durch die Nahrungsaufnahme zugeführt werden muss. Vitamin C hat vielfältige Funktionen in diversen biologischen Prozessen. Es ist ein Kofaktor für mehrere Enzyme, hat anti-oxidative Wirkung, und aktiviert Ten-eleven-translocation Enzyme und beeinflusst damit DNA Methylierung. Ziel der vorliegenden Dissertation war es, den Einfluß des stabileren phosphorylierten Vitamin C (L-ascorbic 2-phosphate, pVC) auf die Differenzierung und Effektorfunktionen von $\gamma\delta$ T-Zellen des Menschen zu untersuchen.

Im ersten Teil der Dissertation wurde der Einfluß von pVC auf die Proliferation, Differenzierung sowie zytotoxische Effektorfunktion von $\gamma\delta$ T-Zellen untersucht. Es zeigte sich, dass pVC die Proliferation *in vitro* signifikant verstärkt. Weitere Untersuchungen ergaben, dass pVC nicht den aktivierungsinduzierten Zelltod von $\gamma\delta$ T-Zellen verhindert, sondern vielmehr den Zellzyklus beeinflusst. Außerdem zeigten $\gamma\delta$ T-Zellen in Gegenwart von pVC eine verstärkte Produktion von sowohl Th1- als auch Th2-Schlüssel-Zytokinen und eine verstärkte Zytotoxizität gegenüber Pankreas Tumorzelllinien. Im zweiten Teil der Dissertation wurde der Einfluß von pVC auf die Expression des für regulatorische T-Zellen spezifischen Transkriptionsfaktor Foxp3 sowie die regulatorische Aktivität von V γ 9V δ 2 T-Zellen untersucht. In Gegenwart von TGF- β wurde durch pVC die Induktion von FoxP3 Expression sowohl auf Ebene der Transkription als auch der Proteinexpression signifikant verstärkt. Ferner stabilisiert pVC die Foxp3 Expression über einen längeren Zeitraum und erhöhte die suppressive Aktivität der $\gamma\delta$ T-Zellen. Die Analyse des Methylierungs-Status der Treg-spezifischen demethylierten Region (TSDR) im *FOXP3* Gen zeigte eine massive Demethylierung nur in Gegenwart von pVC. Im dritten Teil der Dissertation wurde der Einfluß von pVC auf die genomweite Transkription und DNA Methylierung in $\gamma\delta$ T-Zellen nach 8

Tagen *in vitro* Aktivierung untersucht. Zu diesem Zeitpunkt wurden nur geringe Einflüsse von pVC auf Transkriptom und Methylierung festgestellt.

Zusammenfassend zeigen die Ergebnisse dieser Dissertation, dass Vitamin C einen erheblichen Einfluß auf die funktionelle Aktivität von humanen $\gamma\delta$ T-Zellen hat und somit geeignet erscheint, die Effektorfunktion von $\gamma\delta$ T-Zellen für den adoptiven Zelltransfer bei Krebspatienten zu verstärken. Andererseits kann Vitamin C auch die regulatorische Aktivität von $\gamma\delta$ T-Zellen (in Gegenwart von TGF- β) verstärken, was im Rahmen von Autoimmunerkrankungen von Interesse sein könnte.

LIST OF ABBREVIATIONS

µg	microgram
µL	microliter
µM	micromolar
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BrHPP	Bromohydrin pyrophosphate
BTN	butyrophilin
CD	Cluster of differentiation
CGI	CpG islands
CNS	Conserved non-coding sequence
DAMPs	Danger-associated molecular patterns
DC	Dendritic cells
DHA	dehydroascorbate
DMAPP	dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Foxp3	Forkhead box protein 3
GATA-3	GATA-binding protein 3
GSH	glutathione
H ₂ SO ₄	Sulfuric acid
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IFN	Interferon
IL	Interleukin
ILCs	Innate lymphoid cells
IPP	Isopentenyl pyrophosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
IU	International unit
JMJ	Jumonji C (JmjC)-domain-containing histone demethylase
kDA	kiloDalton
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MICA/B	MHC class I-related molecules A/B
min	minute
mL	milliliter
mTOR	mechanistic target of rapamycin
n-BPs	nitrogen-containing bisphosphonates

NK	Natural killer
NKG2D	Natural killer group 2 member D
NKG2DL	Natural killer group 2 member D ligand
pAgs	phosphoantigen
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI	Propidium iodide
PRR	Pattern recognition receptor
PTM	Post-translational modification
pVC	Phospho-modified Vitamin C (L-ascorbic acid 2-phosphate)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute
RRBS	Reduced representation bisulfite sequencing
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SVCT	Sodium-dependent Vitamin C transporter
T-bet	T-box-containing protein expressed in T cells
TCR	T cell receptor
Tet	Ten-eleven translocation
TGF- β	Transforming growth factor beta
TIGIT	T cell Immunoglobulin and Immunoreceptor tyrosine-based inhibitory motif domain
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Tregs	Regulatory T cells
Tris	2-amino-2-hydroxymethyl-propan-1,3-diol
TSDR	Treg-specific demethylated region
VC	Vitamin C (L-ascorbic acid)
WGBS	Whole genome bisulfite sequencing
ZOL	Zoledronic acid

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1. INTRODUCTION

1.1. A brief overview of the immune system

The immune system comprises cells, soluble factors and molecules with specialized roles in the defense against “danger” elicited by viruses, bacteria, or parasites but also by “altered self” like tumor cells to prevent harm of the organism. By analogy to this definition, the mammalian immune system is often associated with “army” terminology as its components are “fighting” enemies like pathogens and do so in a highly orchestrated manner. Based on the subdivision of the defense tasks, two main components of the immune system have been defined: *innate* and *adaptive* immunity. The innate immunity is composed of cellular defense mechanisms including myeloid cells such as macrophages (phagocytic cells), dendritic cells (DCs), and neutrophils as well as molecules such as the complement and coagulation systems. In addition, a previously unappreciated cell type of the innate system, termed innate lymphoid cells (ILCs), has been characterized in mice and human. ILCs play a major role in the guarding and maintenance of the tissue barriers against invading pathogens [1,2]. In general, innate immunity is characterized by the recognition of conserved molecular patterns, which comprise pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), by a limited number of pattern recognition receptors (PRRs). This recognition triggers a cascade of inflammation processes ultimately reducing the pathogen load [3]. However, this is usually not sufficient to eliminate microbes. The innate immune system provides a rapid nonspecific response against intruders and is equipped with a certain degree of immunological memory [4,5]. Indeed, the innate immunological memory (also termed “trained” immunity) is defined as heightened response to a secondary infection that can be exerted toward the same microorganism and a different one. However, this innate memory lacks the high degree of specificity and amplification, two features of the adaptive immunity [5]. Adaptive immunity comprises defense mechanisms mediated by specific immune cells known as lymphocytes (B and T cells) and the specialized molecules (e.g. antibodies) required for their function. Adaptive immunity is characterized by high diversity and specificity of the antigen receptors and long-lasting memory. It is developed by clonal selection from a vast repertoire of lymphocytes bearing receptors with different antigen specificities. The seemingly unlimited diversity of these receptors is not entirely encoded in the germline, but instead results from gene rearrangement of a limited number of germline-encoded gene segments [6]. However, although both components of the immune system seem to act separately, they also cooperate at several points to ensure an optimal protection of the host. Importantly, cellular communication between innate and adaptive immunity as well as with other organs is heavily influenced by soluble mediators (cytokines).

1.2. Conventional versus unconventional T cells

Cells involved in both innate and adaptive immunity arise from pluripotent hematopoietic stem cells in the bone marrow. Particularly, T cell precursors further migrate in the thymus to complete their maturation. The above classic dichotomy (innate versus adaptive immunity) does not give a complete picture of the complexity of the immune system. The two major types of T cells, so-called *conventional* and *unconventional* T cells operate in fundamentally different ways to mediate and coordinate immune responses. On one hand, the “*conventional*” T cells recognize peptide antigens presented by cell-surface proteins of the major histocompatibility complex (MHC) family including MHC class I or MHC class II molecules. These MHC molecules are highly polymorphic and can bind a diverse spectrum of peptides, which provides the basis for the presentation of peptide antigens from nearly any pathogen [7]. The incredible ability of conventional T cells to distinguish a vast array of peptides derived from “non-self” versus “self” proteins is endowed by the elegant process of positive and negative selection within the thymus. T-cell receptor (TCR) gene rearrangements that occur during conventional T-cell differentiation in the thymus are derived from germline-encoded segments, named variable (V), diversity (D), joining (J), and constant (C). The gene rearrangement in the order (V(D)J gives rise to the highly diverse CD4 and CD8 TCR $\alpha\beta$ repertoires as the V region is composed of α and β chains. Rearrangements of genes encoding TCR α and TCR β and random pairing can theoretically generate an expressed diversity close to 5×10^6 TCR $\alpha\beta$ [8]. However, the TCR $\alpha\beta$ repertoire is drastically increased by additional non-germline encoded mechanisms such as inclusion of N nucleotides during gene rearrangement [9]. Functionally, conventional $\alpha\beta$ T cells with their main subsets of CD4 or CD8 expressing cells are responsible for coordinating the adaptive immune responses. On the other hand, “*unconventional*” T cells express CD3-associated TCR molecules like conventional T cells but differ from them in several ways including their limited TCR germline repertoire and the nature of molecules that are recognized by the TCR. Moreover, the principles of the MHC paradigm do not apply to unconventional T cells which are not restricted by conventional MHC class I or class II molecules. Unconventional T cells comprise cells such as the mucosa-associated invariant T (MAIT) cells [10], CD1d-restricted T cells (iNKT cells, [11], both of which express invariant TCR $\alpha\beta$, and gamma/delta ($\gamma\delta$) T cells [12]. Of note, $\gamma\delta$ T cells, named according to the alternatively expressed $\gamma\delta$ TCR, generate TCR heterodimers through somatic V(D)J recombination of corresponding γ and δ genes encoding TCR V, D, J, C. Interestingly, there is a preference for specific pairing of selected TCR γ - and TCR δ V genes [7,13]. The number of V, D and J segments is small for $\gamma\delta$ T cells as compared to $\alpha\beta$ T cells. In contrast to conventional T cells, which are the principal “controllers” of adaptive immunity, the multidimensionality of unconventional T cells is revealed by their ability

to link both innate and adaptive immunity. However, highlighting the fundamental nature of T-cell integration, it has been described that whereas conventional T cells provide clonal antigen-specific responses, unconventional T cells profoundly regulate conventional T cells, often suppressing their activities [14,15].

1.3. Determining $\alpha\beta$ versus $\gamma\delta$ T-cell commitment

TCR gene rearrangements in the process of $\alpha\beta/\gamma\delta$ lineage commitment give rise to $\alpha\beta$ CD4 T cells, $\alpha\beta$ CD8 T cells and $\gamma\delta$ T cells. Within the thymus, stages of T-cell development can be broadly categorized as double negative (DN), double positive (DP) or single positive (SP) according to the expression of the CD4 and CD8 coreceptors. β -selection is the process in which the TCR β chain pairs with the pre-TCR α protein to produce a membrane-localized pre-TCR that signals survival, expansion, and allelic exclusion [16]. Several models have been proposed to explain the $\alpha\beta/\gamma\delta$ lineage choice. The “*instructive*” model suggests that the formation of a functional $\gamma\delta$ TCR instructs the cell to develop into a $\gamma\delta$ T cell, while expression of the pre-TCR complex directs the cell to become $\alpha\beta$ T cell. The “*stochastic*” model proposes that rearrangements of the TCR β , γ , and/or δ genes occur in each developing thymocyte, but only cells that make productive rearrangements of the TCR genes that match the cell fate predetermined by factors including IL-7R expression or Notch signaling are selected to survive [17]. A third model suggests that during thymic development, the “*strength*” of the signal delivered by TCR complexes expressed on the surface of the DN thymocyte progenitors at the β -selection checkpoint determines the $\alpha\beta$ versus $\gamma\delta$ T-cell lineage fate [18,19]. Weak signaling from the pre-TCR promotes the development of $\alpha\beta$ T cells, whereas $\gamma\delta$ TCR, which generally delivers a stronger signal than that of the pre-TCR, drives the development of $\gamma\delta$ T cells.

1.4. TCR signaling

Signaling *via* the TCR plays an essential role in the regulation of T-cell differentiation, survival, activation and proliferation. The TCR is a multi-protein complex including a $\alpha\beta$ TCR heterodimer (or $\gamma\delta$ TCR in $\gamma\delta$ T cells) and CD3 $\gamma\delta\epsilon$ - and ζ -chains. The CD3/ ζ - chains contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are required for signaling [20]. Upon antigen recognition by the TCR/CD3/ ζ complex, a hierarchical signaling cascade is initiated which includes: (i) phosphorylation of the ITAMs on key tyrosine residues by the tyrosine kinase Lck (ii) recruitment of the cytosolic Syk-family tyrosine kinase ζ -chain-associated protein of 70 kDa (Zap70) to the phosphorylated ITAMs, (iii) phosphorylation and activation of Zap70 by Lck, (iv) phosphorylation of the transmembrane adaptor protein LAT

and of others effector molecules [21-23]. Together, these processes result in the activation, proliferation and differentiation of T cells into effectors T cells

The activation (and differentiation) of $\alpha\beta$ T cells depends on the $\alpha\beta$ TCR recognition of antigen peptides presented by the MHC proteins. Upon the recognition of the peptide-MHC (pMHC) complex (signal 1), the cascade of the TCR-dependent signaling cascades as mentioned above is initiated, but full activation and acquisition of appropriate effector T-cell function requires additional signals. Signal 2, also referred to as co-stimulation, is triggered by binding of molecules of B7 family (CD80/CD86) expressed on “professional” antigen-presenting cells (APC) to CD28 on T cells [24]. The CD28/B7 interaction constitutes the predominant pathway of T-cell co-stimulation. However, the presence of alternative pathways was reported. These include the inducible costimulatory (ICOS)/B7-related protein-1 (B7RP-1)- [25], the tumor necrosis factor (TNF)/TNF-receptor (TNFR)- [26] and the CD2/CD58- [27] pathways. Finally, the third signal provided by cytokines induces T-cell differentiation into effector cells that exert cytotoxic or regulatory activity or produce cytokines to activate innate immune cells or B cells [28].

Although most of the mechanisms of $\alpha\beta$ TCR signaling mentioned above are thought to be shared by the $\gamma\delta$ TCR, both the components of the TCR-CD3 complex and receptor-proximal signaling are reportedly different between $\alpha\beta$ T cells and $\gamma\delta$ T cells [19]. In fact, the CD3 δ subunit is not even incorporated into the $\gamma\delta$ TCR complex and is not required for $\gamma\delta$ T-cell development [29,30].

1.5. The role of ROS and mTOR in TCR signaling

Upon stimulation, T cells undergo metabolic reprogramming resulting in an increased production of ATP which supports their proliferation and effector functions. Energy production inevitably generates “unwanted” products, namely reactive oxygen species (ROS). ROS are small short-lived oxygen-containing molecules that are chemically highly reactive, a property that is mainly due to their unpaired electrons (radicals). Superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}), hypochlorous acid (HOCl), lipid peroxides (ROOH), singlet oxygen (1O_2), and ozone (O_3) are some of the most common ROS [31]. The first two species are the most important ROS involved in the regulation of biological processes in which ROS have long been recognized as markers of stress and inducers of cell death [32,33]. However, studies revealed that ROS in low/moderate amounts act as positive regulator of signaling pathways implicated in T-cell activation and proliferation as well as controlling cell death [34,35]. ROS regulate several signaling pathways involved in pluripotency, including mitogen-activated protein kinases (MAPK) and MAPK phosphatases. Intracellular ROS

accumulation can be limited by intracellular antioxidants, of which the ubiquitously expressed glutathione (GSH) is the most abundant. Buffering of ROS by GSH prevents their intracellular accumulation to a toxic concentration. GSH-mediated ROS buffering processes promote the activation of the mechanistical target of rapamycin (mTOR). mTOR is an evolutionarily conserved serine/threonine kinase which consists of two distinct multi-protein mTOR complexes, termed mTORC1 and mTORC2, found in mammalian cells [36]. These complexes share the catalytic mTOR subunit and other associated proteins, including Raptor and Rictor, the two obligate adaptor proteins for mTORC1 and mTORC2, respectively [37,38]. mTOR integrates nutrient inputs, metabolic cues, growth factors, and cytokine signaling to regulate cell growth, metabolism, proliferation, differentiation, and aging [39,40]. mTOR signaling regulate the fate decision between effector and regulatory CD4⁺ T cells and between effector and memory CD8⁺ T cells [38,41]. In $\gamma\delta$ T cells, inhibition of mTOR signaling was reported to promote their anti-tumor effector function [42,43].

1.6. Human $\gamma\delta$ T cells: subsets and ligand recognition

1.6.1. Subsets based on TCR expression

Among the many fascinating and important question that have yet to be solved for $\gamma\delta$ T-cell biology is the amazing difference between species regarding their relative numbers, phenotype, anatomical localization and ligand recognition. In contrast to sheep, cattle, rabbits and chickens whose circulating $\gamma\delta$ T-cell population represents up to 60% of total T-cell pool [44], human $\gamma\delta$ T cells comprise a small population (average 5%) of peripheral CD3⁺ T cells [45]. Despite their relative low levels in peripheral blood, $\gamma\delta$ T cells are found to be enriched at mucosal tissues [45]. In human, distinct subsets of $\gamma\delta$ T cells bearing specific pairs of the γ and δ TCR chains are present in particular locations. Human $\gamma\delta$ T cells are subdivided into V δ 1, V δ 2 and V δ 3 T cells, pairing with distinct V γ gene elements (V γ 2, 3, 4, 5, 8, or 9) [46,47]. Typically, about 50% to 75% of $\gamma\delta$ T cells in peripheral blood express V δ 2 chain usually paired with V γ 9 chain. These cells are referred to as V γ 9V δ 2 T cells [48]. The subset of $\gamma\delta$ T cells expressing the V δ 1 element is more prevalent in tissues than in the peripheral blood [49,50]. Besides V δ 1 and V δ 2 cells, there is a very small subset of V δ 3 T cells, consisting of only 0.2% of circulating T cells. The V γ chain pairing of tissue-resident human V δ 1 T cells is less stringent in comparison to V γ 9V δ 2 T cells, although they are preferentially paired with V γ 4 and V γ 5 and to a lesser degree V γ 9-containing γ -chain [51,52]. This greater diversity of γ and δ chain pairing may imply that there exists a broader range of ligands that are recognized by those $\gamma\delta$ T cells.

1.6.2. Ligands and receptors of human $\gamma\delta$ T cells

Major efforts were made over the years to identify antigens and ligands that are specifically recognized by the $\gamma\delta$ TCR. Some of the identified ligands for non-V δ 2 (i.e V δ 1 or V δ 3) $\gamma\delta$ TCR include CD1d-lipid antigen [53], the endothelial protein C receptor (EPCR) [54], as well as the stress-inducible MHC class I-related molecules A/B (MICA/B) molecules [55]. Ligands such as phosphorylated molecules (phosphoantigens, pAgs) are exclusively recognized by human V γ 9V δ 2 T cells [56]. These pAgs are intermediates of the eukaryotic mevalonate or the prokaryotic non-mevalonate (also termed Rohmer's) pathway of isoprenoid synthesis [57-59]. Ligands for which direct binding to the V γ 9V δ 2 TCR was claimed are the human mutS homolog2 hMSH2 [60] and F1-ATPase together with the delipidated form of apolipoprotein A-I [61] but also super-antigen staphylococcal enterotoxin A and glycolipid components of *Mycobacterium tuberculosis* [62,63] as well as putative tumor-derived antigens such as GroEL homolog and Hsp60 [64,65].

In addition to the TCR, $\gamma\delta$ T cells express other activating cell surface receptors, notably natural killer group 2 member D (NKG2D), which serves as a receptor for MICA/B and the six members of the UL16 binding protein family (ULBP1-6) [66]. Furthermore, $\gamma\delta$ T cells express receptors associated with the innate immune system, notably Toll-like receptors (TLRs), hence corresponding TLR ligands can co-stimulate $\gamma\delta$ T-cell activation [67].

1.6.3. Mechanisms of phosphoantigen-dependent activation of V γ 9V δ 2 T cells

The majority of human V γ 9V δ 2 T cells respond rapidly to small phosphorylated molecules (pAgs). By far the most potent of these compounds is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a phosphorylated metabolite of the microbial non-mevalonate isoprenoid biosynthesis pathway. Classified as "exogenous" pAg, HMBPP is produced by many Gram-positive and Gram-negative bacteria as well as by malaria parasites, *Mycobacterium tuberculosis* and *Toxoplasma gondii* [68,69]. Other compounds with activity on V γ 9V δ 2 T cells, include "endogenous" pAgs intermediates from the eukaryotic mevalonate pathway, such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), synthetic analogues such as monoethyl phosphate, bromohydrin pyrophosphate (BrHPP) [68,70]. Furthermore, V γ 9V δ 2 T cells are activated by aminobisphosphonate drugs such as alendronate, pamidronate and zoledronate (ZOL) and natural and synthetic alkylamines which mechanistically can be explained by blockade of farnesyl pyrophosphate synthase (FPPS) and the subsequent intracellular accumulation of IPP, DMAPP and by-products such as the AMP conjugate of IPP, Apppl, thereby rendering treated cells susceptible to recognition by V γ 9V δ 2 T cells [70,71]. The nature of antigen recognition by $\gamma\delta$ T

cells is distinct from that of $\alpha\beta$ T cells. $V\gamma 9V\delta 2$ T cells do not require MHC molecules for pAg-dependent activation. However, Butyrophilin-3A (BTN3A), a member of the butyrophilin (BTN) protein family, was identified as a necessary component in the pAg-dependent activation of $V\gamma 9V\delta 2$ T cells [72]. There are three related proteins in the BTN3A family, BTN3A1, A2 and A3 each encoded by separate genes but all clustered on the chromosome 6 [73]. All three proteins have two immunoglobulin (Ig)-like extracellular domains (IgC and IgV) that are structurally related to B7 costimulatory molecules. BTN3A1 and BTN3A3 possess an intracellular domain, named B30.2. Regarding the role in $V\gamma 9V\delta 2$ T-cell activation, the BTN3A1 isoform was identified to be indispensable for the pAg-dependent activation [72]. The canonical view of antigen presentation and T-cell activation dictates that antigens are presented extracellularly through binding to the TCR. In line, reports proposed that, consistent with pAg being a “presented” antigen, the extracellular IgV domain of BTN3A1 could bind with low affinity to the pAg (HMBPP and IPP), creating a composite epitope for recognition by $V\gamma 9V\delta 2$ TCR [74,75]. Contradictory studies however indicate that pAgs actually bind to the intracellular B30.2 domain of BTN3A1. These studies demonstrated that pAgs instead interact directly with the intracellular B30.2 domain through a positively charged pocket [76,77]. Thereafter, an “inside-out” signal is triggered and involves the interaction of adaptor proteins such as the cytoskeletal protein periplakin [78] and the small GTPase RhoB [79] with different regions of the BTN3A1 molecule. Ultimately, either by changing the spatial distribution of BTN3A1 or by inside-out signaling and conformational change of the extracellular domain of BTN3A1, binding to the $V\gamma 9V\delta 2$ TCR is induced [80].

1.7. Functional plasticity of human $\gamma\delta$ T cells: many needles in one haystack

Even though $\gamma\delta$ T cells have some properties of innate immune cells (e.g. NK receptors), their functional plasticity resembles the broad spectrum of differentiations observed for $\alpha\beta$ T cells. $\gamma\delta$ T cells integrate features reminiscent of T helper-like (Th1, Th2, Th17, Th9) and regulatory T (Tregs) cells as well as cytotoxic T lymphocytes and APC. Depending on the priming conditions, activated $V\gamma 9V\delta 2$ T cells can produce a range of cytokines and chemokines, kill infected and transformed target cells, regulate survival and differentiation of monocytes and neutrophils, induce maturation of dendritic cells, provide B-cell help and present antigens to CD4 and CD8 T cells [81-83]. Activated $\gamma\delta$ T cells can express high levels of Interferon (IFN)- γ and tumor necrosis factor (TNF)- α [84]. In addition, they can also be polarized *in vitro* into interleukin (IL)-4-, IL-17-, IL-22- and IL-9-producing cells [85-87]. $\gamma\delta$ T cells can also display a dual functional phenotype such as Th1+Th2 or Th1+Th17-like phenotype. For example, activated $V\gamma 9V\delta 2$ T cells can simultaneously produce IFN- γ and IL-13 [88], but also IFN- γ and IL-17 [86]. The broad functional phenotypes of responsive $\gamma\delta$ T

cells enable them to interact with other cells. For example, activated V γ 9V δ 2 T cells produce chemokine such as CXC-chemokine ligand 13 (CXCL13) [89], which regulates the organization of B cells within the follicles of lymphoid tissues [90].

Regulatory T cells (Tregs) are important for the maintenance of immune-balance and self-tolerance. They can be divided into two subpopulations according to their origin; thymic-derived Tregs (tTregs) that arise from immature CD4 $\alpha\beta$ T cells in the thymus, and peripherally-derived Tregs (pTregs) that originate from peripheral tissues. Induced Tregs (iTregs) can also be generated *in vitro* by stimulating naïve CD4 $\alpha\beta$ T cells in the presence of the transforming-growth factor β (TGF- β) [91]. One of the hallmarks of the Treg phenotype is the expression of Foxp3, a transcription factor considered a master regulator of Tregs development and function [92]. Although stable expression of Foxp3 is required for the suppressive activity of Tregs, their effector pathways are heterogenous, which reflects diverse Treg-subphenotypes [93,94]. Tregs utilize inhibitory cytokines such as IL-10, IL-35 or TGF- β ; granzyme B and perforin-mediated cytotoxicity and cell surface inhibitors such as CTLA-4 and T cell Immunoglobulin and Immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) [95,96]. Moreover, adenosine as the product of the CD39/CD73-mediated adenosine triphosphate (ATP) degradation also acts as an immunosuppressive mediator and constitutes a well described mechanism of Treg-mediated suppression [97]. V γ 9V δ 2T cells, in the presence of IL-15 and TGF- β , can also differentiate *in vitro* into cells with regulatory T cell (Treg)-like phenotype which express Foxp3 and can suppress the proliferation of conventional CD4 $\alpha\beta$ T cells [98,99]. Regulatory $\gamma\delta$ T cells ($\gamma\delta$ Tregs) have been described in different contexts and seem to play a role as immunosuppressive cells in cancer, pregnancy, allergy and inflammation [100-103].

An important facet of the $\gamma\delta$ T-cell function is their ability to lyse a broad range of tumor cells including bladder cancer [104], colon cancer [105], glioblastoma multiforme [106], hematological malignancies [107] and multiple myeloma [108]. Moreover, correlation between the numbers of tumor-infiltrating $\gamma\delta$ T cells and a better prognosis was also observed in cancer patients [109-111]. However, tumor-infiltrating $\gamma\delta$ T cells can also promote tumorigenesis through different mechanisms including the production of pro-inflammatory cytokines (e.g. IL-17) or the tumor-driven adoption of immunosuppressive phenotype [112-114].

1.8. Epigenetic control of T-cell differentiation

1.8.1. Epigenetic mechanisms

The epigenome constitutes the interface of a dynamic environment and the genome. Epigenetic is defined as heritable changes in gene expression without changes in the DNA sequence of the genome [115,116]. Among the epigenetic changes that alter chromatin structure and influence gene expression are (i) changes in nucleosome position and conformation; (ii) changes in the histone content of nucleosomes; and (iii) modifications in the histone and DNA sequences [117]. Accordingly, epigenetic mechanisms involve covalent post-translational modification (PTM) of histone including phosphorylation, (de)acetylation, (de)methylation, poly-ADP ribosylation and ubiquitination, and/or (de)methylation of DNA [118,119]. The PTM of the histone target specific amino acids such as lysine and arginine. The PTM change the electric charge of these amino acids and thereby affect the tertiary structure of the histones. By these structural changes, the accessibility to the DNA is regulated and thereby the protein-binding to critical *cis*-regulatory regions such as promoters, enhancers or silencers and many conserved non-coding sequence (CNS) elements. Histone methylation can either activate or repress the transcription, depending on the methylation site. The histone (de)methylation, at lysine and arginine residues, is catalyzed by two groups of enzymes known as histone demethylases: the lysine-specific histone demethylases (LSD1 and LSD2) that can demethylate mono- and di-methylated lysine residues, and the Jumonji C (JmjC)-domain-containing histone demethylase (JMj) that can demethylate mono-, di- and tri-methylated histone at lysine/arginine residues [120,121]. The most extensively studied methylation occurs on histone H3 at lysine (K) 4 (H3K4), H3K9, H3K27, H3K36, H3K79, and H4K20 as well as on histone H3 at arginine (R) 2 (H3R2), H3R8, H3R17, H3R26, and H4R3. The methyl donor in histone methylation is *S*-adenosylmethionine [122]. Functionally, the methylation of H3K4 is associated with active gene-transcription, whereas methylation at H3K27 is a hallmark of silenced chromatin [123,124]. Other post-translational modifications such as acetylation and deacetylation of histone proteins are catalyzed by enzymes such as histone acetyltransferases and histone deacetylases, respectively [125]. In general, histone acetylation is regarded as an activating histone modification: H3K27 acetylation (H3K27ac) was described to mark active promoter and enhancers and is therefore useful to identify *cis*-regulatory elements [126].

Moreover, gene expression can also be regulated by (de)methylation of CpG residues of the DNA. In mammals, the base cytosine is commonly modified by methylation of its carbon at position 5, predominantly in the context of CpG dinucleotides (mCpG) [127]. The methylation at the carbon (C) 5 position of cytosine (5-methylcytosine [5mC]) is the major and best-characterized epigenetic mark of mammalian DNA. The identification of the enzymes and

essential cofactors that catalyze DNA (de)methylation has greatly contributed to understanding the molecular connections within the epigenome. The transfer of a methyl group from the donor S-adenosylmethionine to a cytosine is catalyzed by DNA methyltransferases (DNMT) 1, 3a and 3b. DNMT1 is associated with replication machinery to maintain methylation in dividing cells, whereas DNMT3 and DNMT3b are involved in *de novo* introduction of methyl group on cytosine residues [128]. After the methylation is completed, 5mC, especially in CpG dinucleotide context, can be recognized and then bound with a group of methyl-CpG-binding proteins [129]. In contrast to DNMTs, Ten-eleven-translocation (Tet, including Tet1, Tet2 and Tet3) methylcytosine dioxygenases, which belong to the Fe²⁺ and 2-oxoglutarate-dependent dioxygenase family, were identified to catalyze the hydroxylation of 5mC into 5-hydroxymethylcytosine (5hmC), which then is further oxidized into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [130,131]. Both 5fC and 5caC could be excised by the DNA repair enzyme thymine DNA glycosylase to produce an abasic position, which is eventually replaced by an unmodified C, thus completing the process of DNA demethylation [132,133]. In addition to this enzymatic demethylation also known as active demethylation, there exists another way of demethylation called passive demethylation where the 5mC marks can also be lost by passive demethylation due to dilution effects and the lack of *de novo* methylation during DNA replication [134]. CpG DNA (de)methylation is a key component of the epigenome and is closely associated with either the activation or silencing of transcription. At CpG islands (regions of high CpG density), especially within the promoters, DNA methylation causes repression of transcription, and high methylation supports processes such as X-chromosome inactivation and imprinting [127], whereas a low degree of methylation is found at active gene-regulatory elements that are bound by transcription factors [135]. Hence, using methods of genome-wide DNA-methylation profiling, such as whole-genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS), has been proved very useful to delineate cell-type specific aspects of gene regulation and to identify cis-acting transcription factors.

Well-established crosstalk between histone modifications and DNA methylation exists, which contributes to stable regulation of gene expression [136,137]. Thus, histone and DNA (de)methylation are dynamic processes of the epigenome, ultimately altering processes like T-cell differentiation.

1.8.2. Epigenetic implications in T-cell differentiation

T cells are central to the orchestration of immune responses to invading pathogens, but their activity has to be carefully balanced and controlled to avoid tissue damage and pathology. Following antigenic stimulation, naïve T cells proliferate and differentiate into various T-

helper subsets with different effector, regulatory and memory phenotype, each characterized the expression of a specific gene signature. Epigenetic mechanisms play a crucial role in governing and maintain cellular differentiation. The transition from a naïve T cell to differentiated T cell involves a combination of different mechanisms: consolidation and spreading of DNA methylation and histone modifications; negative feedback loops in which cytokines and transcription factors that activate one pathway inhibit the expression of opposing transcription factors and cytokines; and positive feedback loops in which transcription factors autoactivate their own expression [138]. Several genes controlling immune functions are known to be regulated by epigenetic mechanisms. The fact that histone modifications and DNA methylation can be dynamically modulated during T-cell responses to an antigen is suggested by a number of observations. For instance, the active histone modifications, such as H3K4 methylation and H3K27ac at promoter and enhancer elements, mark key signature cytokine genes such as *IFNG*, *IL4* and *IL17A* [139]. Moreover, in murine T cells, the *IL2*, *IL4* and *IFNG* cytokine genes are known to be regulated by DNA methylation [140-142]. In the absence of DNMT1 and DNMT3a (but not DNMT3b), murine CD4 T cells were unable to silence properly the *IFNG* and *IL4* loci under appropriate culture conditions, resulting in increased and promiscuous cytokine expression [143,144]. T-cell activation under Th2-polarising conditions was shown to substantially reduce the recruitment of DNMT1 to the *IL4-IL13* locus in proliferating cells, eventually leading to reduced DNA methylation of this locus and enhanced gene expression [141]. Moreover, the group of C. Dong generated a genome-wide 5hmC analysis and demonstrated that Tet2-mediated DNA demethylation plays a crucial role in the control of lineage-associated cytokine and transcription factor expression in Th cells [145,146]. Lineage-specific 5hmC modifications during Th1 and Th2 polarization were reported in murine [146] and in human [147] CD4 T cells. A positive correlation was observed between the 5hmC distribution at gene bodies and the lineage-specific transcription factor *TBX21* and *GATA3* for Th1 and Th2, respectively [145]. Additionally, Tet2-mediated CNS demethylation and the expression of DNMT1 (but not DNMT3) were shown to be essential for the differentiation and function of Tregs [148,149]. In the murine system, genome-wide histone (H3) acetylation and methylation profiling have identified distinct molecular programs in IFN- γ versus IL-17-producing $\gamma\delta$ T cells [150]. It is also well established that epigenetic mechanisms regulate the chromatin accessibility of the TCR γ locus during intrathymic T-cell development [151,152].

1.9. Vitamin C: Regulation of immune function

1.9.1. Vitamin C, a general introduction

Vitamin C (VC) or L-ascorbic acid is a six-carbon ketolactone synthesized by a two-step reaction from L-galactose in green plants or from glucose by most animals in the kidney or liver [153]. However, humans - as well as other primates, guinea pigs and fruit bats - are unable to synthesize VC because they harbor inactivating mutations in the gene encoding L-gulonogamma-lactone oxidase (GULO), the enzyme responsible for catalyzing the last step of VC synthesis in the liver [154]. Owing to this inability of endogenous VC synthesis, humans must take up VC from dietary sources. The current recommended daily allowance of VC (75-90 mg per day) can easily be achieved by consuming a balanced nutrition including fruits and vegetables [153]. VC levels are maintained in a range between micromolar in the blood plasma (~50 μM) and millimolar (~1-10 mM) inside the cells [155], with the highest levels found in the brain, the adrenal gland and leukocytes which import VC by a highly specific transport systems, the sodium-dependent vitamin C transporters (SVCT) 1 and 2 (SLC23A1 and SLC23A2) [156,157]. By contrast, sustained malnutrition or low dietary VC intake will lead to plasma levels below 10 μM and result in scurvy, a VC-deficiency disease characterized by bleeding gums, impaired wound healing, anemia, fatigue, depression and, in severe cases, death [153].

VC exists in different redox forms depending on the biological conditions and is considered the most relevant naturally occurring reducing substance [158]. Fully reduced VC (ascorbate) can be oxidized both intracellularly and extracellularly. Extracellular ascorbate is oxidized by free radicals or ROS producing a weak radical intermediate ascorbate radical ($\text{Asc}^{\cdot-}$), which is then fully oxidized into dehydroascorbate (DHA) [157]. DHA, having a short half-life (less than 1 minute) [159], accounts for only approximately 1-5% of VC in the human body [153] and is either transported inside the cells or becomes irreversibly hydrolyzed into 2,3-L-diketoglutarate (2,3-DKG). 2,3-DKG is then degraded into oxalic acid and threonic acid [160]. Inside the cells, DHA is rapidly reduced back to ascorbate by reacting with a reduced glutathione [160]. Moreover, at micromolar concentrations, VC acts as an antioxidant by reducing the harmful ROS levels [88,161]. Conversely, at millimolar plasma concentrations, VC can also function as pro-oxidant [153]. In addition to its redox potential, VC exerts a chelator-like activity [155]; indeed, by reducing ferric to ferrous iron (i.e. $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$), and by generating soluble iron complexes, VC efficiently enhances the absorption of nonheme iron at the intestine level [162]. VC also affects iron metabolism by stimulating ferritin synthesis, inhibiting lysosomal ferritin degradation and cellular iron efflux, and by inducing iron uptake from low-molecular weight iron-citrate complexes [163].

1.9.2. Vitamin C acts as modulator of enzyme activity

Besides its role as antioxidant, VC acts as critical cofactor for numerous enzymes donating its electron (functioning as reducing agent) to prosthetic metal ions to achieve full enzymatic activity [153]. In general, VC-interacting enzymes are categorized into two families: (i) the copper-containing monooxygenases, and (ii) the Fe^{2+} -dependent and α -ketoglutarate (α KG; also known as 2-oxoglutarate (2OG))-dependent dioxygenases (Fe^{2+}/α KGDDs). The monooxygenases include dopamine β -hydroxylase and peptidylglycine α -amidating monooxygenase, whereas the dioxygenases family is composed of prolyl 4-hydroxylases (P4H), prolyl 3-hydroxylase, lysyl hydroxylase, asparaginyl hydroxylase, trimethyllysine hydroxylase, γ -butyrobetaine hydroxylase and 4-hydroxyphenylpyruvate dioxygenase. The anatomical localization and the functions of those enzymes are depicted in Fig. 1a. Fe^{2+}/α KGDDs catalyze a wide range of hydroxylation reactions involved in the collagen synthesis, the hypoxia-inducible factor 1 α (HIF1 α) stability, the carnitine synthesis, the catabolism of tyrosine and the demethylation of protein, DNA and RNA [164,165]. The mechanistic role of VC in these enzymes is exemplified in P4H and its involvement in scurvy. In the absence of VC, the initial collagen hydroxylation catalyzed by P4H can proceed at a maximal rate, albeit less efficient as the conversion of Fe^{2+} to Fe^{3+} during the process, soon results in the inactivation of P4H. This inactivation leads to an incomplete hydroxylation of proline residues in collagen, which in turn causes incomplete crosslinking and eventually the characteristic signs of scurvy [166].

Furthermore, VC has been shown to facilitate DNA demethylation (i.e. enhanced 5hmC production) in cultured cells in a Tet-dependent manner [167,168]. These studies reported that VC-dependent enhanced levels of 5hmC are not due to its activity as an antioxidant, but more likely due to its function as a bound cofactor for Tet enzymes [167-169]. Moreover, several other Fe^{2+}/α KGDDs rely on VC as a bound cofactor [164]. However, the current model implies that VC promotes the Tet-mediated 5hmC production not as a cofactor for Tet, but by converting Fe^{3+} (the most common form of iron in the cell) into Fe^{2+} , which is essential to retain the Tet enzymes in their fully catalytic form [170]. Fig. 1b depicts the involvement of VC in the Tet-mediated DNA demethylation.

Like DNA methylation, also histone methylation is a reversible process, which depends on the activity of the JMJ. VC is required for the optimal catalytic activity of several JMJ, as the demethylation is halted when VC is withdrawn from the *in vitro* assay [120,121]. Hence, VC also acts as a cofactor for the JMJ family (Fig. 1c), thus modulating histone demethylation similarly to the way it does for DNA demethylation.

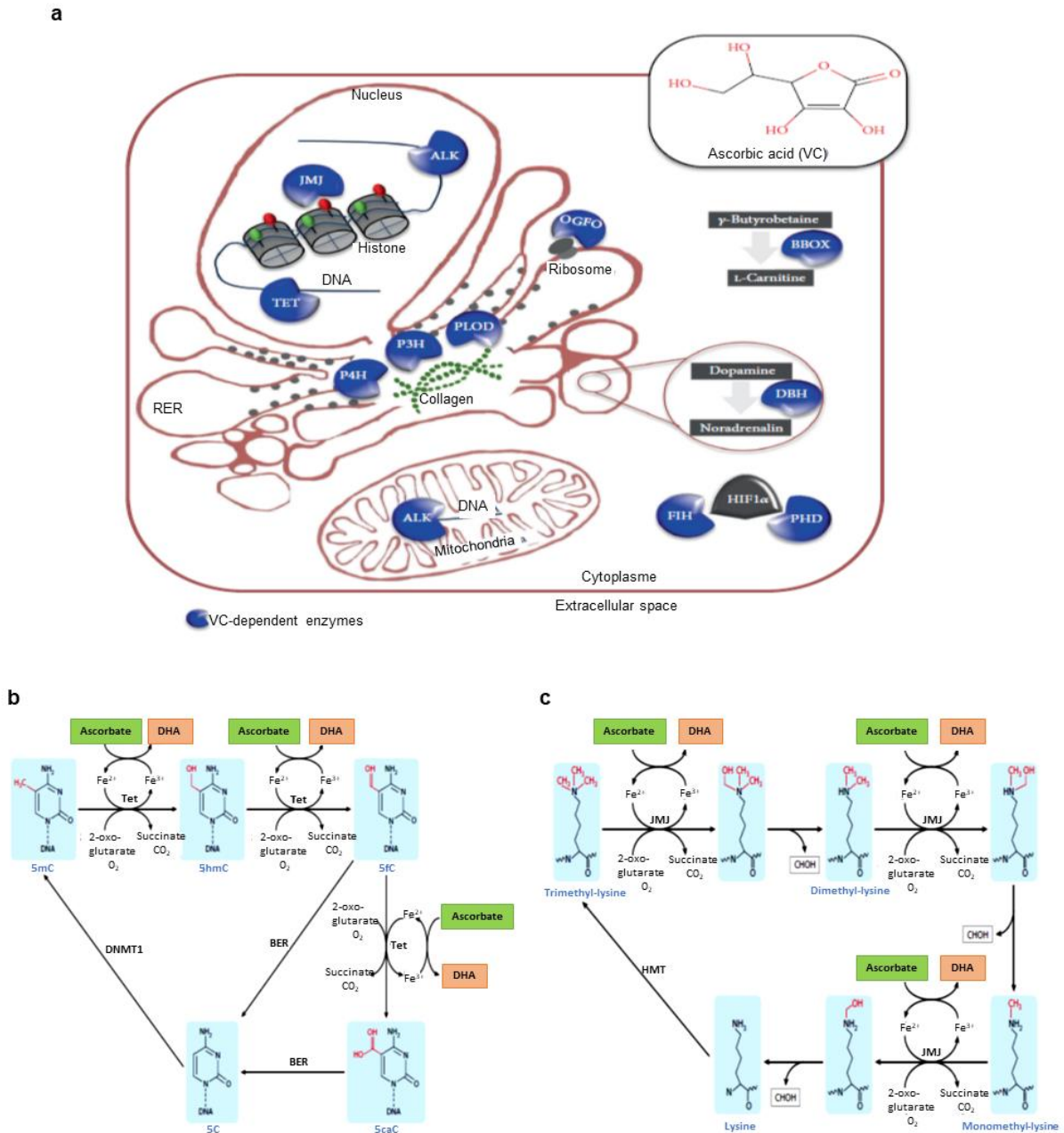


Figure 1. Enzymatic and epigenetic roles of Vitamin C.

(a) Localization and function of VC-dependent mono- and dioxygenases (slightly modified from [155]). **(b, c)** The role of VC in (b) DNA demethylation and in (c) histone demethylation (slightly modified from [122]). ALK: RNA and DNA demethylase family; JMJ: JmjC-domain-containing histone demethylases; OGFO: 2-oxoglutarate- and Fe^{2+} -dependent oxygenase; PLOD: procollagen-lysine α -KG 5-dioxygenases; P3H: collagen prolyl 3-hydroxylase; P4H: collagen prolyl 4-hydroxylases; BBOX: γ -butyrobetaine dioxygenases; DBH: dopamine beta-monooxygenase; PHD: HIF-prolyl hydroxylase; FIH: factor inhibiting HIF5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; BER, base excision repair; DHA, dehydroascorbic acid; DNMT, DNA methyltransferase; TET, Ten-eleven-translocation DNA demethylases.

1.9.3. Influence of Vitamin C on T-cell activation and differentiation

VC is widely regarded as an enhancer of immune functions. Although the underlying mechanisms are still not yet completely elucidated, several studies revealed that VC has multiple effects on the development, proliferation and function of lymphocytes. Studies in the murine and human system demonstrated that VC at physiological concentrations has an enhancing effect on T-cell proliferation and cytokine production, while supraphysiological concentrations are toxic for cells [171-174]. In line, it was found that the restoration of VC-levels in VC-deficient patients positively influenced the T-cell proliferation [175]. The antioxidant activity is the most obvious mechanism by which VC could support immune cell functions, particularly since immune responses are more pronounced in reducing environments [176,177]. Other possible mechanisms by which VC might promote the immune response include modulation of the phosphatase activity of calcineurin [178], the nuclear binding activator protein 1 (AP-1) transcription factor and the phosphorylation of MAPK (p38 and JNK) [179], and epigenetic regulation of gene expression [180,181]. T-cell development occurs in the thymus and can be simulated *in vitro* using fetal thymic organ cultures, in the presence of stromal cells or in feeder-free conditions. In search of factors that could enhance T-cell differentiation, it was reported that, in both humans and mice, VC is required *in vitro* for the early development of T cells as it overcomes a developmental block from double negative to double positive thymocytes. Furthermore, VC speeds up the maturation process of T cells [182,183].

Moreover, it has been demonstrated in murine studies that VC also affects T-cell differentiation outside the thymus. In the presence of VC, Th1 polarization is favored over Th2 polarization [184,185]. T cells isolated from VC-treated mice, displayed increased Th1 cytokines (IL-2, TNF- α and IFN- γ) and produced lower level of Th2 cytokine (IL-4) when activated *in vitro* [184]. Song MH et al. recently reported that Th17 polarization of sorted murine naïve CD4 T cells was more pronounced (enhanced IL-17 production) in the presence of VC. Mechanistically, this effect was not due to enhanced Tet enzyme activity, but to the VC-dependent promotion of the Jmjd2-protein-mediated histone demethylation of the *IL17* promoter and the resulting enhanced expression of the *IL17* gene [186]. Memory T cells constitute a small subset of lymphocytes but provide life-long immunity to previously encountered antigens. The effect of VC on memory T cells is less well investigated. It was found in an *in vitro* mouse model, that VC increased the generation of CD8 memory T cells through increased production of stimulating cytokines (IL-12) by dendritic cells [187]. Tregs require the stable expression of the transcription factor Foxp3 for their suppressive activity. Epigenetic mechanisms were found to be crucial for the stability of Foxp3 expression. Indeed, the activity of the Foxp3-expressing Tregs is dependent of the Tet-mediated

demethylation of CpG-rich Treg-specific demethylated region (TSDR, also known as conserved non-coding sequence 2 [CNS2]) within the first intron of *FOXP3* [148,188]. Recent *in vivo* and *in vitro* studies on murine Tregs found that VC stabilizes the expression of Foxp3 by promoting an active Tet2-mediated CpG demethylation of TSDR in *FOXP3*. Thus, VC is required for the development and function of Tregs [189-191].

1.10. Aim and objectives of the thesis

$\gamma\delta$ T cells are of interest as effector cells for immunotherapy due to their MHC-non-restricted lysis of many different tumor cell types. Because of their broad functional plasticity, they play a crucial role in protection against infection and in cancer immune surveillance. Several clinical trials have been conducted in an effort to harness the functional abilities of human V γ 9V δ 2 $\gamma\delta$ T-cell subset for adoptive immunotherapy. These trials, however reflected an incomplete understanding of the mechanisms of V γ 9V δ 2 $\gamma\delta$ T-cell responsiveness, coupled to the challenge of obtaining sufficient numbers of highly purified $\gamma\delta$ T cells for clinical-scale administration. Thus, efforts are still made to discover new strategies to enhance their proliferation and function. Interestingly, *in vitro* and *in vivo* data highlighted some degree of T-cell plasticity driven by environmental factors such as nutrients. For example, Vitamin C (VC) was found to promote the development of human and murine T cells as well as of hematopoietic progenitor cells. VC also regulates the expression of selected set of genes in the murine lymphocyte differentiation cultures. Studies with human peripheral blood T cells suggest that VC modulates gene expression, most probably by acting as a facilitator of epigenetic modifications. However, possible effects of VC on human $\gamma\delta$ T cells activation, differentiation and function are as yet unknown. The present thesis aimed to investigate the effects of VC on the differentiation and function of V γ 9V δ 2 T cells. In particular, I investigated the effects of VC on:

- The *in vitro* activation and proliferation of human $\gamma\delta$ T cells
- The cytotoxic potential of human $\gamma\delta$ T cells
- The *in vitro* differentiation of human $\gamma\delta$ T cells at the level of cytokine production and transcription factor expression; and
- The transcriptome and DNA methylation of human $\gamma\delta$ T cells

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Blood samples from healthy donors and ethics

Leukocyte concentrates used in this study were collected from healthy donors and were kindly provided by the Institute of Transfusion Medicine, UKSH (Campus Kiel), University of Kiel, Germany. The study was approved by the Ethics Committee of the Medical Faculty of the University of Kiel (Reference D546/16). The number of donors used throughout the study was indicated in the result section. All blood donors have signed informed consent.

2.1.2. Tumor cells

The pancreatic ductal adenocarcinoma cell line Panc89 was kindly provided by Prof. Holger Kalthoff of the Institute for Experimental Cancer Research, UKSH, University of Kiel.

2.1.3. Plastic, glassware, devices and software

2.1.3.1. Plastic and glassware

All plastic and glassware used in this study were supplied by either Eppendorf (Hamburg, Germany), Greiner (Kremsmünster, Austria), Sarstedt AG (Nürnbrecht, Germany), Biozym (Hessisch Oldendorf, Germany), Dunn (Asbach, Germany), Becton Dickinson (Heidelberg, Germany), Nunc (Roskilde, Denmark), BD Diagnostics (Franklin Lakes, USA), Invitrogen (Carlsbad, USA), Qiagen (Hilden, Germany).

2.1.3.2. Devices

Flow cytometer FACSCalibur	BD Biosciences, Heidelberg, Germany
LSRII Fortessa	BD Biosciences, Heidelberg, Germany
FACS Aria	BD Biosciences, Heidelberg, Germany
ELISA microplate reader Infinite TM M200	Tecan, Crailsheim, Germany
CO ₂ -humidified incubator	Thermo Fisher Scientific, Rockford, USA
Megafuge 10 and Biofuge 15R	Heraeus, Hanau, Germany
Inverse light microscope	Carl Zeiss, Jena, Germany
Neubauer Hemocytometer	Fischer, Frankfurt, Germany
Water bath	GFL, Burgwedel, Germany
Rocking device	Fröbel, Lindau, Germany
Developer machine	Agfa, Mortsels, Belgium
Vortex	Scientific Industries, USA
Film cassette	Amersham/GE Healthcare, UK

Nanodrop™ ND-1000 Spectrophotometer	ThermoFisher Scientific, Rockford, USA
SmartSpec™ 3000 Spectrophotometer	Bio-Rad, Hercules, USA
Transfer Tank (mini) Trans-Blot®	Bio-Rad, Hercules, USA
Protean® II Electrophoresis chamber	Bio-Rad, Hercules, USA
Heatblock	Wealtec Corp, USA
Pyromark Q24	Qiagen (Hilden, Germany)
Scintillation counter	PerkinElmer (Waltham, USA)
Pipettes	Eppendorf (Hamburg, Germany) Gilson (Middleton, USA)

2.1.3.3. Software

CellQuest Pro	BD Biosciences, Heidelberg, Germany
FACSDiVA	BD Biosciences, Heidelberg, Germany
FlowJo 10.5.0	TreeStar, USA
Prism	GraphPad, La Jolla, USA
Endnote X6.0.1	Thomson Reuters, Toronto, Canada
Excel	Microsoft Corp, Redmond, USA

2.1.4. Media, buffers and chemicals

The cell culture medium used in this study is complete RPMI 1640 (Gibco, Life Technologies) including 2 mM L-glutamine, 10 mM HEPES and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 IU/mL penicillin and 100 IU/mL streptomycin (ThermoFisher Scientific). It was prepared and kept under sterile conditions at 4°C. The different buffers and chemicals used in this study are described in appendix C.

2.1.5. Antibodies

2.1.5.1. Antibodies for flow cytometry

For Fluorescence activated cell sorting (FACS)-staining of molecules at the cell surface or intracellularly, the following antibodies were purchased and used in concentrations previously determined in the lab. The antibodies were all monoclonal antibodies conjugated with fluorochrome (FITC, AF488, PE, APC, AF647, PECy7, BV605, HV450, BV421) and directed against CD3 (clone SK7), CD80 (clone L307.4), CD45RA (clone L48), CD27 (clone M-T271), CD69 (clone L78), CD62L (clone Dreg-56), GATA-3 (clone L50-823), HLA-DR (clone L243), TCR $\gamma\delta$ (clone 11F2), TCR V δ 2 (clone B6), CD107a (clone H4A3), CD39 (clone A1), and Foxp3 (clone 259D/C7) all from BD Biosciences (Heidelberg, Germany); T-bet (clone 4B10), TCR V δ 2 (clone B6), Ki-67 (clone Ki-67), CD73 (clone AD2) and CD4 (clone SK3) from

Biolegend (San Diego, CA, USA); CD86 (clone 2331(FUN-1)) from R&D Systems (Wiesbaden, Germany); TIGIT (clone MBSA43), NKG2D (clone 1D11) and CD56 (clone CMSSB) from eBioscience (San Diego, CA, USA) and TCR V γ 9 (clone 7A5; [192]).

2.1.5.2. Antibodies for ELISA

For the ELISA assays, antibodies used for the detection of IFN- γ , IL-13 and granzyme B were purchased from R&D Systems; anti-perforin antibody was obtained from Abcam (Cambridge, UK).

Matching pairs of antibodies contained a primary unlabeled antibody and a biotinylated detection antibody that were used in previously titrated dilutions according to the manufacturers guide.

2.1.5.3. Antibodies for Western blot

Anti-mTOR Ab	Cell Signaling Technology (Beverly, USA)
Anti-phospho-mTOR Ab	Cell Signaling Technology (Beverly, USA)
HRP-anti- β -actin Ab	Proteintech, Inc (Manchester, UK)
HRP-mouse anti-rabbit IgG	Abcam (Cambridge, UK)

2.1.5.4. Microbeads

Activation/ Expander T cell beads (A/E-beads) purchased from Miltenyi (Bergisch-Gladbach, Germany) were coated with 10 μ g/mL anti-CD3, 10 μ g/ mL anti-CD28 and 0.5 μ g/mL anti-CD2 mAbs and were used as stimulus where indicated at the ratio cell/beads 1:1. Magnetic cell sorting beads were purchased from Miltenyi Biotec and Dynabeads (Life Technologies, Carlsbad, CA, USA).

2.1.6. Antigens and recombinant human proteins

2.1.6.1. Antigens

Synthetic phosphoantigen bromohydrin pyrophosphate (BrHPP) was kindly provided by Innate Pharma (Marseille, France). The most potent microbial phosphoantigen, (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) was purchased from Echelon Biosciences (Salt Lake City, USA). For both antigens, a sterile filtered stock was prepared, and aliquots were stored at -20°C. Nitrogen-containing aminobisphosphonate (zoledronic acid, ZOL) used as stimulus was kindly provided by Novartis (Basel, Switzerland).

2.1.6.2. Recombinant human proteins

The following recombinant cytokines: rhIL-2 was obtained from Novartis (Basel, Switzerland) and rhTGF- β 1 was purchased from Peprotech (Hamburg, Germany). Sterile stock solutions were prepared, and aliquots were stored at -80°C.

2.1.7. L-ascorbic acid (Vitamin C, VC) and L-ascorbic 2-phosphate acid (phospho-modified Vitamin C, pVC)

VC and pVC, purchased from Sigma-Aldrich/Merck (Cat N° A4544 and A8960), were prepared in sterile water and aliquoted stock solutions were stored at -80°C. Both chemicals were used in concentrations as described in the result section.

2.2. Methods

2.2.1. Cell isolation procedure

2.2.1.1. Isolation of PBMC of healthy donors

Peripheral blood mononuclear cells (PBMC) were isolated from leukocytes concentrate of healthy donors by Ficoll-Hypaque (Biochrom, Cambridge, UK) density gradient centrifugation. The blood was diluted (1:2) in PBS. 30 mL of the diluted blood was gently transferred onto 15 mL of ficoll in 50 mL falcon tube and spun down for 20 min, 2000 rpm, without brake. The resulting white layer containing the leukocytes was then carefully collected into a new 50 mL falcon tube and washed twice with PBS to remove the remaining ficoll. Thereafter the cell pellet was resuspended in complete RPMI medium. Aliquots of the isolated PBMC were cryopreserved for later CD4 T cell isolation and the rest were used for total $\gamma\delta$ (or V δ 2) T cell isolation.

2.2.1.2. Magnetic cell sorting of $\gamma\delta$ T cells, V δ 2 T cells, CD4 T cells

Total $\gamma\delta$ T cells as well as V δ 2 T cells were positively isolated by magnetic cell sorting (MACS) following the manufacturer's instructions (Miltenyi Biotec). PBMC were washed with MACS buffer and blocked with FcR blocking reagent (Miltenyi Biotec) to avoid unspecific binding of the antibodies. $\gamma\delta$ T cells were positively isolated using the anti-human TCR $\gamma\delta$ microbeads kit (Miltenyi Biotec). V δ 2 T cells were positively isolated using the PE-conjugated anti-V δ 2 mAb (clone B6; BD Pharmingen) and the anti-PE microbeads (Miltenyi Biotec). After the use of two consecutive MACS columns, the purity of each cell type was routinely > 98 %.

CD4 T cells were negatively isolated by MACS separation system (CD4 T Cell Isolation Kit II, Miltenyi Biotec) followed by the depletion of CD25⁺ Treg using Dynabeads (Life

Technologies, Carlsbad, CA, USA). The isolated cells were counted and resuspended at the concentration of 1×10^6 cells/mL in complete RPMI medium.

2.2.1.3. Cell viability and counting

The number of the living cells was determined using eosin dye-based exclusion of dead cells method. Unless otherwise stated, cell suspensions were diluted 1:5 or 1:10 with a solution of eosin. 10 μ L of the mixed solution was then loaded onto a Neubauer cell counting chamber under light microscope magnification, and the living cells (non-colored) were counted.

2.2.2. Cell culture procedure

2.2.2.1. $\gamma\delta$ T-cell cultures

To investigate the influence of pVC on human $\gamma\delta$ T-cell activation and proliferation, PBMC and MACS-purified $\gamma\delta$ T cells were stimulated with pAgs (HMBPP or BrHPP) or with ZOL and 50 IU/mL IL-2 in 96-well round-bottom microculture plates. PBMC (2×10^5 cells/well) and MACS-purified $\gamma\delta$ T cells (50×10^3 cells/well) were cultured in complete RPMI medium and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Short-term (12-14 days) lines of V γ 9V δ 2 T cells (ZOL-expanded V γ 9V δ 2 T cells) were established by stimulating PBMC with 2.5 μ M ZOL in the presence of 50 IU/mL IL-2. IL-2 was added every 2-3 days over a culture period of 14 days. Lines were used for experiments when $\gamma\delta$ T cells as determined by the proportion of V γ 9 or V δ 2 T cells represented more than 90% of the total cell population. The short-term expanded $\gamma\delta$ T-cell lines (4×10^4 cells/well) were left unstimulated or re-stimulated in 96-well round-bottom plates with 300 nM BrHPP and 50 IU/mL IL-2 in the presence or absence of 173 μ M (50 μ g/mL) pVC.

To investigate the effect of pVC on the transcription factor expression and cytokine induction, MACS-purified $\gamma\delta$ T cells (50×10^3 cells/well) were stimulated with 300 nM BrHPP or 50×10^3 A/E-beads for 8 days with 50 IU/mL recombinant human IL-2 and 2 ng/mL TGF- β (where indicated) in the presence or absence 173 μ M pVC.

To further test the stability of Foxp3 protein-expression, $\gamma\delta$ T cells were expanded for 8 days under different conditions as mentioned afore. Thereafter, cells were washed twice, transferred into new 96-well round-bottom plates and cultured in the presence of 50 IU/mL IL-2 only. After additional 6 days, cells were analyzed for Foxp3 protein-expression by flow cytometry.

2.2.2.2. Tumor cell culture

Panc89 adenocarcinoma cell line used as tumor cell line in this study was cultured in complete RPMI medium at 37°C in a humidified atmosphere of 5% CO₂ in air. When the cells reached more than 80% confluence in the cell culture flask, cells were harvested using Trypsin-EDTA (0.05%-0.02%) and were reseeded into a new cell culture flask containing complete RPMI medium. Panc89 tumor cell lines were kept in culture not longer than 2 months.

2.2.2.3. *In vitro* suppression assay

For the *in vitro* suppression assay, $\gamma\delta$ T cells were first stimulated for 14 days in the presence of IL-2 and TGF- β , and the additional presence of pVC where indicated. On day 14, the expanded $\gamma\delta$ T cells (20 x 10³ cells/well) were co-cultured with magnetically isolated autologous CD25-depleted CD4 responder T cells (20 x 10³ cells/well) for 5 days. The absolute numbers of viable CD4 and $\gamma\delta$ T cells were determined by SCDA (see 2.2.3.4.)

2.2.3. Flow cytometry analysis

2.2.3.1. Surface marker analysis

To investigate the effect of pVC treatment on the expression of surface molecules, cells were harvested from cultures, transferred into 96-V bottom plates and washed with wash buffer (Appendix C). Thereafter cells were stained with fluorochrome-conjugated monoclonal antibodies directed against the analyzed cell surface molecules and were incubated for 30 min at 4°C. Cells were then washed twice with wash buffer and resuspended in wash buffer. Cells were immediately acquired on LSRII Fortessa flow cytometer. At least 10,000 events were acquired for the analysis of the different surface molecules. Data were then analyzed with FlowJo v. 10.

2.2.3.2. Intracellular transcription factors, Ki-67 and ROS detection

For detection of transcription factors, the cells were fixed and permeabilized using the Foxp3 transcription factor staining buffer (eBioscience; ThermoFisher, Waldham, MA, USA) according to the manufacturer's instructions. The cells were washed and blocked for 15 min at 4°C using the blocking solution (1:50, FCS:Perm buffer). Thereafter, the cells were washed and stained with fluorochrome-conjugated mAbs against Foxp3, T-bet and GATA-3 for 30 min at 4°C. After two additional washing steps, the cells were immediately acquired on LSRII Fortessa.

For the intracellular detection of Ki-67 antigen, cells were subjected to ethanol fixation. The cells were fixed by adding cold ethanol (70%) and were incubated 1h at -20°C. The cells were then washed thrice with staining buffer (Appendix C) and stained with FITC-labeled Ki-67 mAb (clone Ki-67) for 30 min at room temperature. After two additional washing steps, the cells were immediately acquired on LSRII Fortessa.

The level of intracellular ROS was measured using a ROS detection reagent (ThermoFisher Scientific) according to the manufacturer's guidelines. For all stainings, at least 10,000 events were acquired using LSRII Fortessa and data were analyzed with FlowJo v. 10.

2.2.3.3. Analysis of cell death

Activation-induced cell death (AICD) of re-stimulated ZOL-expanded V γ 9V δ 2 T cells was analyzed using Annexin V/PI staining. ZOL-expanded V γ 9V δ 2 T cells were preincubated (or not) for 20h with pVC before re-stimulation with BrHPP (or medium). After 20h, cells were harvested and washed with Annexin V binding buffer (Appendix C). Thereafter cells were resuspended in Annexin V binding buffer and stained with 100 μ L of Annexin V-FITC (1:50; MabTag GmbH, Friesoythe, Germany) and 1 μ g/mL of Propidium iodide (PI; Serva, Heidelberg, Germany) for 15 min at room temperature in the dark and immediately subjected to flow cytometry analysis.

2.2.3.4. Assessment of cell proliferation by SCDA

For the determination of $\gamma\delta$ (or V γ 9 in some experiments) and CD4 responder T-cell expansion, a previously described flow cytometry-based method [193], termed standard cell dilution assay (SCDA) was used to measure the absolute numbers of proliferating $\gamma\delta$ and CD4 responder T cells per microculture well. Briefly, expanded cells were harvested at the indicated time points, washed and stained with FITC-labeled anti- $\gamma\delta$ TCR (or AF488-V γ 9) and PE-conjugated anti-CD4 mAbs for 30 min. Shortly before analysis, PI (0.2 μ g/mL) and a known number of APC-labeled and fixed standard cells were added. Purified CD4 T cells, labeled with APC-conjugated anti-HLA class I mAb (clone W6/32) and anti-TCR $\alpha\beta$ mAb (clone BMA031) and fixed in 1 % paraformaldehyde, served as standard cells (Appendix D). The absolute cell number of viable $\gamma\delta$ and CD4 responder T-cell was calculated from the ratio of FITC⁺ $\gamma\delta$ T cells/APC⁺ standard cells and PE⁺ CD4 T cells/APC⁺ standard cells.

In some experiments, the absolute number of viable $\gamma\delta$ T cells was counted microscopically after eosin dye exclusion of dead cells.

2.2.3.5. Cell cycle analysis

ZOL-expanded V γ 9V δ 2 T-cells were stimulated at 1×10^6 cells/mL with 300 nM BrHPP for 3 days after 20h pretreatment with pVC. Thereafter, the cells were washed twice and resuspended in 0.5 mL of cold 5 mM EDTA-containing PBS. Cells were fixed by adding 0.5 mL ethanol (100%). After 30 min incubation at room temperature, cells were washed and resuspended in 5mM EDTA-containing PBS. Cells were incubated with 1mg/mL RNase A (Qiagen, Hilden, Germany) and 50 μ g/mL PI for 1h at room temperature. DNA distribution was analyzed with FACSCalibur flow cytometer (BD). At least at least 8,000 events were acquired, and data were analyzed with FlowJo v. 10.

2.2.4. *In vitro* cytotoxicity assay (chromium-release assay)

Before the assay, Panc89 tumor cell line (50×10^4 cells/well) were cultured for 3 days in 6-well plate in complete RPMI medium in the presence or absence of pVC. On the day of the co-culture, tumor cell lines were labeled with radioactive sodium chromate (Na^{51}Cr ; Amersham, Karlsruhe, Germany) by incubating 1×10^6 cells in 150 μ Ci of Na^{51}Cr for 90 min at 37°C. Cells were washed three times with complete RPMI medium, resuspended in the same medium and plated in triplicates in 96-well V-bottom plates at a final concentration of 2000 cells/well. V γ 9V δ 2 T cells (effector cells) were added at 80:1, 40:1, 20:1 and 10:1 E/T ratios. Plates were incubated at 37°C for 4h. Supernatants were collected and the released ^{51}Cr was quantified using a gamma counter. Tumor cells incubated without effector cells were used to measure spontaneous ^{51}Cr release. Target cells were treated with 1% Triton X (Sigma-Aldrich) as a measure of maximal lysis. Counts from triplicate wells were averaged and the percentage of specific lysis was calculated using the following equation: %Specific lysis = $100 \times [(\text{test } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})] / [(\text{maximal } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})]$.

2.2.5. Measurement of cytokine production

2.2.5.1. Bead-based immunoassay

At day 8, the supernatants from the $\gamma\delta$ T cells, cultured in different combinations with BrHPP and pVC, were collected and assessed for cytokines content using the LEGENDplex™ Human Th cytokine panel kit (Biolegend) following the manufacturer's guidelines. Measurements of cytokines including IL-5, IL-13, IL-6, IL-9, IL-10, IFN- γ , TNF- α , IL-17a, IL-17f, IL-4, IL-21 and IL-22 were determined using LSRII Fortessa. The LEGENDplex™ assay is a bead-based immunoassay using the same basic principle as sandwich immunoassay. In brief, beads are differentiated by size and internal fluorescence intensity. Each bead set

conjugated with a specific antibody on its surface served as the capture beads for the particular analyte (cytokine). A selected panel of capture beads was mixed and incubated with supernatant sample containing target analytes specific to the capture antibodies. After washing, a biotinylated detection antibody cocktail was added, which led to the formation capture bead-analyte-detection antibody complex. Streptavidin-phycoerythrin (SA-PE) was subsequently added. Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of particular analyte was determined using a standard curve generated in the same assay.

2.2.5.2. ELISA

Based on the results of the bead-based immunoassay, levels of IFN- γ and IL-13 in the culture supernatants were additionally quantified by sandwich-ELISA. Moreover, to investigate the effect of pVC on the release of the soluble mediators of cytotoxicity, supernatants from the co-culture of $\gamma\delta$ T cells with Panc89 was collected and the production of mediators such as granzyme B, perforin and IFN- γ was quantified using specific kits. Briefly, ELISA plates (Nunc) were coated with 100 μ L/well of the specific anti-human capture antibody diluted in PBS. Plates were incubated overnight at room temperature (RT). After incubation, plates were washed thrice with PBS containing 0.05% Tween-20 and blocked for 1h with 300 μ L/well of the blocking buffer (PBS/1% BSA). Supernatants and the recombinant cytokine standards were then diluted using specific reagent diluents (Appendix C) and added in a volume of 100 μ L. Plates were then incubated for 2h at RT, and thereafter, were washed as previously stated. The corresponding biotinylated anti-human detection antibody was diluted and added at a volume of 100 μ L for 2h at RT. Following a further washing step, 100 μ L/well of streptavidin-horseradish peroxidase (HRP), diluted 1:40, was added and plates were incubated 20 min at RT. After a final washing step, 100 μ L/well of the substrate solution (1:1 H₂O₂: Tetramethylbenzidine, TMB) was added. After 5-10 min of incubation in the dark at RT, the reaction was stopped by adding 50 μ L/well of 2N, H₂SO₄ (R&D Systems). The plates were immediately read at 450 nm using Infinite MT200 microplate reader (Tecan) calibrated according to the manufacturer's specifications. Data were analyzed with Excel (Microsoft Corp). The standard curve was fitted to the absorbance observed for the standards value by a second order polynomial curve. Test sample values were interpolated from the standard curve and were subsequently corrected for supernatant dilution factor to finally obtain the concentration in the original samples.

2.2.6. Immunoblotting assay

2.2.6.1. Cell lysate preparation

For the analysis of mTOR and the respective phosphorylated form (p-mTOR) by Western blot, ZOL-expanded V γ 9V δ 2 T cells were adjusted to 2×10^6 cells/mL and were preincubated with 173 μ M pVC for 1h. Thereafter, the cells were left unstimulated or re-stimulated with 300 nm BrHPP for 20 min and 60 min at 37°C. Following stimulation, the cells were washed with cold PBS and lysed in 25 μ L of standard NP40 lysis buffer (1% (v/v) Nonidet[®] P40 (Sigma-Aldrich), 20 mM Tris–buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA) supplemented with protease/phosphatase inhibitors including sodium orthovanadate, sodium fluoride, sodium pyrophosphate, phenylmethylsulfonyl fluoride, and pepstatin A (all from Sigma-Aldrich). After a centrifugation step (14,000 rpm at 10 min), the supernatants containing the lysate (protein) was collected into a fresh 1.5 mL tube. Protein concentration was estimated on spectrophotometer SmartSpec 3000 (Bio-Rad) using Bradford method.

2.2.6.2. Bradford assay

Protein content in cellular lysates was determined by a colorimetric Bradford assay which is based on an absorbance shift of the dye Coomassie Brilliant Blue (Life Technologies) once bound to protein. 5 μ L of the sample were mixed with 995 μ L of Coomassie reagent. The absorbance was immediately measured at 595 nm with the SmartSpec 3000 (Bio-Rad).

2.2.6.3. SDS-PAGE

To perform SDS-PAGE, 5 μ g of protein together with 3x sample loading buffer containing β -mercaptoethanol (Appendix C) were run on NuPAGE 4-12 % Bis-Tris Gel (Life Technologies) placed into gel apparatus containing MES running buffer. The protein samples were then separated by vertical electrophoresis at 200 V for approximately 45 min.

2.2.6.4. Protein transfer and immunoblotting

After gel electrophoresis, the gel was soaked in a protein transfer buffer (appendix) alongside nitrocellulose membrane (Hybond[™] C, GE Healthcare). The separated proteins were then transferred onto 0.45 μ m nitrocellulose membrane using the electrophoretic transfer tank (Bio-Rad). After 1h of transfer at 100V, membrane was dissembled from transfer cassette and was stained with Ponceau S staining solution (Sigma-Aldrich) to monitor the efficiency and the quality of the transfer. After a washing step in TBS (Appendix C), the membrane was blocked with blocking buffer (Appendix C) for 1h at room temperature with agitation on a rocker platform, and appropriate primary polyclonal antibodies against mTOR or phospho-mTOR (cat N^o 9862 and 2971; Cell Signaling Technology) diluted (1:1000) were added

overnight. After 15 min washes with TBST, specific HRP-labeled anti-rabbit secondary antibody was diluted (1:5000) and added for 45 min. After further washing steps with TBST (Appendix C), the membrane was placed in the correct orientation inside plastic coverings and enhanced chemiluminescence (ECL) western blot detection reagent (GE Healthcare) was added for 1 min. After different periods of exposure time to Hyper Film (GE Healthcare), the films were developed using the developer machine (Agfa).

2.2.7. Molecular biology procedure

2.2.7.1. RNA and DNA Isolation

RNA and DNA isolation were performed using the miRNeasy Mini and the DNeasy® Blood & Tissue Kits, respectively (both from Qiagen). In brief, V δ 2 T cells were expanded for 8 days in the presence of IL-2 and BrHPP with different combinations of TGF- β and pVC. Cells were collected from culture plates and pelleted by centrifugation and were subjected to the nucleic acids' isolation according to the manufacturer's protocols. The concentration and the purity of the isolated RNA and DNA were determined using the Nanodrop™ ND-1000 spectrophotometer at 260/280 nm, the ratio was routinely between 1.7 and 2.

2.2.7.2. RNA sequencing and bioinformatic analysis

To investigate the influence of pVC on the gene expression profile of human V δ 2 T cells, RNA sequencing was performed at the Institute of Clinical Molecular Biology (University of Kiel, Germany) in cooperation with Dr. Robert Häsler. Subsequent bioinformatic analyses were performed by Dr. Daniela Esser (Institute of Experimental Medicine, UKSH, University of Kiel). For all sequencing, reads in good quality according to the program `fastq_illumina_filter` version 0.1 (downloaded from <http://cancan.cshl.edu>), quality trimming was performed with `prinseq-lite` version 0.20, while adapter sequences were cropped with `cutadapt` version 1.12 [194]. All filtered reads were mapped against the human genome reference hg19 using `TopHat` version 2.1.0 with the sensitive option [195]. Read counts per transcript were calculated with the Python script `HTSeq` version 0.6.1p1 for reverse stranded libraries [196]. Reads overlapping with more than one feature were handled with the mode 'union'. Reads with an alignment quality lower 20 were skipped. The p-values for differentially expressed genes were calculated with `DeSeq2` version 1.22.2 [197]. To improve stability, a shrinkage estimation for dispersions and fold changes were applied. The function 'replaceOutliersWithTrimmedMean' was used to detect and remove outliers for each gene.

The distances between samples were calculated using the R function 'dist' with Euclidean distance based on regularized logarithm transformed ('rlog') read counts per gene.

Heatmaps were created with the R function `heatmap.2` (package `gplots` version 3.0.1.1) using z-scores based on the read counts after normalized counts transformation. The dendrograms were based on hierarchical clustering performed with the R function `'hclust'`. The input matrix for the hierarchical clustering was calculated with the R function `'dist'` using Euclidean distance.

Networks were created with the `igraph` version 1.2.2 using the Fruchterman-Reingold algorithm. Connections were based on the annotation of the String database [198] with medium reliance.

Gene set enrichment analyses based on pathways annotated in the KEGG database were executed with the online tool InnateDB [199], whereby the correction for multiple testing was performed with the method developed by Benjamini and Hochberg [200] using the R function `'p.adjust'`.

2.2.7.3. DNA methylation analysis

For all methylation analyses, cells from male donors were used.

2.2.7.3.1. Reduced representation bisulfite sequencing (RRBS)

To investigate the effect of pVC on the genome-wide DNA methylation, magnetically isolated V δ 2 $\gamma\delta$ T cells were stimulated with BrHPP or with A/E-beads in IL-2 and TGF- β containing complete RPMI medium in the presence or absence of pVC. On day 8, genomic DNA was extracted from expanded $\gamma\delta$ T cells using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and subjected to RRBS performed at BGI Genomics Institute (Shenzen, China). Bioinformatic analyses were performed by Qiwei Sun (BGI Genomics Institute, Shenzhen, China). In brief, the isolated DNA was cut by restriction enzyme `MspI` to produce CpG-rich fragments. Processing DNA-end repair and 3'-dA overhang, 40-220 bp fragments were selected and subjected to bisulfite treatment using the EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's instructions. After PCR amplification, the qualified libraries were sequenced using Illumina high-throughput bisulfite sequencing (MethylC-Seq). After sequencing, methylation level was determined by the reads which covered in cytosine (C) [201], and the methylation level equal to the mC reads number/total C reads number at each reference cytosine as described [202].

2.2.7.3.2. Analysis of FOXP3 methylation using pyrosequencing

To investigate the effect of pVC on the *FOXP3* CNS2 demethylation, expanded $\gamma\delta$ T cells were stained with anti-Foxp3 mAb (clone 259D/C7) and were sorted according to their intracellular Foxp3 expression into Foxp3⁺ V δ 2 and Foxp3⁻ V δ 2 T cells. Cell sorting was performed on a FACSAria II cell sorter (BD Biosciences). The purity of the sorted cell populations is shown for one representative experiment in Fig. 2. Genomic DNA was prepared from unsorted input cells (purified V δ 2 T cells) as well as from FACS-sorted, cultured Foxp3⁺ and Foxp3⁻ cells using the NucleoSpin Tissue kit (Macherey & Nagel, Düren, Germany) and was subjected to pyrosequencing performed by Dr. Stefan Floess (Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany). An additional step was added to the manufacturer's protocol to remove formaldehyde-induced crosslinking. Briefly, Chelex-100 beads (Bio-Rad) were added after the lysis step and incubated at 95°C for 15 min in a shaker. The beads were spun down and the supernatant was transferred to a fresh tube. After addition of an adjusted amount of 99.8% ethanol (Merck), the subsequent purification steps were performed according to the manufacturer's protocol. Genomic DNA was converted with bisulfite using the EZ DNA Methylation-Lightning kit (Zymo Research) according to the manufacturer's instructions. The human Treg-specific demethylated region was amplified by PCR using bisulfite-converted DNA, the primers hTSDR-for (5'-GAGATGATTTGTTTGGGGGTAGAGGA-3'), hTSDR-rev (5'-bio AACACCCATATCACCCACCT-3') and the ZymoTaq PreMix (Zymo Research) according to the manufacturer's protocol. The amplicate was sequenced by pyrosequencing using the sequencing primer hTSDR-seq (5'-ATAGTTTTAGATTTGTTTAGATTTT-3') on a Pyromark Q24 (Qiagen, Hilden, Germany) and analyzed following the manufacturer's instructions.

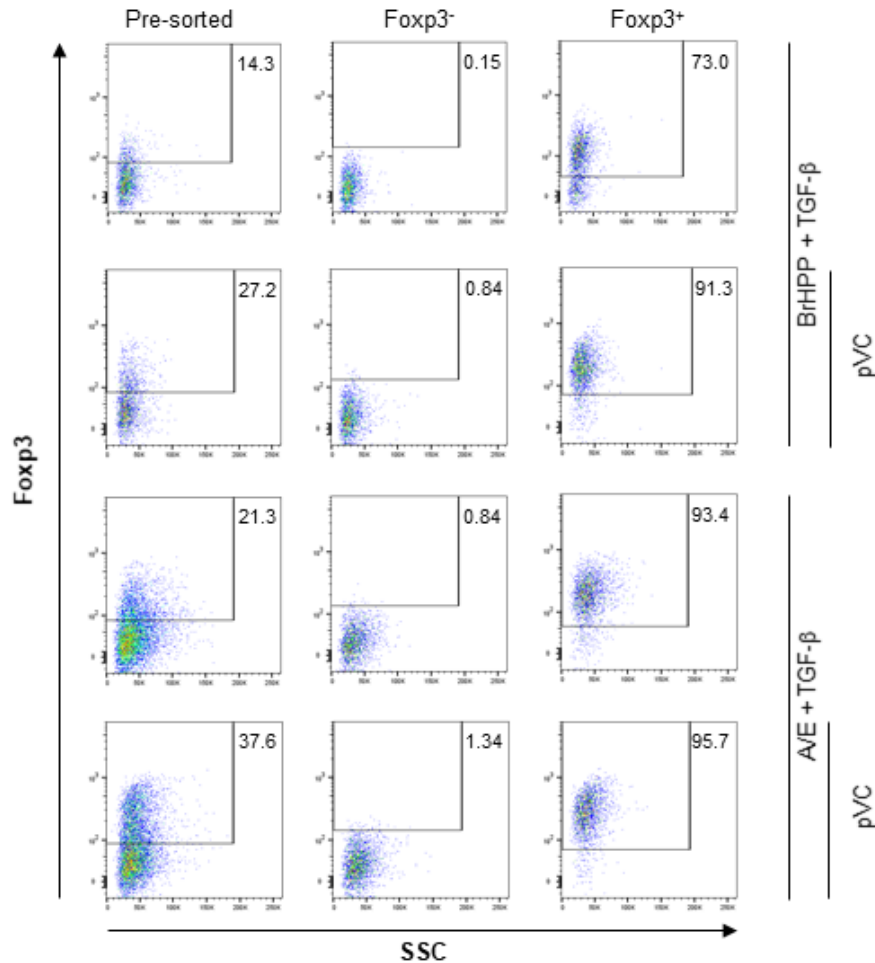


Figure 2. Purity of sorted Foxp3⁺ and Foxp3⁻ subpopulations from the TGF-β/pVC-expanded Vδ2 T cells.

Purified Vδ2 T cells were activated with BrHPP or A/E-beads and cultured in the presence of IL-2 and different combinations of TGF-β and pVC as indicated. After 8 days, cells were sorted into Foxp3⁺ and Foxp3⁻ subpopulations. Dot plots of one representative experiment out of four are shown. Numbers indicate the percentage of Foxp3⁺ cells in the pre-sorted expanded Vδ2 T cells, the sorted Foxp3⁻ and Foxp3⁺ cell fractions.

2.3. Statistical analysis

Results of *in vitro* cell culture experiments and FACS data were analyzed with Excel (Microsoft Corp). Statistical significance was calculated with the paired two-tailed Student's *t* test. *p* values < 0.05 were considered significant and are displayed as * for *p* < 0.05, ** for *p* < 0.01, *** for *p* < 0.001

3. RESULTS

3.1. Modulation of human $\gamma\delta$ T-cell activation by Vitamin C

3.1.1. Differential effects of Vitamin C (VC) and phospho-modified Vitamin C (pVC) on $\gamma\delta$ T-cell expansion upon primary stimulation

L-ascorbic acid (Vitamin C, further referred to as VC) is an established constituent of cell culture media used for stem cell differentiation. In these systems, the phospho-modified derivative L-ascorbic acid 2-phosphate (further referred to as pVC) is generally used because it is more stable and less toxic at high concentrations [203]. In order to experimentally explore the effect of VC and pVC on the viability and proliferative capacity of human $\gamma\delta$ T cells, it was necessary to establish the concentration of Vitamin C that would be tolerable to cells. To this end, in a first set of experiments the effects of VC and pVC were compared over wide concentration ranges on the selective activation and short-term expansion of V γ 9V δ 2 T cells within PBMC. PBMC from healthy donors containing 2-4% $\gamma\delta$ T cells were stimulated with optimal concentrations of the synthetic V γ 9V δ 2 T cell-specific antigen HMBPP (10 nM) or the aminobisphosphonate zoledronate (ZOL, 2.5 μ M) and IL-2 (50 IU/mL). The selective expansion of $\gamma\delta$ T cells was determined by SCDA measuring the number of viable V γ 9 T cells after 7 days of culture. As shown in Fig. 3, V γ 9 T cells within PBMC strongly expanded in response to both HMBPP (left panel) and ZOL (right panel). The overall V γ 9 T-cell expansion was not significantly influenced by pVC tested over a concentration range from 35 to 692 μ M. In contrast, high concentrations of VC (i.e. 284 μ M to 1136 μ M) inhibited the HMBPP- and ZOL-induced $\gamma\delta$ T-cell expansion. Taken together, these results suggest that VC has a narrow window of concentration for *in vitro* use while the phospho-modified derivative pVC can be applied over a wider concentration range without toxic effects.

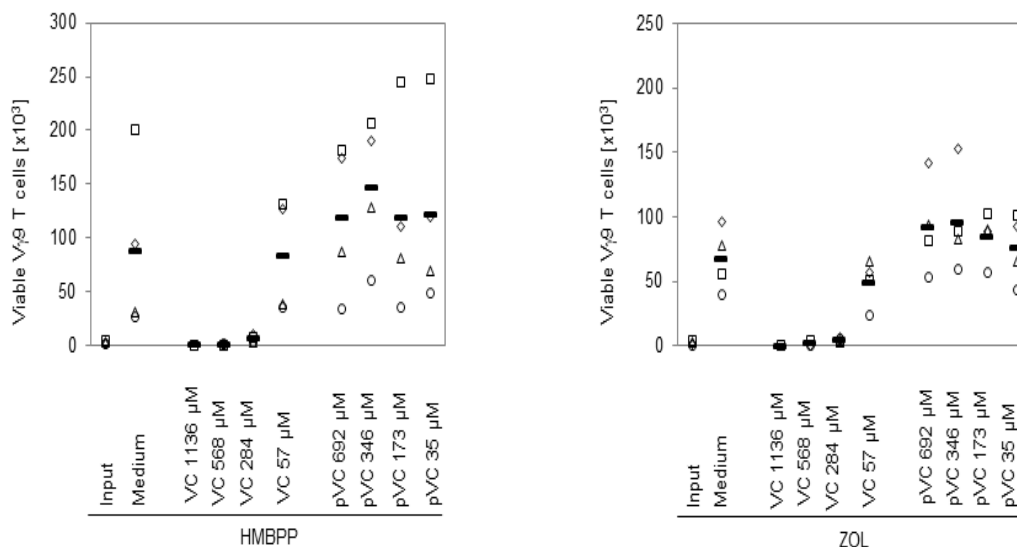


Figure 3. Effects of VC and pVC on the *in vitro* expansion of V γ 9V δ 2 T cells.

PBMC obtained from four healthy donors were stimulated with HMBPP (left panel) or ZOL (right panel) in the presence of IL-2. VC or pVC were added at the indicated concentrations. The number of viable V γ 9 T cells per microculture well was determined in triplicates by flow cytometry after 7 days. Each symbol represents an individual healthy donor. Horizontal bars represent the mean values.

From these observations, only pVC at the concentration of 173 μ M (corresponding to 50 μ g/mL) was used in all subsequent experiments.

3.1.2. Effects of pVC on the $\gamma\delta$ T-cell expansion upon initial $\gamma\delta$ T-cell activation

In addition to its anti-oxidant properties, pVC has been reported to promote DNA synthesis and mammalian cell differentiation [203,204]. To better define the influence of pVC on human $\gamma\delta$ T-cell activation and proliferation, we activated PBMC and magnetically isolated $\gamma\delta$ T cells with HMBPP or BrHPP in IL-2-containing medium in the presence or absence of pVC. After 7 days of culture, the selective expansion of viable V γ 9 T cells was determined by SCDA and by microscopic count. Results depicted in Fig. 4 indicate that pVC did not clearly modulate V γ 9 T-cell proliferation when PBMC were stimulated with pAg BrHPP (Fig. 4a left part). However, a consistent growth-promoting effect of pVC was observed when PBMC were stimulated with HMBPP (right part). Moreover, the proliferation of magnetically isolated $\gamma\delta$ T cells in response to both pAgs was increased by pVC, even though this did not reach statistical significance (Fig. 4b). Measurement of the cell numbers by microscopic cell count after eosin dye exclusion verified a significant positive effect of pVC on the expansion of purified $\gamma\delta$ T cells in response to BrHPP (Fig. 4c).

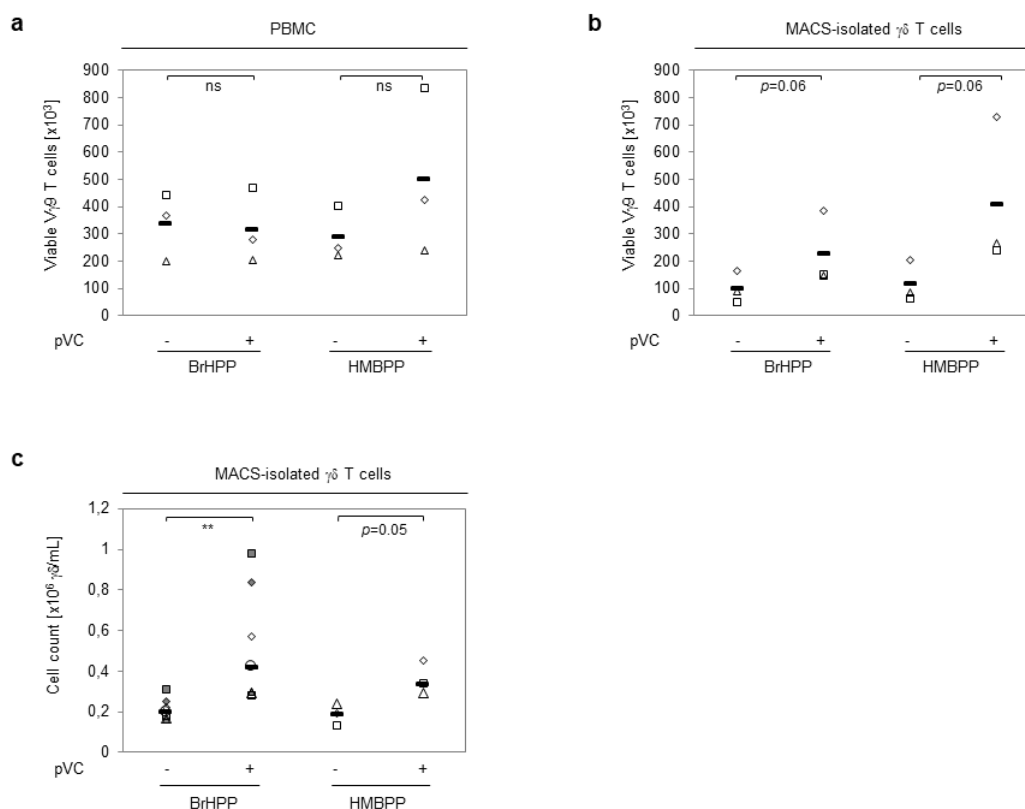


Figure 4. Effect of pVC on the $\gamma\delta$ T-cell expansion upon initial stimulation with phosphoantigens.

(a) PBMC from three healthy donors were stimulated with BrHPP or HMBPP and IL-2 in the absence or presence of 50 $\mu\text{g/mL}$ (173 μM) pVC. The number of viable $V\gamma 9$ T cells per microculture well was determined in triplicates by flow cytometry after 7 days of culture. **(b)** Purified $\gamma\delta$ T cells (50×10^3 cells/well) from the same donors as in (a) were stimulated with BrHPP or HMBPP and IL-2 in the absence or presence of 50 $\mu\text{g/mL}$ (173 μM) pVC. The number of viable $V\gamma 9$ T cells per microculture well was determined in triplicates by SCDA after 7 days of culture. **(c)** absolute cell counts were determined microscopically after exclusion of dead cells by eosin dye. Each symbol indicates an individual healthy donor. Horizontal bars represent the mean values. BrHPP, $n = 3-7$; HMBPP, $n = 3$. * $p < 0.05$, ** $p < 0.01$, ns: not significant.

3.1.3. pVC promotes the expansion of $V\gamma 9V\delta 2$ T cells upon BrHPP re-stimulation

Because pVC was found to promote the proliferation of $V\gamma 9V\delta 2$ T cells upon initial stimulation we next investigated the effect of pVC on ZOL-expanded $V\gamma 9V\delta 2$ T cells in combination with BrHPP-re-stimulation. The experimental strategy is illustrated in Fig. 5a. In brief, $\gamma\delta$ T-cell lines were established by culturing PBMC with ZOL and repetitive addition of IL-2 for 14 days. The purity of such ZOL-expanded short-term $\gamma\delta$ T-cell lines (as determined by the proportion of $V\gamma 9$ or $V\delta 2$ T cells) routinely exceeded 90%. These ZOL-expanded $V\gamma 9V\delta 2$ T cells were washed and then re-cultured in IL-2-containing medium in the absence or presence of BrHPP and/or pVC. The number of viable $V\gamma 9$ T cells was determined by SCDA. As expected, re-stimulation with BrHPP induced activation-induced cell death [192], and thus

inhibited the proliferation of $V\gamma 9$ T cells, whereas the additional presence of pVC rescued and, significantly augmented the $V\gamma 9$ T cell-expansion (Fig. 5b). Microscopic inspection of cell cultures indicated that this effect was associated with increased cell aggregate formation when pVC was present together with BrHPP (Fig. 5b, lower part). pVC had no effect on $V\gamma 9$ T-cell expansion when the cells were left unstimulated.

To investigate the kinetics of rescuing effect of pVC on cell growth and to clearly establish the time window during which pVC contributes to $V\gamma 9V\delta 2$ T-cell proliferation the most, three additional ZOL-expanded $V\gamma 9V\delta 2$ T-cell lines were generated from three donors. Thereafter, the lines were left unstimulated or BrHPP-re-stimulated in IL-2-containing medium. Aliquots of the culture were supplemented with pVC. Cell proliferation quantified at different time points (day 3, 5 and 7) showed that the growth-promoting effect of pVC was most obvious when cellular expansion (i.e., the number of viable $V\gamma 9$ T cells per microculture well) was analyzed at day 7 after re-stimulation (Fig. 5c).

Next, we asked whether pVC supplementation to the $V\gamma 9V\delta 2$ T-cell cultures during their initial ZOL-induced expansion could prime these cells for higher proliferative activity upon re-stimulation. Therefore, ZOL-expanded $V\gamma 9V\delta 2$ T cells \pm pVC were harvested, washed twice and re-cultured in IL-2-containing medium with or without BrHPP-re-stimulation (in the absence of pVC). After 7 days of expansion, the proliferation of $V\gamma 9$ T cells was assessed by SCDA. As shown in Fig. 5d, upon BrHPP-re-stimulation, [ZOL + pVC]-expanded $V\gamma 9V\delta 2$ T cells ($\gamma\delta$ [pVC]) displayed higher cell numbers than ZOL-expanded $V\gamma 9V\delta 2$ T cells ($\gamma\delta$ [med]). Strikingly, in the absence of re-stimulation, $\gamma\delta$ [pVC] expanded to higher numbers compared to $\gamma\delta$ [med] (Fig. 5d).

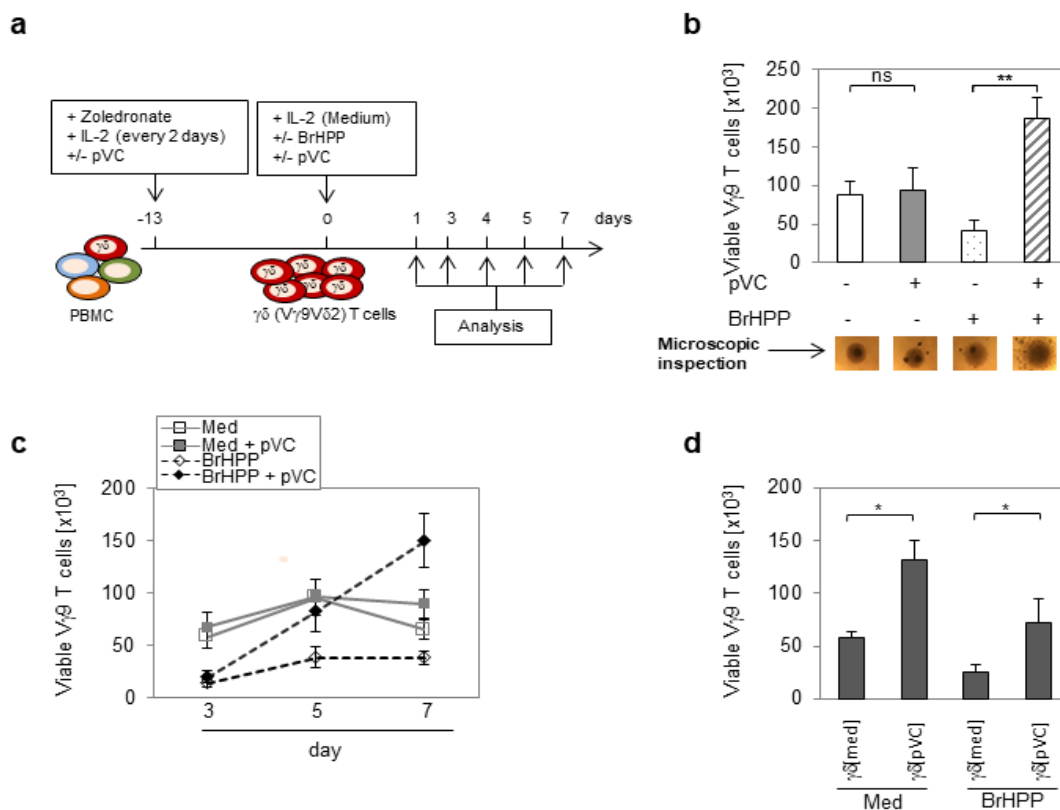


Figure 5. pVC promotes the proliferation of BrHPP-re-stimulated $\gamma\delta$ T-cell lines.

(a) Experimental setup: ZOL-expanded $V\gamma 9V\delta 2$ T cells (4×10^4 cells/well) were left unstimulated or were re-stimulated with BrHPP in the presence (or not) of $173 \mu\text{M}$ ($50 \mu\text{g/mL}$) pVC. On d7 the number of viable $V\gamma 9$ T cells was determined by SCDA. **(b)** The bar diagram shows the mean values \pm SD of the numbers of viable $V\gamma 9$ T cells from six independent experiments. Lower part: Microscopic picture of $\gamma\delta$ T-cell micro-cultures corresponding to the upper panel with 50x magnification. **(c)** Kinetics of $V\gamma 9$ T-cell expansion, represented by mean values \pm SD of viable cell numbers from three independent experiments. **(d)** ZOL-expanded $V\gamma 9V\delta 2$ T cells were generated in the presence ($\gamma\delta[\text{pVC}]$) or absence ($\gamma\delta[\text{med}]$) of pVC. The cells were harvested, washed and re-cultured in IL-2-containing medium w/o BrHPP. The number of viable $V\gamma 9$ T cells was assessed by SCDA on d7. Bar diagram depicting the mean values \pm SD of viable $V\gamma 9$ T-cell numbers from four independent experiments. * $p < 0.05$, ** $p < 0.01$, ns: not significant.

3.1.4. Only concurrent pVC treatment and TCR-stimulation result in enhanced $V\gamma 9V\delta 2$ T-cell proliferation

Next, we determined the time window during which pVC exerted its growth-promoting effect. To this end, ZOL-expanded $V\gamma 9V\delta 2$ T cells were left unstimulated or were re-stimulated with BrHPP in IL-2-containing medium. pVC was added at different time points, i.e. day 0, day 3, or day 0 plus day 3. Proliferation of $V\gamma 9$ T cells was again quantified after 7 days. When pVC was provided simultaneously with BrHPP re-stimulation, there was a significant increase of $V\gamma 9$ T-cell numbers (Fig. 6). Additionally, if pVC was supplemented at d0 and d3, the $V\gamma 9$ T-cell numbers were only slightly elevated compared to d0 treatment. In contrast, when pVC treatment was started on d3 after BrHPP-re-stimulation, the pVC-induced proliferation was

slightly increasing, however less than when pVC was added at d0 and without reaching statistical significance. These results indicate that pVC had to be present at the initiation of the cell culture (day 0) to exert its enhancing effect on proliferation, adding a new restriction point for the effect of pVC on $\gamma\delta$ T-cell differentiation.

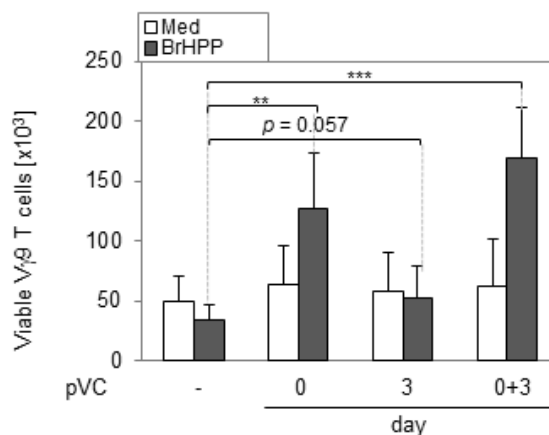


Figure 6. Effect of pVC-supplementation at different time points on the proliferation of BrHPP-re-stimulated $\gamma\delta$ T-cell lines.

ZOL-expanded V γ 9V δ 2 T cells were left unstimulated or were re-stimulated with BrHPP in IL-2-containing medium. pVC was added at different time points, i.e. d0, d3, or d0+3. Numbers of viable V γ 9 T cells determined on d7 by flow cytometry using the SCDA-method. The bar diagram shows the mean values \pm SD of the numbers of viable V γ 9 T cells from six independent experiments. ** $p < 0.01$, *** $p < 0.001$

3.1.5. pVC does not prevent activation-induced cell death of V γ 9V δ 2 T cells

To further elucidate the mechanism by which pVC enhances V γ 9V δ 2 T-cell numbers, we considered the possibility, that pVC might prevent cell death of V γ 9V δ 2 T cells upon BrHPP re-stimulation, because TCR-dependent re-stimulation of activated $\gamma\delta$ T cells is known to induce activation-induced cell death (AICD, [192]). To this end, we analyzed cell death induction by flow cytometry using a combination of Annexin V and PI staining [205]. Cells in early apoptotic phase are Annexin V⁺/PI⁻, those in necroptotic phase are Annexin V⁻/PI⁺ while already dead cells (late apoptosis and necroptosis) stain positively for both markers [205,206]. ZOL-expanded V γ 9V δ 2 T cells, pretreated (or not) with pVC, were left unstimulated or were re-stimulated with BrHPP in IL-2-containing medium. Cell death analysis was performed after 20h. Dot blots of one representative experiment are presented in Fig. 7a, and a summary of four experiments in Fig. 7b. As expected, BrHPP induced significant cell death (approximately 77% PI⁺/Annexin V⁺ cells) which, however, was not inhibited by pVC. Moreover, we also did not see any effect of pVC on the appearance of early apoptotic (PI⁻/Annexin V⁺) or necrotic cells (PI⁺/Annexin V⁻).

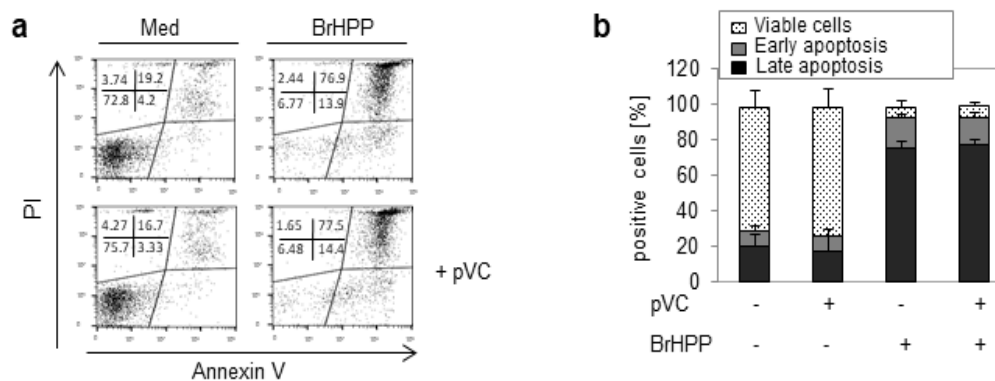


Figure 7. pVC does not prevent AICD in BrHPP-re-stimulated $\gamma\delta$ T cells.

ZOL-expanded $V\gamma 9V\delta 2$ T cells were pre-treated (where indicated) with pVC for 20h followed by BrHPP stimulation for another 20 h. Thereafter, cells were stained with Annexin V-FITC/PI and were analyzed by flow cytometry. **(a)** Dot plots of one representative experiment are shown. Numbers indicate the percentage of positive cells. **(b)** Bar diagram from four independent experiments showing frequency (mean values \pm SD) of viable cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺).

3.1.6. pVC promotes the cell cycle progression of re-stimulated $V\gamma 9V\delta 2$ T cells

Results presented in the previous section suggested that pVC increases $V\gamma 9V\delta 2$ T-cell proliferation upon TCR (re-)stimulation by a mechanism independent of inhibition of AICD. Therefore, we explored further mechanisms underlying the pVC-mediated increased proliferation, including cell cycle analysis by flow cytometry. To this end, ZOL-expanded $V\gamma 9V\delta 2$ T cells were pre-treated (or not) for 20h with pVC before re-culture with BrHPP or medium only. The cell cycle analysis was performed after 3 days. The cell cycle phase was determined according to the DNA-content, which was quantified by flow cytometry using PI-staining. Histograms of a representative experiment are depicted in Fig. 8a, and a summary of four experiments in Fig. 8b. In the presence of pVC, we observed a significantly higher proportion of cells in the G₂/M phase in the BrHPP-re-stimulated cells. In addition to the cell cycle analysis, we also analyzed the expression of the cell proliferation marker Ki-67 which is expressed by cycling cells, i.e. in S, G₂ and M phases, but absent in G₀ phase [207,208]. Interestingly, we observed a higher proportion of Ki-67⁺ $V\delta 2$ T cells upon pVC treatment irrespective of BrHPP-re-stimulation (Fig. 8c, d). Taken together, these results suggest that pVC promotes the proliferation of re-stimulated $V\gamma 9V\delta 2$ T cells not by preventing AICD but rather by inducing cell cycle progression.

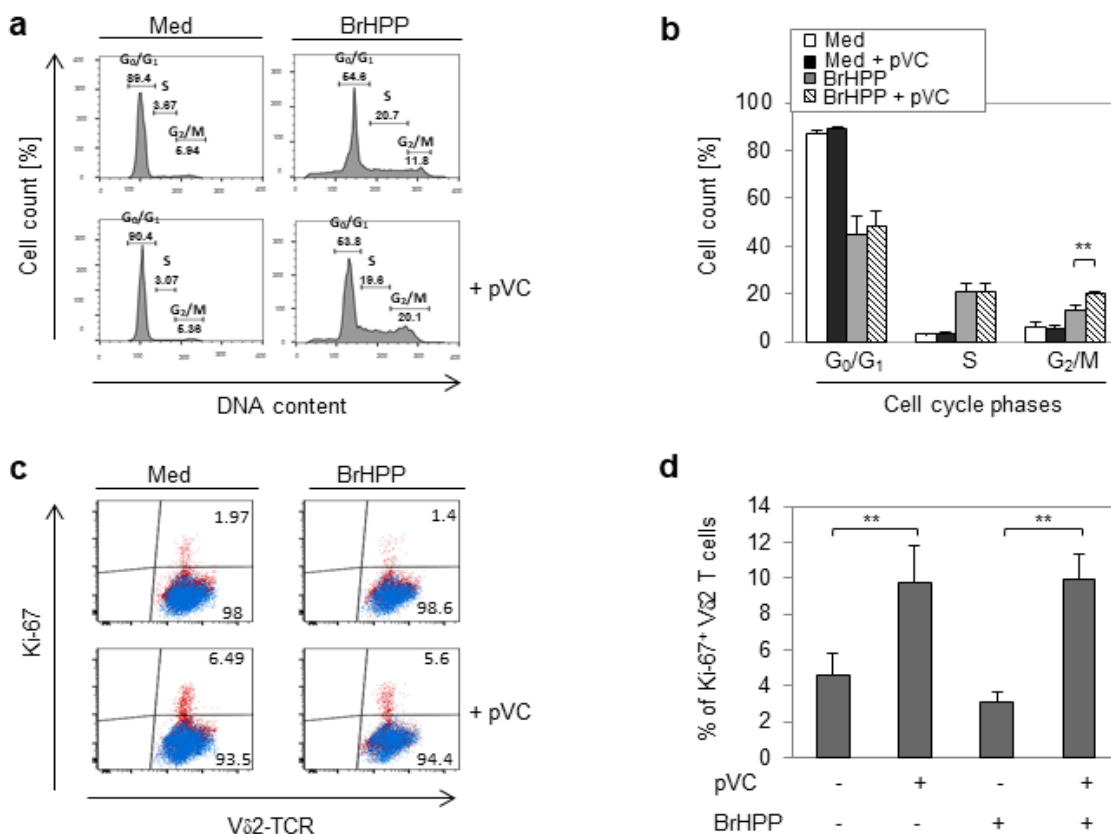


Figure 8. pVC promotes the proliferation of BrHPP-re-stimulated $\gamma\delta$ T cells by inducing cell cycle progression.

(a) ZOL-expanded V γ 9V δ 2 T cells were left unstimulated or were re-stimulated with BrHPP for 3 days after a 20h pretreatment with pVC. Cell cycle distribution of living cells obtained by gating on forward/sideward scatter was determined using PI-staining and flow cytometry. Histograms of one representative out of four independent experiments are shown. (b) Bar graphs represent the percentage (mean values \pm SD) of expanded cells in each phase of the cell cycle; n=4. (c) dot plots of one representative experiment showing the Ki-67 protein-expression in viable V δ 2 T cells (determined by FACS gating) on day 7 after re-stimulation. (d) Bar charts showing the percentage (mean values \pm SEM) of Ki-67⁺ V δ 2 T cells from five independent experiments. ***p* < 0.01

3.1.7. pVC treatment enhances both the T-bet- and the GATA-3 protein-expression in IL-2-expanded $\gamma\delta$ T cells

Next, we asked whether the influence of pVC could also be extended to T-cell subset-related functions. To this end, magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or anti-CD2/CD3/CD28 Ab-coated microbeads (activation/expander beads, A/E beads) in IL-2-containing medium (non-polarizing conditions) in the presence or absence of pVC for 8 days. Thereafter, the protein-expression of transcription factors known to be crucial for T helper (Th) subset differentiation, i.e. transcription factor T-box-containing protein expressed in T cells (T-bet for Th1) and GATA-binding protein 3 (GATA-3 for Th2) was assessed by flow cytometry. As shown in Fig. 9a, b, both GATA-3 and T-bet were co-expressed in IL-2-

expanded $\gamma\delta$ T cells upon stimulation. Interestingly, the addition of pVC led to a significant increased number of cells co-expressing GATA-3 and T-bet.

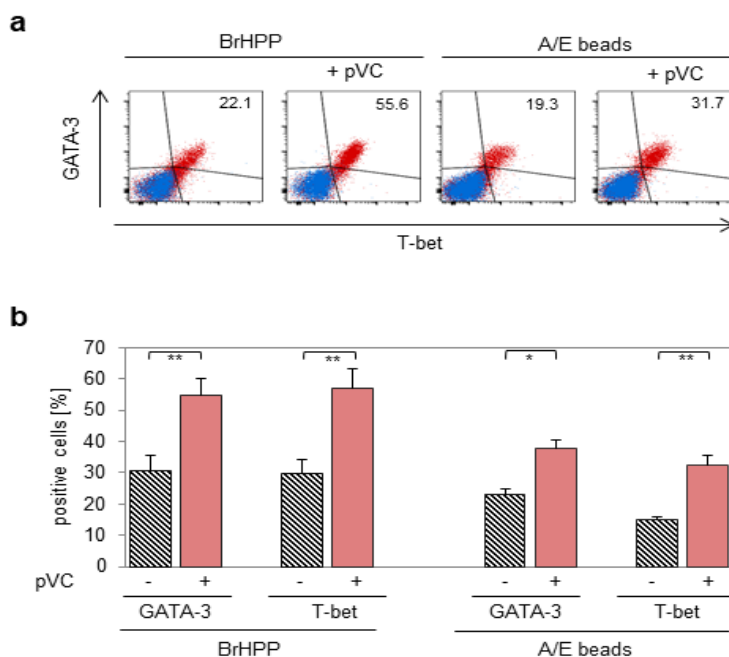


Figure 9. Modulation of transcription factor expression by pVC in IL-2-expanded $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or with anti-CD2/CD3/CD28 Ab-coated microbeads (activation/expander beads, A/E beads) and expanded in IL-2-containing medium in the presence or absence of pVC. On day 8, cells were harvested, stained intracellularly for GATA-3 (clone L50-823) and T-bet (clone 4B10) (red) and their corresponding isotype controls (blue). **(a)** Dot plots of one representative experiment are shown. Numbers indicate the percentage of GATA-3⁺ T-bet⁺ cells. **(b)** Bar graphs represent the percentage (mean \pm SD) of GATA-3⁺ and T-bet⁺ $\gamma\delta$ T cells from six independent experiments.

3.1.8. pVC modulates the cytokine profile of IL-2-expanded $\gamma\delta$ T cells

Because of the observed concomitant T-bet and GATA-3 protein-expression which was further enhanced upon pVC supplementation, we analyzed if pVC would modulate the Th1/Th2 bias of the $\gamma\delta$ T-cell cytokine-profile. To this end, the secretion of a broad panel of 12 cytokines of differentially expanded $\gamma\delta$ T cells was measured in cell culture supernatants on day 8 after stimulation using a bead-based cytokine array. Among these, the most abundant cytokines secreted in IL-2-expanded $\gamma\delta$ T cells were IFN- γ and IL-13 and to a lesser extent IL-5. Upon addition of pVC the secretion of IFN- γ , IL-13 and IL-5 was clearly enhanced (Fig. 10a). Using ELISA to verify the results of the cytokine array, we were able to confirm the observations regarding the IFN- γ and IL-13 secretion and also found a significantly enhanced production in the pVC-treated cells (Fig. 10b, c). While IL-2-expanded $\gamma\delta$ T cells produced low levels of IFN- γ (BrHPP: 431.3 \pm 410 pg/mL; A/E beads: 274 \pm 223

pg/mL) and IL-13 (BrHPP: 434.6 ± 63 pg/mL; A/E beads: 387.8 ± 84.3 pg/mL), the addition of pVC significantly increased the levels of IFN- γ (BrHPP: 737.6 ± 644.4 pg/mL; A/E beads: 435.8 ± 429 pg/mL) and IL-13 (BrHPP: 2486.6 ± 508.5 pg/mL; A/E beads: 1108.7 ± 350.6 pg/mL). Taken together, pVC modulated both, Th1- and Th2-specific transcription factor expression and the related cytokine production in IL-2-expanded $\gamma\delta$ T cells.

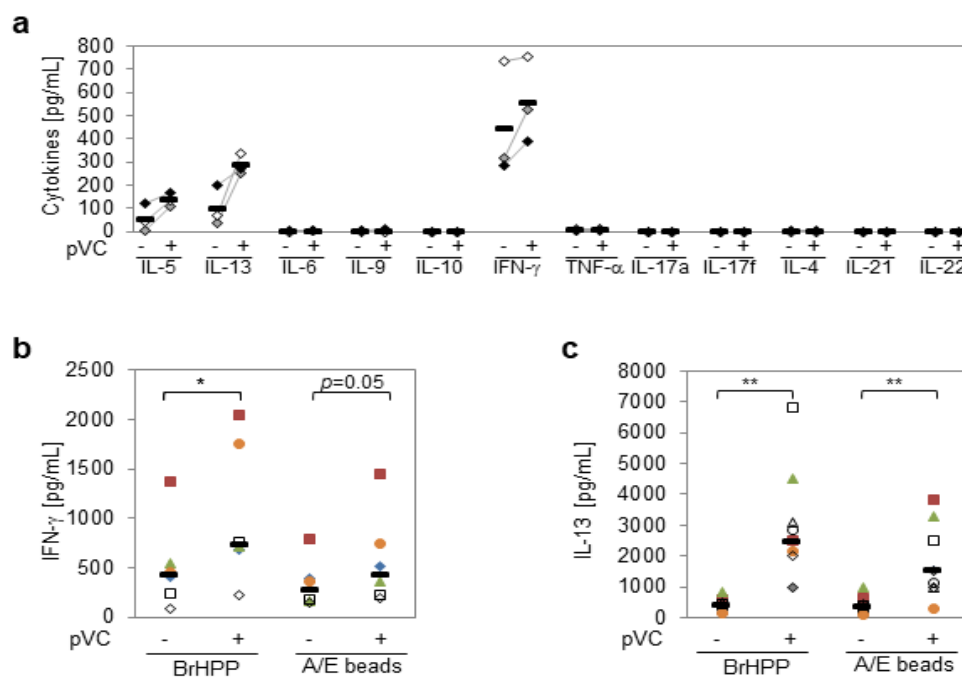


Figure 10. Modulation of cytokine production by pVC in IL-2-expanded $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E-beads and expanded in IL-2-containing medium +/- pVC. **(a)** At day 8, supernatants were collected, and the indicated cytokines were measured by LEGENDplex™ bead-based immunoassay ($n = 3$). **(b, c)** detection of IFN- γ ($n = 6$) and IL-13 ($n = 9$) in the supernatants obtained on day 8 after initial stimulation using ELISA. Each symbol represents an individual donor. Horizontal bars indicate the median value * $p < 0.05$ and ** $p < 0.01$

3.2. Modulation of $\gamma\delta$ T cell-mediated cytotoxic potential by pVC

$\gamma\delta$ T cells exhibit cytotoxic effector functions against a variety of tumor cell lines. VC, on the other hand, affects tumor cell metabolism and directly kills some tumor cells [209,210]. We therefore analyzed the interplay of pVC and $\gamma\delta$ T cells in the $\gamma\delta$ T-cell mediated anti-tumor cell cytotoxicity.

3.2.1. pVC enhances tumor cell lysis activity by ZOL-expanded V γ 9V δ 2 T cells

The positive effect of pVC on the proliferation of V γ 9V δ 2 T cells prompted us to analyze its effects in the context of $\gamma\delta$ T cell-based cytotoxicity. The effect of pVC on the cytotoxic potential of ZOL-expanded V γ 9V δ 2 T cells was experimentally analyzed using three different approaches; (i) pVC was directly added during the short-term cytotoxicity assay, (ii) $\gamma\delta$ T cells

were pre-incubated with pVC prior to the cytotoxicity assay and (iii) tumor cells were pre-incubated with pVC. Using the *in vitro* chromium release cytotoxicity assay, we measured the effect of pVC on the specific lysis of tumor cells by V γ 9V δ 2 T-cell lines. A scheme of the different experimental approaches is depicted in Fig. 11a. In brief, V γ 9V δ 2 T-cell lines were first established by culturing PBMC with ZOL in the presence or absence of pVC and repetitive addition of IL-2 for 14 days. Pancreatic ductal adenocarcinoma cell line (Panc89), used as target cell, were incubated for 72 h in the presence or absence of pVC. At the time of the cytotoxicity assay, the tumor cell lines were labelled with radioactive sodium chromate (Na⁵¹Cr) and were co-cultured with ZOL-expanded V γ 9V δ 2 T cells in the presence or absence of BrHPP. After 4h of incubation at 37°C, the release of ⁵¹Cr into the supernatant subsequent to the lysis of the tumor cell lines was measured.

We found that addition of BrHPP during the *in vitro* killing assay strongly increased the cytotoxic activity against Panc89. Interestingly, when pVC was additionally present during the co-culture (i), the cytotoxicity in the presence of BrHPP was significantly further increased (Fig. 11b).

Using the second approach (ii), we observed that, ZOL-expanded V γ 9V δ 2 T cells that were initially pre-treated with pVC, exhibited an enhanced cytotoxic activity against Panc89 (Fig. 11c). Notably, this observation was most significant at high effector target ratios (i.e. 80:1, 40:1) upon BrHPP re-stimulation.

Finally, with the third approach (iii), regarding the pVC pre-treatment of the tumor cell, there was a tendency towards increased sensitivity of pVC-pre-treated tumor cells which, however, was not statistically significant (Fig. 11d).

Taken together, it became clear that pVC can enhance the cytotoxic activity of pAg-re-stimulated V γ 9V δ 2 T cells, which was most pronounced when pVC was added during the 4h cytotoxicity assay.

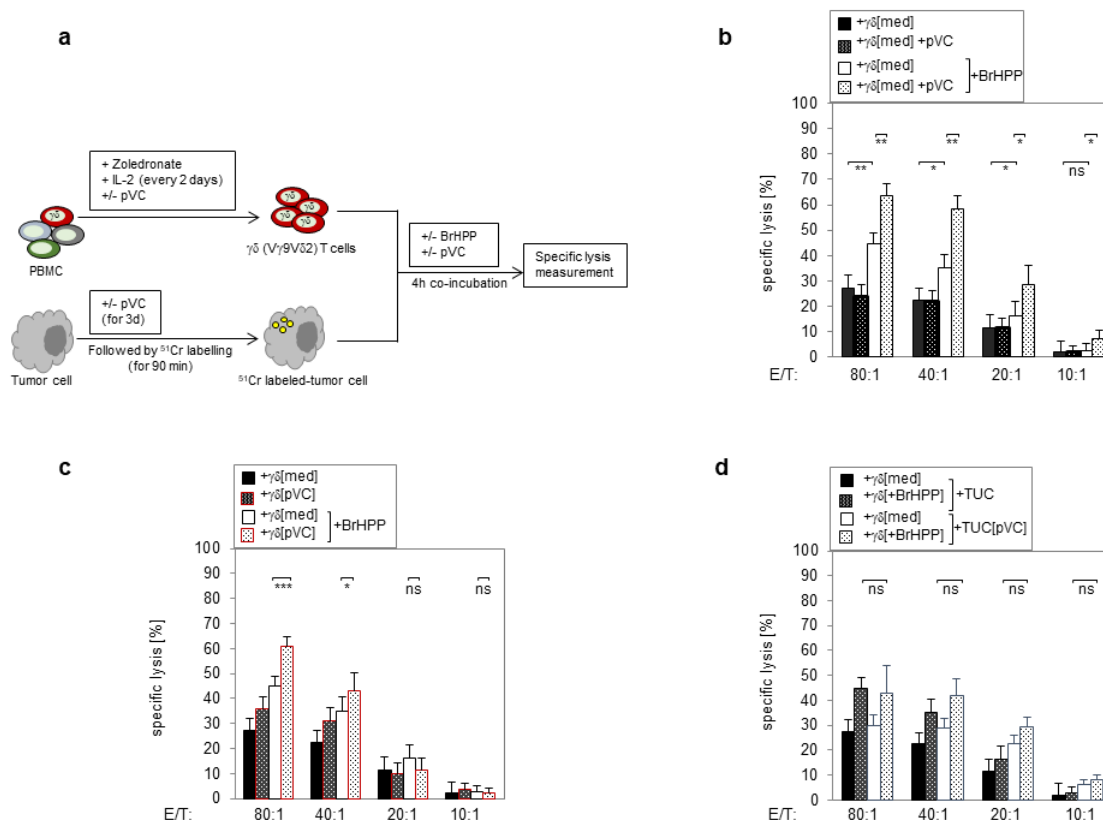


Figure 11. pVC enhances the anti-tumor cell cytotoxic activity of V γ 9V δ 2 T cells.

(a) Experimental design: ZOL-expanded V γ 9V δ 2 T-cell lines were established in the presence or absence of pVC. Pancreatic ductal adenocarcinoma tumor cell line (Panc89), after 3 days incubation at 37°C in the presence or absence of pVC, was labelled with radioactive sodium chromate (Na⁵¹Cr) for 90 min at 37°C and was thereafter washed twice and used as target cells. **(b-d)** ZOL-expanded V γ 9V δ 2T cells (effector cells), left unstimulated or re-stimulated with BrHPP in the presence (or not) of pVC, were added at different effector/target ratios [E/T] (80:1, 40:1, 20:1 and 10:1). **(b)** pVC was added to the co-culture. **(c)** Untreated and pVC-pretreated ZOL-expanded V γ 9V δ 2T cells were cocultured with ⁵¹Cr labelled Panc89 cells in the presence or absence of BrHPP. **(d)** untreated and pVC-pretreated Panc89 were ⁵¹Cr labelled and co-cultured with unstimulated or BrHPP-re-stimulated V γ 9V δ 2 T cells. The cytotoxicity was measured using the ⁵¹Cr-release assay. The bar diagram shows the quantification of the released ⁵¹Cr as percentage of specific lysis. Data are shown as mean values (\pm SEM) of four independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, ns not significant, TUC: Tumor cells (Panc89), med: medium

Because the cytotoxicity-enhancing effect of pVC was most pronounced upon short-term co-culture with Panc89, we focused on this co-culture for the further analysis of the effector/target interaction.

3.2.2. Effect of pVC on the surface expression of activation, effector, costimulatory and memory differentiation markers

Next, we wanted to know whether pVC enhanced the V γ 9V δ 2 T-cell-mediated cytotoxicity by altering the expression of cell surface markers related to V γ 9V δ 2 T-cell activation. V γ 9V δ 2 T

cell lines were re-stimulated (or not) with BrHPP in IL-2-containing medium in the presence or absence of pVC. After 4h, cells were harvested and stained with monoclonal antibodies for CD69 (marker of early activated cells) and CD62L (marker of naïve cells). V γ 9V δ 2 T cells enhanced the expression of CD69 upon re-stimulation. CD69 expression was further enhanced when pVC was added to the BrHPP-re-stimulated cells, although this did not reach statistical significance (Fig. 12a). As expected, BrHPP re-stimulation induced a decrease in CD62L expression. However, addition of pVC did not further alter CD62L expression of BrHPP-re-stimulated V γ 9V δ 2 T cells (Fig. 12b).

The antitumor effect of $\gamma\delta$ T cells is induced by the interaction of $\gamma\delta$ TCR with tumor cells. Additionally, activating receptors, such as the natural killer (NK) group 2D (NKG2D) receptor, expressed on $\gamma\delta$ T cells can recognize NKG2D ligands (NKG2DLs), which can also trigger the cytotoxic function of $\gamma\delta$ T cells. As shown in Fig. 12c, NKG2D was highly expressed on unstimulated as well as BrHPP-re-stimulated cells, and the expression remained unchanged in the presence of pVC. It was reported that activated V γ 9V δ 2 T cells can acquire features of antigen-presenting cells (APC) [83]. Therefore, we wanted to know whether pVC altered the cell surface markers associated with APC function. We tested the expression of MHC-II (HLA-DR), CD80 and CD86 4h following re-stimulation with BrHPP in the presence or absence of pVC. Results depicted in Figs. 12d-f showed that HLA-DR was highly expressed on unstimulated as well as BrHPP-re-stimulated cells. However, pVC did not alter the expression of HLA-DR, CD86 and CD80 neither at steady state nor upon BrHPP re-stimulation. Interestingly, however, the expression of CD80 and CD86 at later time point (i.e., d4 and d6) was significantly higher on BrHPP-re-stimulated V δ 2 T cells treated with pVC than on untreated cells (data not shown).

CD56 (Neural cell adhesion molecule-I) expression correlates with cytotoxic effector activity. In line, it was reported that CD56-expressing V γ 9V δ 2 T cells displayed potent cytotoxicity against tumor cells [211,212]. Analyzing the effect of pVC on the CD56 expression of V γ 9V δ 2 T cells, we observed that CD56 was highly expressed on unstimulated as well as BrHPP-re-stimulated V γ 9V δ 2 T cells, which is in accordance with previous study [211]. However, addition of pVC did not change the expression at all (Fig. 12g).

Finally, we analyzed whether pVC affected the distribution of $\gamma\delta$ T-cell memory phenotypes. To this end, from the same experimental set up as applied to the surface markers expression, differentiation into either type of memory T cells was analyzed based on the expression of CD27 and CD45RA [213]. Unstimulated V γ 9V δ 2 T cells contained equal proportions of CD45RA⁻CD27⁺ (Tcm) and CD45⁺CD27⁻ (Tem) subsets. BrHPP-re-stimulation

induced an asymmetric distribution of both subsets, however with increased CD45RA⁺CD27⁻ subset. This memory phenotypic distribution was not affected by pVC treatment (Fig. 12h).

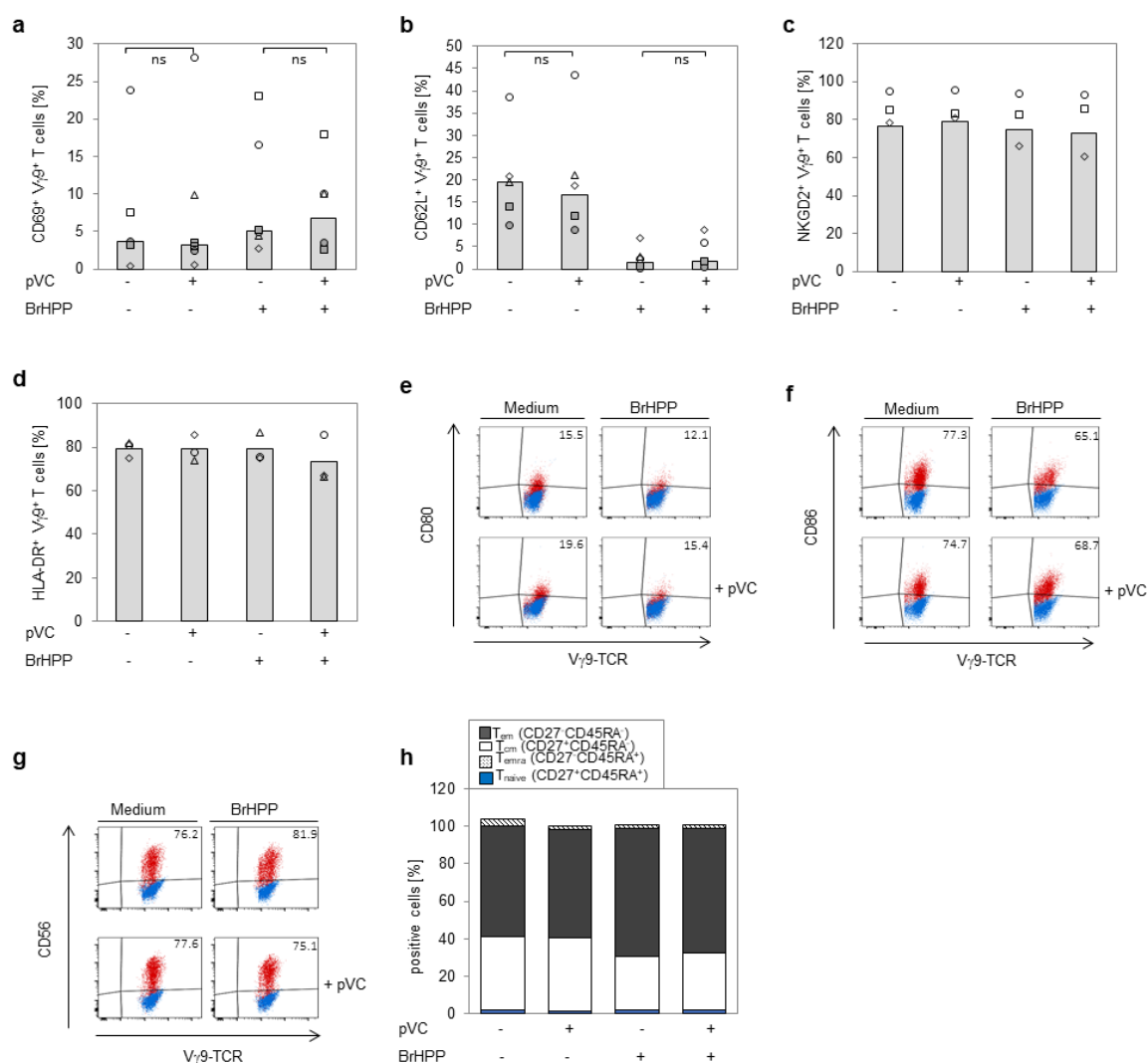


Figure 12. Modulation of surface marker expression by pVC.

ZOL-expanded V_γ9V_δ2 T cells were left unstimulated or re-stimulated with BrHPP in the presence or absence of 173 μM (50 μg/mL) pVC. After 4h of incubation, cell surface expression of (a) CD69 (clone L78), (b) CD62L (clone Dreg-56), (c) NKG2D (clone 1D11), (d) HLA-DR (clone L243), (e) CD80 (clone L307.4), (f) CD86 (clone 2331(FUN-1)) and (g) CD56 (clone NCAM16.2) was measured by flow cytometry. (h) cells were stained with mAbs for V_γ9-TCR (clone 7A5), CD45RA (clone L48) and CD27 (clone M-T271). Upon flow cytometric analysis on gated V_γ9⁺ T cells, memory subsets were identified as CD45RA⁺CD27⁺ (T_{cm}), CD45⁺CD27⁻ (T_{em}), CD45RA⁺CD27⁺ (T_n) and CD45⁺CD27⁻ (T_{emra}). (a-d) Each symbol represents an individual donor. Bar chart represents the mean value. (e-g) dot plots of one representative experiment out of two are shown. (h) Bar charts depict mean value of the percentage of positive cells among gated V_γ9 T cells from four independent experiments.

3.2.3. Effect of pVC on the release of cytotoxic mediators

The cytotoxic activity of γδ T cells against tumor cells can be mediated by death receptor / ligand interactions or by the release of cytolytic mediators. The enhanced V_γ9V_δ2 T-cell

cytotoxicity mediated by pVC prompted us to examine typical mediators of cytotoxic activity. To this end, we analyzed the degranulation of $V\gamma 9V\delta 2$ T cells in co-culture with Panc89. As a marker for degranulation the surface mobilization of CD107a was quantified. Dot plots of a representative experiment and a summary of four experiments are depicted in Fig. 13a. Co-culture of Panc89 and $V\gamma 9V\delta 2$ T -cells induced a weak surface mobilization of CD107a. Upon BrHPP-re-stimulation, the cell surface expression of CD107a was significantly increased. However, the addition of pVC did not further affect the CD107a mobilization of unstimulated or BrHPP-re-stimulated $V\gamma 9V\delta 2$ T cells.

The release of granzyme B and perforin, soluble mediators of cytotoxicity, was measured in parallel by ELISA. BrHPP-re-stimulated $V\gamma 9V\delta 2$ T cells, co-cultured with Panc89, produced higher amount of granzyme B and perforin when compared to unstimulated cells (Fig. 13b, c). The additional presence of pVC induced a slight increase of perforin and granzyme B, however without reaching statistical significance.

Finally, we analyzed the effect of pVC on the IFN- γ production of $V\gamma 9V\delta 2$ T cells co-cultured with Panc89 in the presence or absence of BrHPP and found a strong BrHPP-induced IFN- γ secretion, which was further significantly increased in the presence of pVC (Fig. 13d).

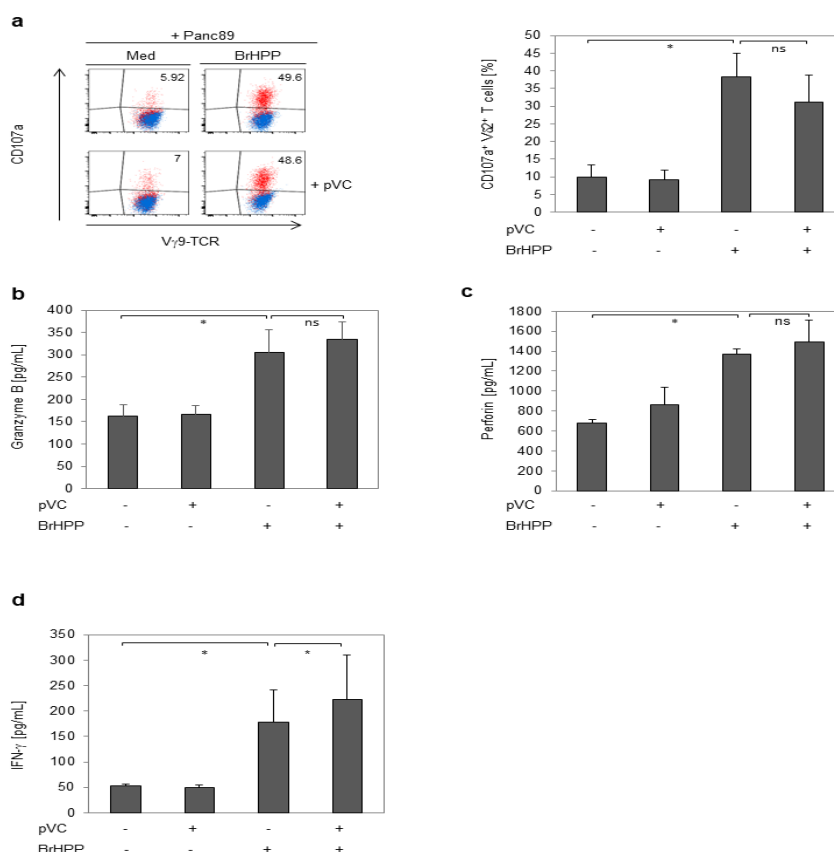


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Figure 13. Modulation of CD107a expression, Granzyme B, Perforin and IFN- γ production by pVC.

(a) ZOL-expanded V γ 9V δ 2 T cells were co-cultured with Panc89 (E:T ratio of 40:1) in medium or with 300 nM BrHPP in the presence or absence of pVC. The CD107a surface mobilization on V δ 2 T cells was analyzed after 4h of co-culture by flow cytometry using mAbs specific for V δ 2 TCR (clone B6) and CD107a (clone H4A3). Left: dot plots of one representative experiment are shown. Right: Bar graphs showing mean values \pm SEM of four independent experiments. **(b, c)** Using the same culture conditions as in (a), the release of granzyme B and perforin in the supernatants was quantified using specific ELISAs (after 4 hours of co-culture). Bar graph depicts mean values \pm SEM of six independent experiments. **(d)** The release of IFN- γ in the supernatants was quantified using specific ELISA after 4 hours of co-culture. Bar graphs showing mean values \pm SEM of six independent experiments. * $p < 0.05$, ns not significant

3.2.4. pVC modulates V γ 9V δ 2 T-cell receptor signaling**3.2.4.1. pVC reduces ROS production in V γ 9V δ 2 T cells**

Following activation, T cells exhibit a heightened metabolic activity that drives increased production of reactive oxygen species (ROS) by the mitochondrial electron transport chain mainly [34,214]. Whereas low concentrations of ROS support cell survival and proliferation, high concentrations of ROS correlate with DNA damage and cell death [34,215]. To test the effect of pVC on the production of ROS, ZOL-expanded V γ 9V δ 2 T cells were left unstimulated or re-stimulated with BrHPP in IL-2-containing medium in the presence or absence of pVC. After 4h of incubation, the intracellular ROS production was analyzed using flow cytometry. In line with the well-known antioxidant activity of Vitamin C, we observed that spontaneous as well as BrHPP-induced ROS production was significantly reduced by pVC treatment (Fig. 14a, b).

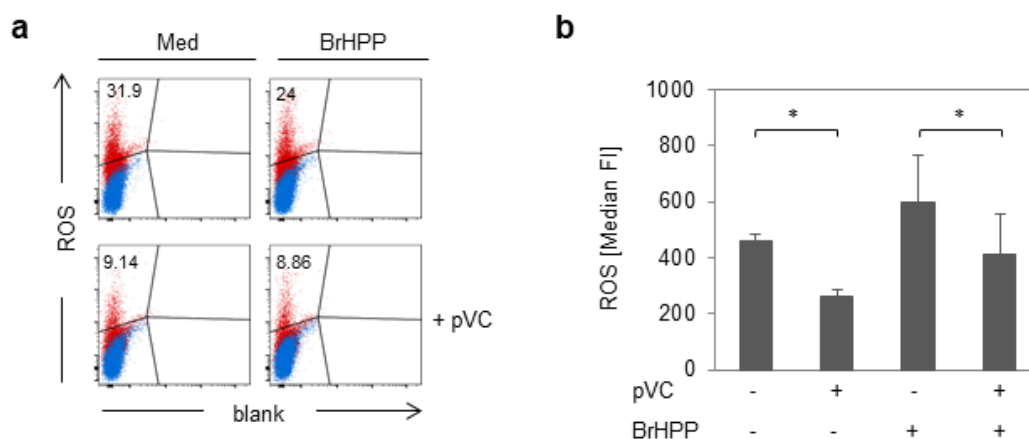


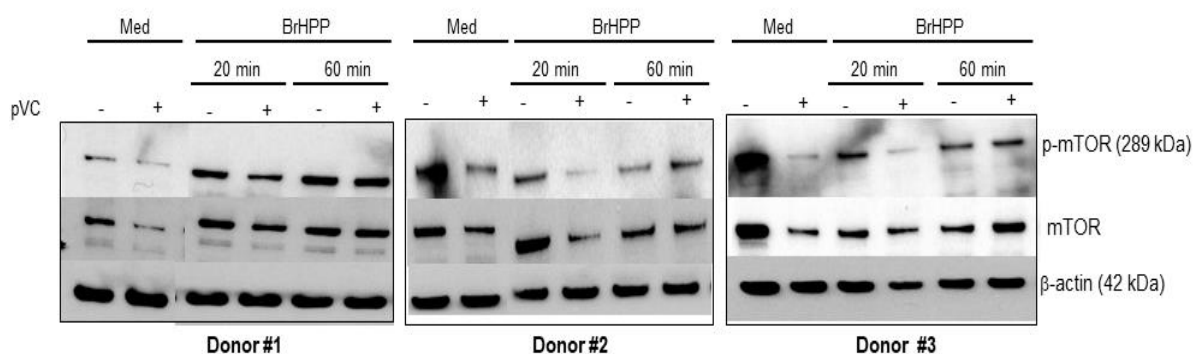
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Figure 14. pVC decreases the level of intracellular ROS.

ZOL-expanded V γ 9V δ 2 T cells were left unstimulated or were re-stimulated with BrHPP for 3h following 1h pretreatment with 50 μ g/mL (173 μ M) pVC (where indicated). The level of intracellular ROS was measured by flow cytometry using a ROS detection kit (Molecular probes, life technologies). **(a)** Dot plots of one representative experiment are shown. Numbers indicate the percentage of positive cells. **(b)** Bar graphs showing mean (\pm SEM) of isotype-subtracted median fluorescence intensity [Median FI] from four independent experiments. * $p < 0.05$

3.2.4.2. The effect of pVC on mTOR activity/expression

mTOR is a serine/threonine kinase whose inhibition was previously described to potentiate the yield and cytotoxic effector function of human V δ 2 [43] and murine V γ 4 [42] $\gamma\delta$ T-cell subsets. Here, we investigated whether pVC could enhance V γ 9V δ 2 T-cell mediated cytotoxicity by counteracting mTOR signaling. To this end, ZOL-expanded V γ 9V δ 2 T cells were cultured in complete RPMI medium for 24h. Then, V γ 9V δ 2 T cells were pre-treated with pVC (where indicated) for 1h followed by BrHPP-re-stimulation (or not). After 20 min and 60 min of incubation, cells were collected for Western blotting assays. Fig. 15 shows the protein expression as well as the phosphorylation state (at Ser2448) of mTOR. mTOR signaling is active in V γ 9V δ 2 T cells as previously reported [43]. We observed, particularly at 20 min, that pVC pretreatment resulted in substantially attenuated steady-state and BrHPP-induced mTOR phosphorylation as well as the mTOR protein-expression. The experiment was performed three times to ensure reproducibility and provided evidence of inhibitory effect of pVC on mTOR.

**Figure 15. pVC reduces mTOR protein-expression.**

ZOL-expanded V γ 9V δ 2 T-cell lines were washed twice and cultured in fresh medium for 24 h. Thereafter, they were treated with pVC for 1h followed by the addition of medium only or BrHPP for 20 min and 60 min. After stimulation, cells were harvested and lysed. Equal amount of protein from whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. Western blotting assay were performed with specific polyclonal antibodies for p-mTOR (cat N° 2971, Cell Signaling Technology) and, mTOR (cat N° 9862); and β -actin as loading control.

3.3. Influence of pVC on the generation of regulatory human V γ 9V δ 2 T cells

Regulatory T cells (Tregs) are critical for the maintenance of immune homeostasis and self-tolerance and can be therapeutically used for prevention of unwanted or exacerbated immune responses. $\gamma\delta$ T cells in different microenvironments can assume features reminiscent to Tregs. *In vitro* generation of " $\gamma\delta$ Tregs" can easily be achieved by $\gamma\delta$ -TCR stimulation of PBMC (or freshly isolated $\gamma\delta$ T cells) cells in the presence of TGF- β and IL-2/IL-15 [98,99,216]. However, these induced- $\gamma\delta$ Tregs display an unstable Treg phenotype and loss of suppressive activity, reminiscent to induced- $\alpha\beta$ Tregs. Since VC is known to stabilize Foxp3 protein-expression in $\alpha\beta$ T cells, we analyzed the influence of pVC on the induction of $\gamma\delta$ Tregs.

3.3.1. pVC promotes suppressive activity in TGF- β -expanded $\gamma\delta$ T cells

To investigate the influence of pVC on the suppressive activity of human $\gamma\delta$ T cells, we first activated magnetically isolated $\gamma\delta$ T cells with BrHPP or A/E beads in the presence of IL-2 plus TGF- β with/without pVC for 14 days. To assess the suppressive activity of the differentially expanded $\gamma\delta$ T cells, we co-cultured them with magnetically purified CD4⁺CD25⁻ responder T cells in an autologous *in vitro* co-culture assay stimulated with A/E beads in the absence of antigen-presenting cells. Thereafter, the proliferation of the responder cells as well as $\gamma\delta$ T cells was measured by SCDA after 5 days. Figs. 16a-c show the determination of the numbers of responder as well as the differentially expanded $\gamma\delta$ T cells. Compared to solo-culture of responder CD4 T cells, there was a decrease in the numbers of viable responder cells in co-culture with TGF- β -expanded $\gamma\delta$ T cells, whereby A/E beads-expanded $\gamma\delta$ T cells displayed a stronger suppressive activity compared to BrHPP-activated $\gamma\delta$ T cells (Fig. 16a, b). Interestingly, this suppressive effect was more pronounced for $\gamma\delta$ T cells which had been generated in the presence of pVC (i.e. $\gamma\delta$ [A/E + TGF- β + pVC] and $\gamma\delta$ [BrHPP + TGF- β + pVC]). Reciprocally, the cell number of $\gamma\delta$ [BrHPP + TGF- β + pVC] T cells was strongly enhanced compared to the non-pVC-treated cells (i.e. $\gamma\delta$ [BrHPP + TGF- β]) (Fig. 16c). Such an effect was not observed for the A/E-beads expanded $\gamma\delta$ T cells. Taken together, these results demonstrate that $\gamma\delta$ T cells, exposed to the strong TCR stimulus of A/E beads and expanded in the presence of TGF- β and pVC, can potently suppress autologous CD4 T-cell proliferation.

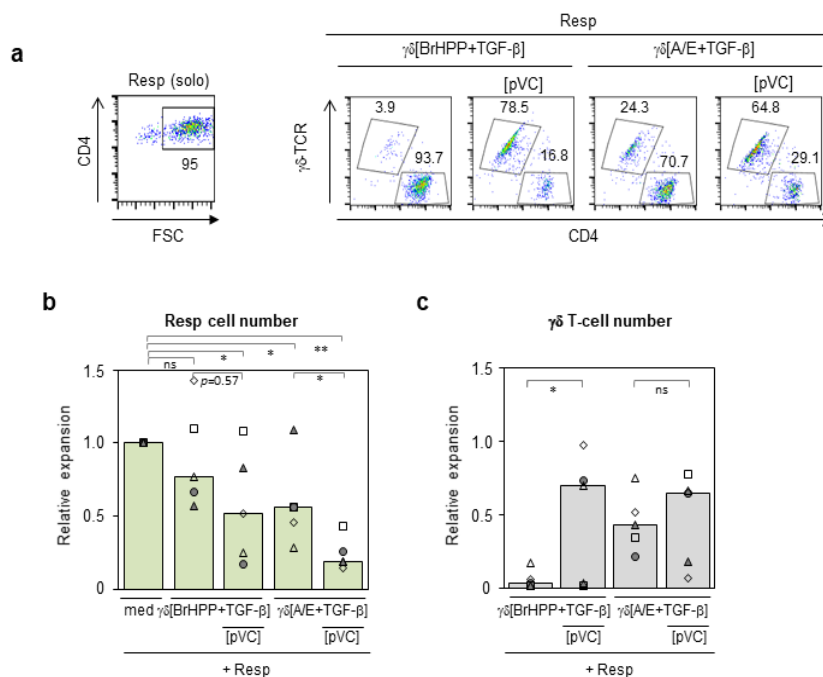


Figure 16. Vitamin C enhances the suppressive activity of TGF- β -expanded $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were differentiated for 14 days as indicated in the figure (i.e. $\gamma\delta$ [BrHPP + TGF- β], $\gamma\delta$ [BrHPP + TGF- β + pVC], $\gamma\delta$ [A/E + TGF- β], $\gamma\delta$ [A/E + TGF- β + pVC]). Thereafter, autologous CD4⁺CD25⁻ T cells (Resp, responders) were co-cultured with the differentially expanded $\gamma\delta$ T cells (suppressors) in the presence of A/E beads at a responder/suppressor ratio of 1:1. The numbers of viable responder- and $\gamma\delta$ T cells per micro-culture was measured by SCDA after 5-7 days. **(a)** Dot plots of one representative out of five independent experiments are shown. Numbers indicate the percentage of positive cells **(b, c)** The relative expansion of (b) responder CD4 T cells and (c) $\gamma\delta$ T cells are depicted as a quotient of viable cells *versus* the cell number of CD4 T cells in solo-culture (med) of the respective experiment. Each symbol represents an independent experiment. The bar chart represents mean values. * $p < 0.05$, ** $p < 0.01$, ns not significant, med: medium

3.3.2. Foxp3 expression in TGF- β -expanded $\gamma\delta$ T cells is upregulated upon pVC treatment

Tregs are characterized by the expression of the master transcription factor forkhead box P3 (Foxp3). Foxp3 is not only a marker for Tregs, but also critically required for their suppressive capacity [92,217]. Loss of Foxp3 expression or dysfunction can lead to severe autoimmune disorders [218,219], making the stability of Foxp3 expression a critical issue, which must be ensured before employing Foxp3⁺ Tregs in therapeutic applications.

To investigate whether the observed enhanced suppressive activity in pVC-treated TGF- β -expanded $\gamma\delta$ T cells correlated with a higher or more stable Foxp3 protein-expression, we analyzed the expression of Foxp3 by flow cytometry in differentially expanded $\gamma\delta$ T cells. On day 8, the expression of Foxp3 was below the level of detection as judged by intracellular staining and remained unchanged in IL-2-cultured cells (Fig. 17a). Foxp3 protein-expression was upregulated when the same cells were activated for 8 days in the presence of TGF-

β (i.e. $\gamma\delta$ [BrHPP + TGF- β], $\gamma\delta$ [A/E + TGF- β]). Interestingly, the addition of pVC led to a significant further increase of Foxp3 protein-expression in TGF- β -expanded $\gamma\delta$ T cells (Fig. 17a right part; Fig. 17b). The proportion of Foxp3⁺ $\gamma\delta$ T cells in cultures supplemented with TGF- β only (BrHPP: $3.7 \pm 3.6\%$; A/E beads: $4 \pm 1.5\%$) was increased to $16.1 \pm 7.5\%$ (BrHPP) and $12.9 \pm 4.8\%$ (A/E beads) upon additional treatment of pVC. Moreover, the population size of the TGF- β -expanded $\gamma\delta$ T cells was increased upon pVC treatment (Fig. 17c). These data suggest that pVC can enhance the TGF- β -induced Foxp3 protein-expression in $\gamma\delta$ T cells.

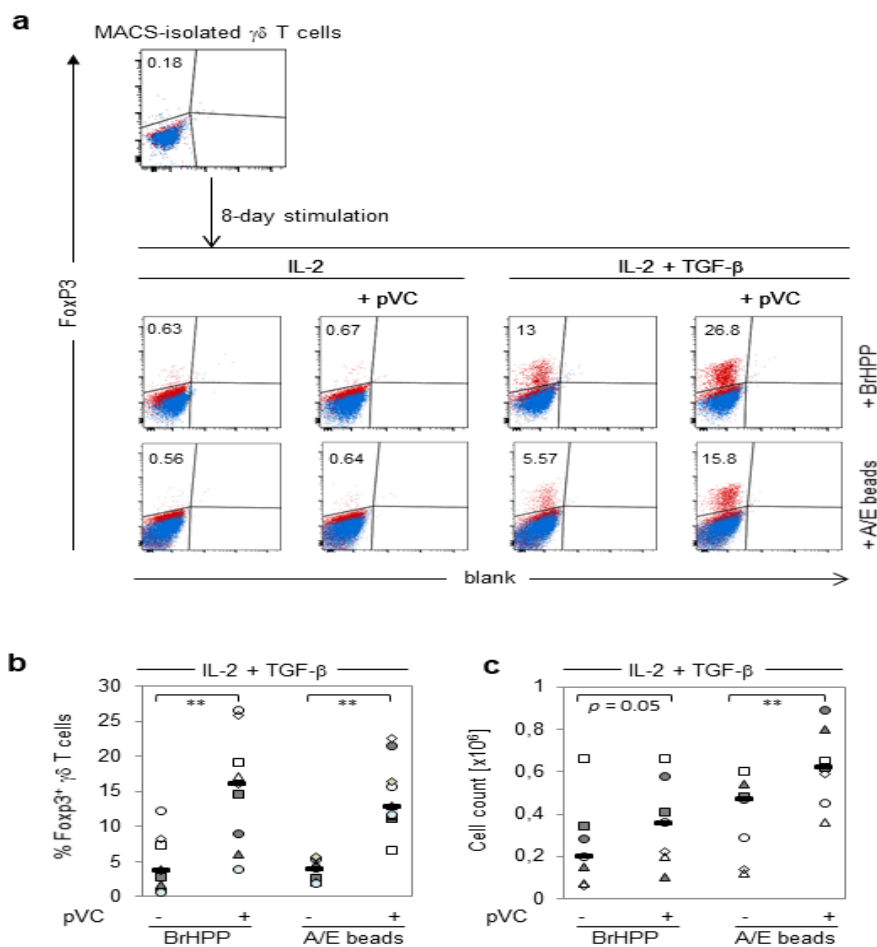


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Figure 17. pVC enhances the Foxp3 protein-expression in human $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E beads in complete RPMI medium supplemented with IL-2 +/- TGF- β in the presence (or not) of pVC. On d8, cells were harvested, stained with the specific mAbs (red color) for Foxp3 (clone 259D/C7) and the respective isotype control (blue color) and were analyzed by flow cytometry. **(a)** Dot plots of one representative out of eight independent experiments are shown. Numbers indicate the percentage of Foxp3⁺ cells. **(b)** The graph shows the frequency of Foxp3⁺ $\gamma\delta$ T cells from TGF- β -expanded $\gamma\delta$ T cells \pm pVC. Horizontal bar represents the median value **(c)** The cell numbers of TGF- β -expanded $\gamma\delta$ T cells were assessed by microscopy, excluding cell death by eosin staining. Each symbol represents an individual donor. ** $p < 0.01$

3.3.3. Concomitant TCR-stimulation and pVC supplementation are required to augment the Foxp3-induction

As already investigated for the pVC-dependent enhanced proliferation, we analyzed the requirement for concomitant TCR-stimulation and pVC-treatment to augment the Foxp3-protein-expression. To this end, magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E beads in [IL-2 + TGF- β]-containing medium. pVC was supplemented at two different time points, d0 and d3. Foxp3 protein-expression was assessed by flow cytometry on d8. Dot blots of one representative experiment are depicted in Fig. 18a, and a summary of three experiments in Fig. 18b. Two effects were notable: first, when pVC was provided at the same time with TCR stimulation, there was a marked increase in Foxp3 protein-expression of the TGF- β -expanded $\gamma\delta$ T cells. Second, when pVC treatment started on d3 after TCR stimulation the Foxp3-upregulation was less pronounced. Indeed, when pVC treatment started on d3, a peak of only (BrHPP: $12 \pm 2.9\%$; A/E beads: $9.64 \pm 1.4\%$) Foxp3⁺ $\gamma\delta$ T cells was observed; in contrast to d0 treatment inducing a peak of (BrHPP: $19.7 \pm 5.3\%$; A/E beads: $19.1 \pm 1.6\%$) Foxp3⁺ $\gamma\delta$ T cells (Fig. 18b). These results indicate that pVC must be present concomitant with TCR-stimulation to effectively augment the Foxp3-induction.

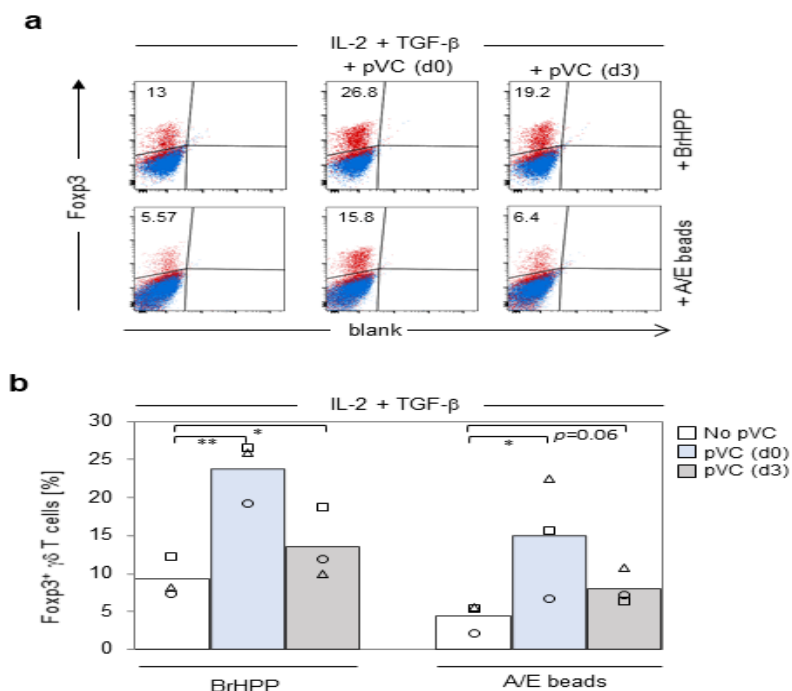


Figure 18. Effect of pVC-application at different time points on the TGF- β -induced Foxp3 protein-expression in $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were A/E- or BrHPP-stimulated in the presence of IL-2 + TGF- β . Additionally, the cells were left untreated or were treated with pVC on d0 and d3. On d8, cells were harvested, stained with the specific mAbs (red color) for Foxp3 (clone 259D/C7) and the respective isotype control (blue color); expression of Foxp3 was analyzed by flow cytometry. **(a)** Dot plots of one representative out of three independent experiments. Numbers indicate the percentage of Foxp3⁺ $\gamma\delta$ T cells. **(b)** Each symbol represents an individual donor. The bar chart represents the mean value; * $p < 0.05$, ** $p < 0.01$

3.3.4. pVC-treatment enhances the stability of the TGF- β -induced Foxp3 protein-expression

TGF- β -expanded $\gamma\delta$ T cells displayed a significant increase of Foxp3 expression in the presence of pVC. Next, we analyzed whether pVC-treated TGF- β -expanded $\gamma\delta$ T cells maintain Foxp3 expression for extended time. Therefore, Foxp3⁺ $\gamma\delta$ T cells were generated with TGF- β in the presence or absence of pVC. At d8 of the culture, the differentially expanded $\gamma\delta$ T cells were split and reseeded in IL-2-containing medium for additional 6 days (i.e. d14). Thereafter, Foxp3 protein-expression was checked by flow cytometry. In TGF- β -expanded $\gamma\delta$ T cells cultured in the absence of pVC (i.e. $\gamma\delta$ [BrHPP + TGF- β] and $\gamma\delta$ [A/E + TGF- β]), the Foxp3 protein-expression was unstable and gradually decreased up to d14 (Fig. 19a, b). As observed in previous experiments, the presence of pVC led to an increase in Foxp3⁺ $\gamma\delta$ T-cell numbers on d8. In $\gamma\delta$ [A/E + TGF- β + pVC] T cells, the Foxp3 protein-expression remained stable up to d14, whereby in $\gamma\delta$ [BrHPP + TGF- β + pVC] T cells, Foxp3 was less stable and declined to be expressed in only half of the cells (Fig. 19a, b) by

d14. These results indicate that pVC supports the stability of Foxp3 protein-expression in TGF- β -expanded human $\gamma\delta$ T cells cultured with A/E beads.

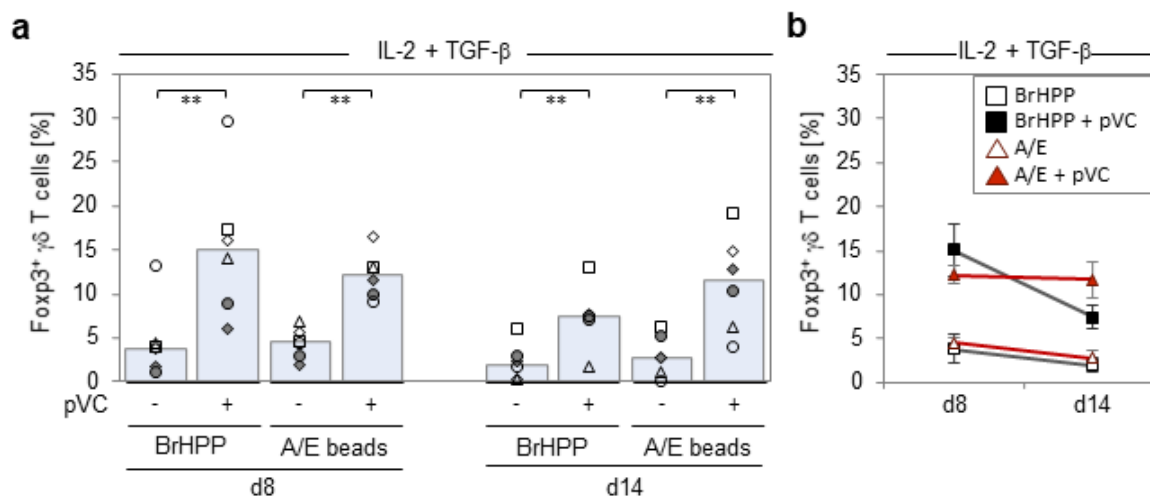


Figure 19. Vitamin C induces Foxp3 stability in TGF- β -expanded $\gamma\delta$ T cells.

(a) $\gamma\delta$ T cells were stimulated with BrHPP or A/E Beads in complete RPMI medium supplemented with IL-2 + TGF- β in the presence (or not) of pVC. At d8, cells were washed and re-seeded in IL-2 containing medium. Thereafter, Foxp3 protein-expression was analyzed by flow cytometry on day 8, and on day 6 (i.e. d14) after re-culture. Each symbol represents an individual donor. Bar chart represents the median value. **(b)** The kinetic of the Foxp3 protein-expression is depicted as trend of the median values \pm SEM from six independent experiments * $p < 0.01$

3.3.5. pVC induces an enhanced GATA-3 expression in TGF- β -expanded $\gamma\delta$ T cells

Previous work showed that activated mouse and human $\alpha\beta$ Tregs express GATA-3, which is required to suppress T helper type 2 (Th2) responses [220,221]. Moreover, previous work from our group reported that suppressive V δ 2 $\gamma\delta$ T cells (initially expanded with TGF- β) showed an increased expression of GATA-3, which can be correlated with their suppressive activity [99]. Therefore, we hypothesized that elevated Th2 responses (i.e. GATA-3 expression) might correlate with the suppressive activity of TGF- β -expanded $\gamma\delta$ T cells. To test, magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E beads in IL-2-containing medium with TGF- β in the presence or absence of pVC. On d8, flow cytometric analyses revealed that $9.7 \pm 9\%$ $\gamma\delta$ [BrHPP + TGF- β] and $12.3 \pm 3.9\%$ $\gamma\delta$ [A/E + TGF- β] T cells expressed GATA-3-protein. Interestingly, GATA-3 protein-expression was significantly enhanced among pVC-treated TGF- β -expanded $\gamma\delta$ T cells (Fig. 20).

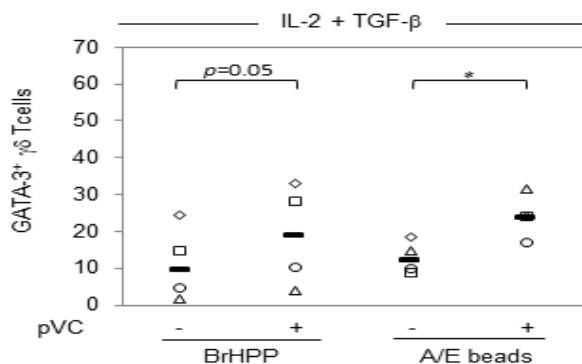


Figure 20. pVC induces an enhanced expression of GATA-3 in TGF- β -expanded $\gamma\delta$ T cells.

Magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E-beads in complete RPMI medium supplemented with IL-2 + TGF- β in the presence (or not) of pVC. On d8, cells were harvested, labeled with mAb specific for GATA-3 (clone L50-823) and analyzed by flow cytometry. Graph shows the percentage of GATA-3⁺ $\gamma\delta$ T cells. Each symbol represents individual donor. Horizontal bars indicate the median value, * $p < 0.05$

3.3.6. pVC promotes the surface-expression of markers associated with regulatory T cells

Based on the findings that pVC augments the Foxp3 protein-expression as well as the suppressive activity of $\gamma\delta$ Tregs, we analyzed the expression of cell surface molecules associated with regulatory function (i.e. CTLA-4, TIGIT (T cell Immunoglobulin and Immunoreceptor tyrosine-based inhibitory motif domain) and CD39/CD73). Our data showed that, on d8, CTLA-4 was only slightly expressed on stimulated human $\gamma\delta$ T cells and the expression was not significantly influenced by the presence of pVC (data not shown). TIGIT was highly expressed on the surface of A/E bead- and BrHPP-stimulated $\gamma\delta$ T cells expanded in presence of IL-2 as well as in the presence of IL-2 + TGF- β , as determined by flow cytometry 8 days after initial stimulation. Notably, this expression was higher on IL-2-expanded cells than on TGF- β -expanded cells. The addition of pVC further augmented the TIGIT surface-expression under all culture conditions (Fig. 21a).

Moreover, we found that, independently of cytokine milieu and influence of pVC, CD39 is upregulated upon activation of $\gamma\delta$ T cells (Fig. 21b). We observed that CD73, present on magnetically isolated $\gamma\delta$ T cells, drastically decreased upon TCR-stimulation, whereby CD73 remained on the surface of pVC-treated $\gamma\delta$ T cells, which was most striking on $\gamma\delta$ [A/E + pVC] T cells (Fig. 21b). In all cases, the remaining CD73 surface-expression was only present on CD39⁺ cells. These findings indicate that, depending on the initial stimulation and the cytokine milieu, pVC can counteract the activation-induced down-modulation of CD73.

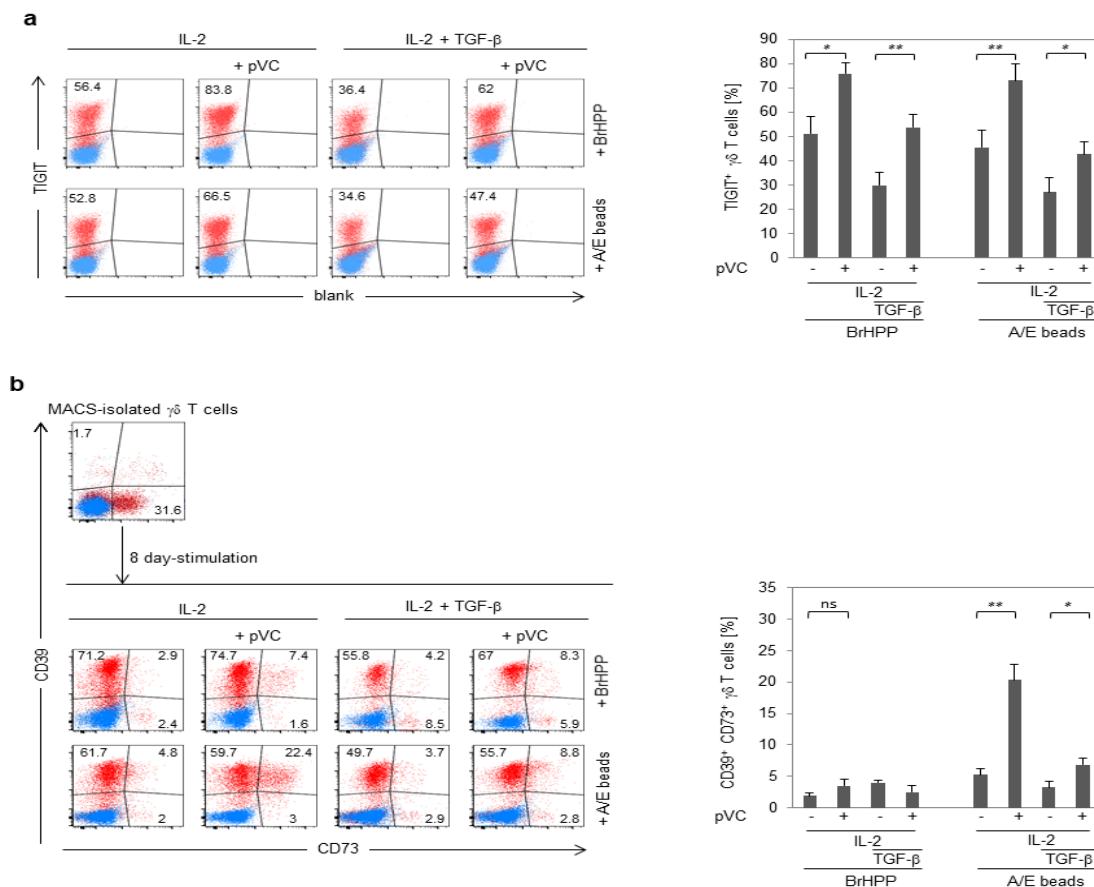


Figure 21. Vitamin C upregulates the expression of TIGIT and CD39/CD73.

Magnetically isolated $\gamma\delta$ T cells were stimulated with BrHPP or A/E beads in complete RPMI medium supplemented with IL-2 +/- TGF- β in the presence (or not) of pVC. At d8, cells were harvested and stained with the mAbs (red color) specific for **(a)** TIGIT (clone MBSA43) and **(b)** CD39 (clone A1) and CD73 (clone AD2) and their respective isotypes control (blue color); The fluorescence intensity of the indicated cell surface molecules was analyzed by flow cytometry. Left panel: Dot plots of one representative experiment are shown. Numbers indicate the percentage of positive cells. Right panel: Bar graphs showing the percentage (mean \pm SEM) of (upper) TIGIT⁺ cells and (lower) cells co-expressing CD39 and CD73 from five independent experiments. * p < 0.05, ** p < 0.01, ns not significant

3.4. Influence of pVC on the transcriptome and epigenetic marks of expanded human $\gamma\delta$ T cells

Epigenetic mechanisms play a key role in cell differentiation by controlling gene-expression programs that are stable over time and through cellular generations. Previous studies revealed that the effect of Vitamin C on the differentiation of immune cells is connected to transcriptional and epigenetic re-programming. In order to decipher how pVC modulates the cellular differentiation process in expanded $\gamma\delta$ T cells, we performed analyses of transcriptomic and epigenomic datasets.

3.4.1. RNA sequencing-based gene expression profile of expanded V δ 2 T cells

3.4.1.1. Influence of pVC on the transcriptome of IL-2-expanded V δ 2 T cells

To explore whether pVC induces a unique transcriptional programs in human $\gamma\delta$ T cells, we analyzed the gene-expression by RNA sequencing in highly purified V δ 2 T cells (magnetically isolated with 98% purity). We cultured purified V δ 2 T cells from two donors with BrHPP in IL-2-containing medium in the presence or absence of pVC. On day 8, cells were harvested, and total RNA was extracted and subjected to RNA sequencing performed at the Institute of Clinical Molecular Biology (University of Kiel, Germany) in cooperation with Dr Robert Häsler. Bioinformatic analyses performed by Dr. Daniela Esser (Institute of Experimental Medicine, UKSH, University of Kiel) identified up to 10,000 transcripts among all culture conditions. Gene network analysis (Fig. 22a) was performed focusing on the top 250 differentially expressed genes and a heatmap of top 100 differentially regulated genes ($p < 0.05$) was generated (Fig. 22b). In the network analysis, genes are clustered according to their connections in the String database (which can be direct physical interactions or more indirect modulations). The topology of the network showed 4 small clusters that were separated among themselves and from a major cluster. From the major cluster which contained a consistent number of genes, Cyclin Dependent Kinase Inhibitor 1 (*CDKN1A*) and Mitogen-Activated Protein Kinase 8 (*MAPK8*; JNK) were centrally positioned and linked to many other genes, pointing to a central role. Importantly, we observed that, while *CDKN1A* expression and TNF Receptor Superfamily Member 10b (*TNFRSF10B*; TRAIL-R2) along with Dual-Specificity Phosphatase 1 (*DUSP1*) were downmodulated, in contrast, *MAPK8* was significantly upregulated by pVC (Fig. 22a, b). pVC also upregulated TIMP Metalloproteinase Inhibitor 1 (*TIMP1*) (Fig. 22b). Gene ontology analysis based on the pathways annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, showed that, among genes whose expression was significantly affected by pVC, *CDKN1A* and *MAPK8* were found to be present in several pathways (Table 1). Altogether, these results suggest that *MAPK8* may be an important factor for pVC-mediated $\gamma\delta$ T-cell differentiation.

Finally, to test the assumption that genes whose expression was induced at the protein level are also modulated at the transcriptional level, we extracted a list of genes whose protein-expression was significantly increased upon pVC treatment or might be involve in pVC-enhanced $\gamma\delta$ T-cell differentiation (genes of interest). To this end, we compared the transcriptome of pVC-treated-expanded V δ 2 T cells versus the untreated group. Notably, genes which had already been verified on the level of protein-expression were found to have an increased expression upon pVC treatment (Fig. 22c). These genes include *ENTPD1* (CD39 encoding gene), *TIGIT*, *GATA3* and *IL13*. *TBX21* (encoding T-bet) was expressed but

Figure 22. pVC modulates the transcriptome of IL-2-expanded V δ 2 T cells.

Magnetically isolated V δ 2 T cells from two male donors (#1, #2) were stimulated with BrHPP in complete RPMI medium supplemented with IL-2 in the presence (or not) of pVC. On day 8, total RNA was isolated and subjected to RNA-seq analysis. **(a)** Co-expression network based on the top 250 most variable genes. Yellow and blue nodes represent upregulated and downregulated genes, respectively. Connections were based on the annotation of the String database [198] with medium reliance. **(b)** Heatmap showing z-scores of expression of the top 100 most differentially regulated genes, clustered hierarchically. **(c)** Heatmap showing z-scores of expression of genes of interest. Genes are shown as upregulated or downregulated according to the color key.

Table 1. List of the genes affected by pVC treatment in IL-2-expanded $\gamma\delta$ T cells (KEGG database)

Pathway	Affected genes (N°)	Genes in the pathway (N°)	p-value	adj. p-value	Gene names
Cytokine-cytokine receptor interaction	14	258	2.99451E-06	0.000122775	<i>CCR3; CCR8; CSF2RB; CXCL9; CXCR7; EDA2R; IL9R; TNFRSF10B; TNFSF11; TNFSF13; TNFSF14; TNFSF4; TNFSF8; TNFSF9</i>
Chemokine signaling pathway	7	181	0.006758214	0.130519605	<i>CCR3; CCR8; CXCL9; GNG8; PXN; RASGRP2; TIAM1</i>
p53 signaling pathway	4	68	0.009550215	0.130519605	<i>CDKN1A; MDM2; TNFRSF10B; ZMAT3</i>
Adherens junction	3	73	0.0601442	0.450514709	<i>ACTN3; CSNK2A2; FER</i>
Alanine, aspartate and glutamate metabolism	2	35	0.06816649	0.450514709	<i>GAD1; GFPT2</i>
Bladder cancer	2	38	0.07867718	0.450514709	<i>CDKN1A; MDM2</i>
Type I diabetes mellitus	2	41	0.08965581	0.450514709	<i>GAD1; ICA1</i>
Ubiquitin mediated proteolysis	4	140	0.09287819	0.450514709	<i>ERCC8; MDM2; UBE2J1; UBE2O</i>
Amino sugar and nucleotide sugar metabolism	2	47	0.112831	0.450514709	<i>GFPT2; MPI</i>
Type II diabetes mellitus	2	49	0.1208698	0.450514709	<i>IRS2; MAPK8</i>
Focal adhesion	5	210	0.1153662	0.450514709	<i>ACTN3; MAPK8; MYLK; PXN; SPP1</i>
Toll-like receptor signaling pathway	3	103	0.1323032	0.452035933	<i>CXCL9; MAPK8; SPP1</i>
Leukocyte transendothelial migration	3	120	0.1820908	0.476830473	<i>ACTN3; NCF4; PXN</i>
Adipocytokine signaling pathway	2	70	0.2115611	0.476830473	<i>IRS2; MAPK8</i>
RIG-I-like receptor signaling pathway	2	70	0.2115611	0.476830473	<i>MAPK8; TMEM173</i>

RESULTS

Osteoclast differentiation	3	133	0.2230415	0.476830473	MAPK8 ; NCF4; TNFSF11
Chronic myeloid leukemia	2	74	0.22962	0.476830473	CDKN1A ; MDM2
Glioma	2	66	0.1936596	0.476830473	CDKN1A ; MDM2
Melanoma	2	72	0.2205745	0.476830473	CDKN1A ; MDM2
Bacterial invasion of epithelial cells	2	77	0.2432335	0.476830473	ARHGAP10; PXN
Neuroactive ligand-receptor interaction	5	275	0.2477218	0.476830473	GRIN3B; P2RY8; PTGER4; S1PR1; S1PR4
Regulation of actin cytoskeleton	4	217	0.2755174	0.476830473	ACTN3; MYLK; PXN; TIAM1
ErbB signaling pathway	2	89	0.2978421	0.476830473	CDKN1A ; MAPK8
Apoptosis	2	88	0.2932998	0.476830473	CSF2RB; TNFRSF10B
Cell adhesion molecules (CAMs)	3	144	0.258989	0.476830473	L1CAM; SELL; TIGIT
Prostate cancer	2	90	0.3023803	0.476830473	CDKN1A ; MDM2
Protein processing in endoplasmic reticulum	3	171	0.3494591	0.516279352	CKAP4; MAPK8 ; UBE2J1
Chagas disease (American trypanosomiasis)	2	103	0.3607484	0.516279352	GNAO1; MAPK8
Pyrimidine metabolism	2	104	0.3651732	0.516279352	CMPK2; NME7
MAPK signaling pathway	4	257	0.3867455	0.528552183	DUSP1; DUSP6; MAPK8 ; RASGRP2
Toxoplasmosis	2	119	0.4299759	0.563365324	GNAO1; MAPK8
Cell cycle	2	126	0.4590343	0.563365324	CDKN1A ; MDM2
Systemic lupus erythematosus	2	126	0.4590343	0.563365324	ACTN3; HIST1H2BK;
Axon guidance	2	128	0.467181	0.563365324	L1CAM; PLXNB2;
Hepatitis C	2	136	0.4990347	0.567421334	CDKN1A ; MAPK8
Tight junction	2	135	0.4951188	0.567421334	ACTN3; CSNK2A2
Wnt signaling pathway	2	140	0.5145054	0.567421334	CSNK2A2; MAPK8
Insulin signaling pathway	2	143	0.5259027	0.567421334	IRS2; MAPK8
Jak-STAT signaling pathway	2	158	0.5801634	0.594667485	CSF2RB; IL9R
Pathways in cancer	4	329	0.577325	0.594667485	CDKN1A ; GLI1; MAPK8 ; MDM2;
Purine metabolism	2	174	0.6329271	0.6329271	NME7; PDE6G

3.4.1.2. Influence of pVC on the transcriptome of TGF- β -expanded V δ 2 T cells

We used the same approach as in the previous section to identify gene(s) that were up-regulated upon pVC-treatment in TGF- β -expanded V δ 2 T cells. Gene network analysis of the top 250 significantly modulated genes, showed the upregulation of the genes such as the Integrin Subunit Alpha E (*ITGAE*) and Histone deacetylase inhibitor 9 (*HDAC9*) (Fig. 23a). The heatmap generated from the top 100 most differentially expressed genes showed that 62 genes were upregulated, while 38 were downmodulated in the presence of pVC. In line with observation from the gene network, the data further indicated that *ITGAE* and *HDAC9* upregulation was increased in the presence of pVC (Fig. 23b). The KEGG pathway analysis did not reveal any significant modulated pathways in TGF- β expanded $\gamma\delta$ T cells upon pVC-treatment (Table 2).

To analyze the expression of important Th lineage associated genes, including genes typically modulated in Tregs, we generated a heatmap from genes of interest. Particularly, in line with flow cytometric data on Foxp3 protein-expression, *FOXP3* upregulation was increased in the presence of pVC (Fig. 23c). pVC also upregulated the expression of genes such as *ENTPD1* and *TIGIT*.

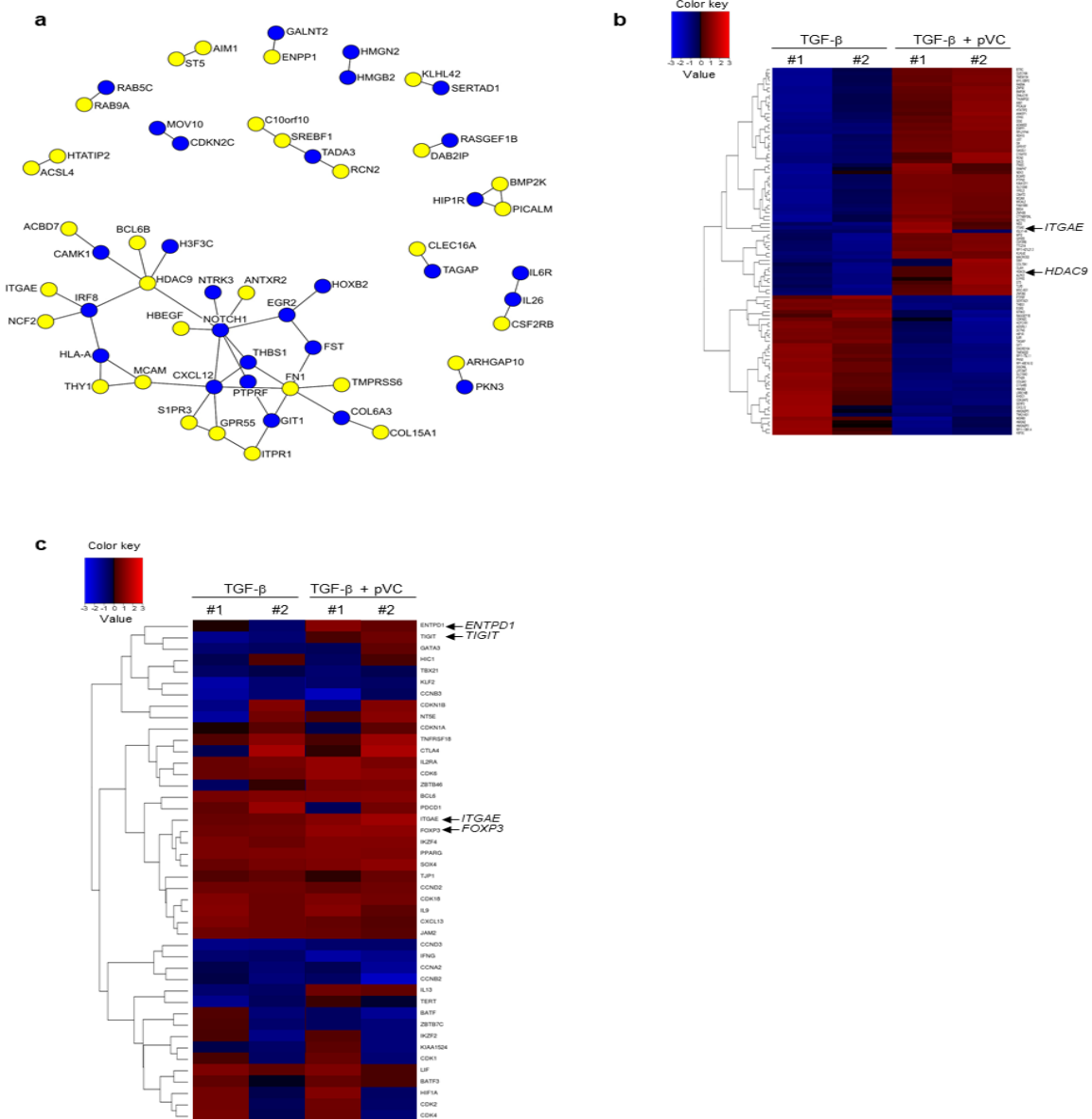


Figure 23. pVC modulates the transcriptome of TGF-β-expanded Vδ2 T cells.

Magnetically isolated Vδ2 T cells from two male donors (#1, #2) were stimulated with BrHPP in complete RPMI medium supplemented with IL-2 + TGF-β in the presence (or not) of pVC. On day 8, total RNA was isolated and subjected to RNA-seq analysis. **(a)** Co-expression network based on the top 250 most regulated genes. Yellow and blue nodes represent upregulated and downregulated genes, respectively. Connections were based on the annotation of the String database [198] with medium reliance. **(b)** Heatmap showing z-scores of expression of the top 100 most differentially regulated genes, clustered hierarchically. **(c)** Heatmap showing z-scores of expression of handpicked genes. Genes are shown as upregulated or downregulated according to the color key. Data of two independent experiments are shown.

Table 2. List of the genes affected by pVC treatment in TGF- β -expanded CD4⁺ T cells (KEGG database)

Pathway	Affected genes (N°)	Genes in the pathway (N°)	p-value	adj. p-value	Gene names
Fatty acid biosynthesis	3	12	0.000147737	0.004875328	<i>ACSBG1; ACSL4; OLAH;</i>
Glycosaminoglycan biosynthesis	2	21	0.01515704	0.125045581	<i>DSE; UST;</i>
Pantothenate and CoA biosynthesis	2	17	0.010047886	0.110526742	<i>ENPP1; PANK3;</i>
Apoptosis	3	88	0.045137977	0.177951603	<i>BIRC3; CSF2RB; IL1RAP;</i>
ECM-receptor interaction	3	88	0.045137977	0.177951603	<i>COL6A3; FN1; THBS1;</i>
Cytokine-cytokine receptor interaction	6	258	0.029010766	0.159559211	<i>CSF2RB; CXCL12; IL1RAP; IL26; IL6R; TNFSF8;</i>
PPAR signaling pathway	3	69	0.024326224	0.159559211	<i>ACSBG1; ACSL4; GK;</i>
Phagosome	4	152	0.048532255	0.177951603	<i>HLA-A; NCF2; RAB5C; THBS1;</i>
Cell adhesion molecules (CAMs)	5	144	0.009663373	0.110526742	<i>CDH3; HLA-A; ICOSLG; PTPRF; TIGIT;</i>
Fatty acid degradation	2	44	0.059776131	0.193963845	<i>ACSBG1; ACSL4;</i>
Intestinal immune network for IgA production	2	46	0.064654615	0.193963845	<i>CXCL12; ICOSLG;</i>
Glycerolipid metabolism	2	55	0.088120623	0.23968152	<i>AGPAT4; GK;</i>
Leukocyte trans-endothelial migration	3	120	0.094419993	0.23968152	<i>CXCL12; NCF2; THY1;</i>
Retinol metabolism	2	66	0.11959595	0.243542155	<i>CYP1A2; RDH10;</i>
Adipocytokine signaling pathway	2	70	0.131650264	0.243542155	<i>ACSBG1; ACSL4;</i>
Regulation of actin cytoskeleton	4	217	0.132841176	0.243542155	<i>FGF22; FN1; GIT1; ITGAE;</i>
Epithelial cell signaling in <i>Helicobacter pylori</i> infection	2	67	0.122582635	0.243542155	<i>GIT1; HBEGF;</i>
Focal adhesion	4	210	0.121854741	0.243542155	<i>BIRC3; COL6A3; FN1; THBS1;</i>
Small cell lung cancer	2	88	0.188721734	0.274404028	<i>BIRC3; FN1;</i>
Protein digestion and absorption	2	89	0.191993107	0.274404028	<i>COL15A1; COL6A3;</i>
Bacterial invasion of epithelial cells	2	77	0.153365851	0.266372267	<i>ARHGAP10; FN1;</i>
TGF-beta signaling pathway	2	80	0.162878102	0.268748868	<i>FST; THBS1;</i>
GnRH signaling pathway	2	92	0.201851827	0.274404028	<i>HBEGF; ITPR1;</i>
Purine metabolism	3	174	0.207881839	0.274404028	<i>AK3; ENPP1; PDE4A;</i>

Jak-STAT signaling pathway	3	158	0.17133696	0.269243794	<i>CSF2RB; IL26; IL6R;</i>
Lysosome	2	123	0.305495412	0.34763271	<i>GALC; PPT2;</i>
Endocytosis	3	214	0.304957882	0.34763271	<i>GIT1; HLA-A; RAB5C;</i>
Amoebiasis	2	109	0.258542408	0.32814998	<i>FN1; RAB5C;</i>
Oocyte meiosis	2	114	0.275330953	0.33651561	<i>BTRC; ITPR1;</i>
Ubiquitin mediated proteolysis	2	140	0.36175298	0.395495544	<i>BIRC3; BTRC;</i>
Insulin signaling pathway	2	143	0.371526117	0.395495544	<i>PTPRF; SREBF1;</i>
Pathways in cancer	3	329	0.575373945	0.593354381	<i>BIRC3; FGF22; FN1;</i>
Neuroactive ligand-receptor interaction	2	275	0.715318129	0.715318129	<i>PTH2R; S1PR3;</i>

3.4.2. Genome-wide DNA methylation analysis of human $\gamma\delta$ T cells expanded in the presence (or not) of pVC

Previous studies reported that Vitamin C-mediated T-cell differentiation and function are correlated to DNA and chromatin structural changes [186,189,190].

To assess the impact of pVC on the gene (de)methylation status of differentially expanded V δ 2 T cells purified from the same donors used for the RNA sequencing analysis, we determined genome-wide DNA-methylation profiles by Reduced Representation Bisulfite Sequencing (RRBS). To this end, genomic DNA was isolated from 8-day untreated and pVC-treated expanded V δ 2 T cells expanded with BrHPP in IL-2-containing medium in the presence or absence of TGF- β . Bioinformatic analyses were performed by Qiwei Sun (BGI Genomics Institute, Shenzhen, China). The isolated DNA was digested by restriction enzyme MspI to produce CpG-rich fragments. These fragments were ligated to adapters, recovered, subjected to bisulfite conversion and sequencing (see RRBS experimental process in 2.2.7.3.1). After sequencing, changes in methylation, at single-nucleotide resolution, were quantified and used to determine the differential methylation patterns in untreated versus pVC-treated expanded V δ 2 T cells.

Target Bisulfite Sequencing (TBS) was performed on 2 biological replicates/per culture condition of untreated versus pVC-treated expanded V δ 2 T cells. From sequencing, the unique mapping rate to the reference genome hg19 was > 95%. After filtering, clean reads were mapped to the reference using BSMAP [222]. Furthermore, the bisulfite conversion rate was ~ 99%, indicating that the false-negative (discovery) rate was less than 1%.

3.4.3. Global DNA methylation pattern in pVC-treated versus untreated expanded V δ 2 T cells

To understand the impact of pVC on DNA methylation events, we first profiled global DNA methylation changes of expanded V δ 2 T cells left untreated or treated with pVC. A circos plot depicting methylation levels across all chromosomes in the differentially expanded $\gamma\delta$ T cells is presented in Fig. 24a. Results showed that pVC modestly modulated CpG methylation patterns at multiple sites across chromosomes. This is exemplified for chromosome 1 with focus on promoter and CpG islands (CGI) (Fig. 24b). Next, we quantified the average methylation level of methylated cytosine at specific genomic sites such as promoters and CGI. Results depicted in Fig. 24c showed that there were no global changes for the average CpG methylation in any of these regions, although a discrete reduced methylation was observed in pVC-treated V δ 2 T cells (i.e. pVC and TGF- β + pVC). We also analyzed the average global changes to CHG and CHH (where H is A, C or T) methylation contexts across the promoter and CGI. For both CHG and CHH contexts, we observed a slight but not significant decrease in average global methylation at promoters and CGI in pVC-treated V δ 2 T cells when compared to the untreated group (Fig. 24d, e). Notably, this pVC-mediated decreased methylation was more pronounced in medium (with IL-2)-expanded V δ 2 T cells than in TGF- β -expanded cells. Furthermore, considering only cytokine condition, and not pVC treatment, TGF- β reduced the CHG and CHH methylation levels (Fig. 24d, e).

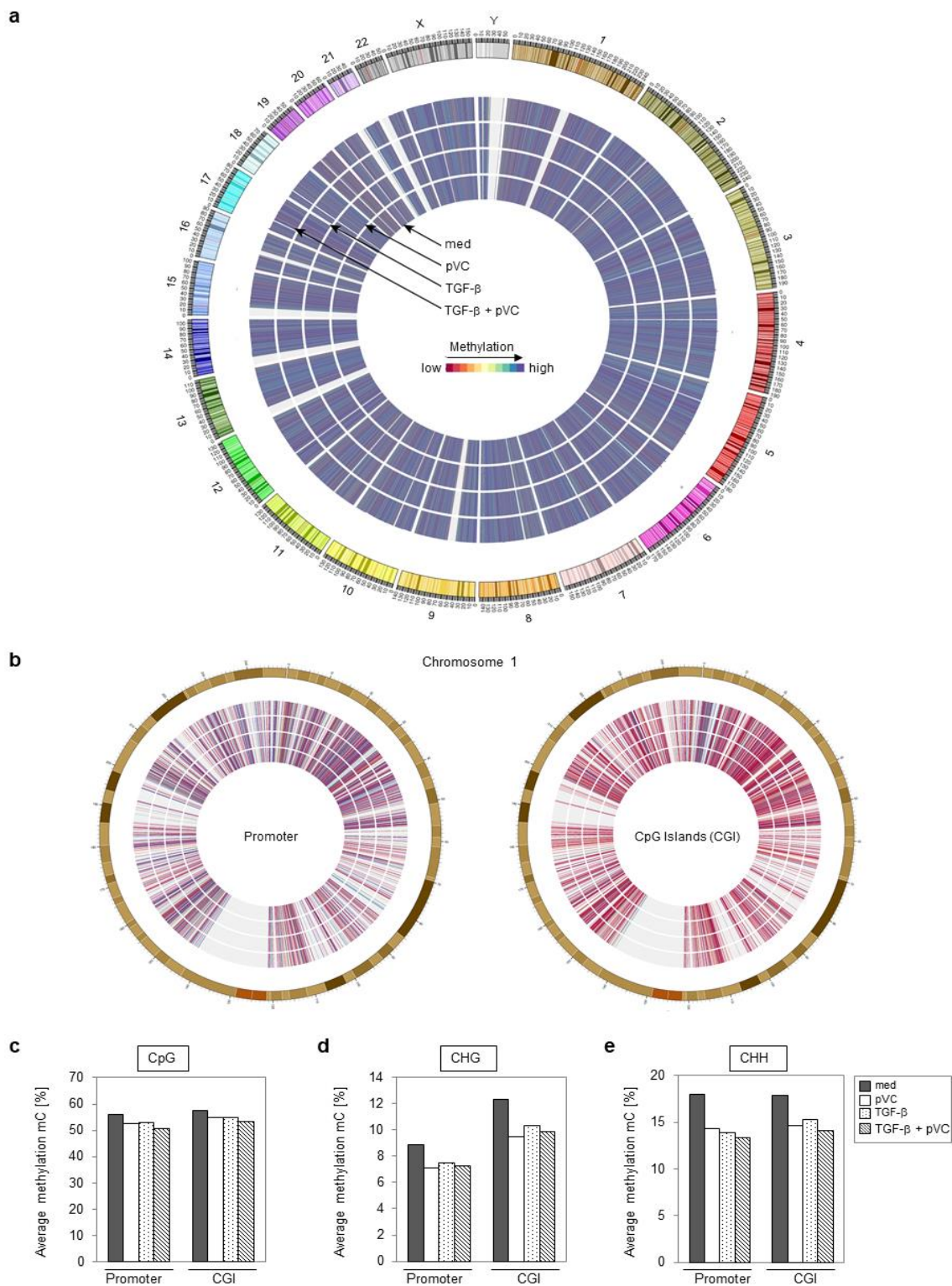


Figure 24. Modulation of DNA methylation by pVC.

MACS-sorted V δ 2 T cells were stimulated with BrHPP and differentiated as indicated in the figure (i.e. med, pVC, TGF- β and TGF- β + pVC) for 8 days. Thereafter, genomic DNA was isolated and subjected to RRBS to determine the methylation level. **(a)** Circos plot of RRBS data depicted as heatmap representing the CpG methylation level across all chromosomes. Methylation levels are depicted as color-coded bars (red-pink). **(b)** Exemplary higher magnification for chromosome 1 showing average CpG methylation pattern at the promoter and CpG Islands (CGI). Data are pooled from two independent experiments. **(c-e)** Bar diagram showing the average methylation levels in (c) CpG, (d) CHG and (e) CHH contexts across the promoter and the CGI; n = 2

3.4.4. pVC treatment induces DNA hypomethylation within *FOXP3* locus

VC is known to activate Tet enzymes and thus to influence the epigenome. In view of the stabilizing effect of pVC on Foxp3 protein-expression (Fig. 19), we analyzed DNA methylation at the *FOXP3* gene locus. Zooming into the *FOXP3* locus based on RRBS data, we observed less methylation in $\gamma\delta$ T cells exposed to pVC, both in cultures exposed to TGF- β or not (Fig. 25a). We also analyzed whether the effects of pVC observed on *FOXP3* methylation could also be extended to other Treg signature genes such as *TNFRSF18* (GITR) and *IKZF2* (HELIOS). However, similar methylation patterns were detected in all culture conditions, which remained unchanged in the presence of pVC (Fig 25b, c).

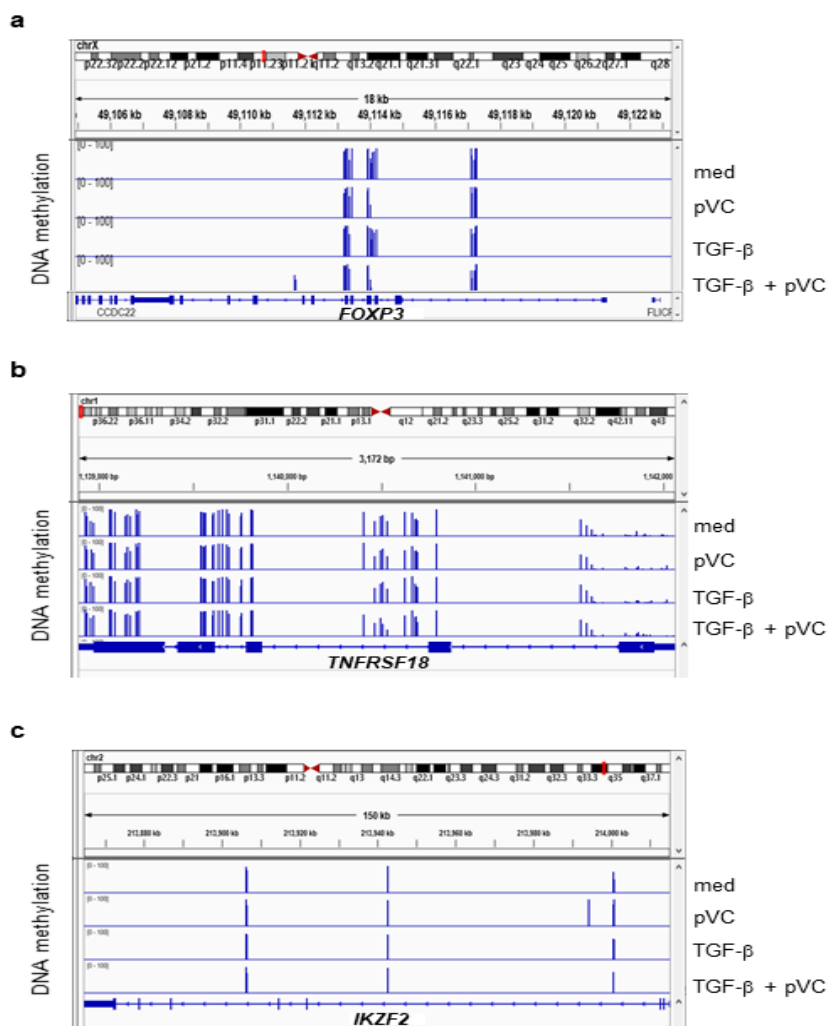


Figure 25. Influence of pVC on *FOXP3*, *TNFRSF18* and *IKZF2* CpG demethylation in expanded V δ 2 T cells.

MACS-sorted V δ 2 T cells were stimulated with BrHPP and differentiated for 8 days as indicated in the figure. Thereafter, genomic DNA was isolated and subjected to RRBS to determine the methylation level in the CpG context. **(a-c)** Exemplary genomic view plots, drawn from two pooled independent experiments, indicate the CpG methylation patterns of (a) *FOXP3*, (b) *TNFRSF18* and (c) *IKZF2* loci

Next, we specifically quantified DNA methylation within *FOXP3* CNS2. First, we expanded V δ 2 T cells with BrHPP or A/E beads in the presence of IL-2 plus TGF- β , and the additional presence (where indicated) of pVC. After 8 days of culture, expanded V δ 2 T cells were harvested, stained with fluorochrome-labeled mAb against Foxp3 (clone 259D/C7) and sorted into Foxp3⁺ and Foxp3⁻ cell fractions. Cell sorting was performed on FACSaria II (BD Biosciences). Subsequently, genomic DNA was isolated from both fractions and DNA methylation analysis of the CNS2 was performed using pyrosequencing. The analyses were performed by Dr. Stefan Floess (Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany). Among the 14 CpG motifs of the *FOXP3* CNS2, seven motifs were considered for these analyses. As shown in the heatmap in Fig. 26a, Foxp3⁺

4. DISCUSSION

4.1. Modulation of human $\gamma\delta$ T-cell activation and effector functions by pVC

4.1.1. pVC enhances the proliferation of human $\gamma\delta$ T cells upon activation

Several studies have demonstrated that VC, in addition to its role in cancer treatment [209,223,224], promotes mammalian cell differentiation and DNA synthesis [183,203,206]. However, the impact of VC on the differentiation and effector function of human $\gamma\delta$ T cells has not been addressed to date. Results from human hematopoietic stem cell differentiation as well as human corneal endothelial cell culture studies have demonstrated that L-ascorbic acid, if present in the less stable non-phosphorylated form (VC), has lower potency to induce cell proliferation than the phospho-modified derivative L-ascorbic acid 2-phosphate (pVC), which is also more stable in cell culture at 37°C [225].

In the first part of this study, we used both forms of VC and analyzed their effects on $\gamma\delta$ T-cell proliferation during primary activation of PBMC or of purified $\gamma\delta$ T cells with $\gamma\delta$ T-cell selective stimuli (the pAgs HMBPP and BrHPP, and the aminobisphosphonate ZOL), as well as during re-stimulation of ZOL-expanded $V\gamma 9V\delta 2$ T cells with BrHPP. We observed that at concentrations above 57 μ M, VC actually inhibited the proliferation of $\gamma\delta$ T cells when PBMC were stimulated with HMBPP or ZOL. In contrast, pVC showed no toxicity even at the highest tested concentration of 692 μ M (Fig. 3). Although previous studies reported a cytotoxic effect of VC [153,226], careful analyses in the literature revealed that this VC-mediated cytotoxicity was only observed when the cell culture was performed with low cell numbers and/or high (millimolar) concentrations of VC [226,227], and not if confluent cells as demonstrated using human fibroblasts [226,228,229]. From our setting, we could confirm that concentration of VC above 57 μ M inhibited the proliferation of $\gamma\delta$ T cells here activated at low density (1×10^5 PBMC containing 2-4% $\gamma\delta$ T cells). Mechanistically, VC is quickly oxidized in culture medium into dehydroascorbate (DHA), generating toxic levels of ascorbyl radical and ROS products (mainly H_2O_2), which inhibit cell growth [230]. Duarte et al. demonstrated that the genotoxic effect of VC occurred via the formation of extracellular H_2O_2 and the presence of intracellular labile ferrous iron, but not extracellular transition metal ions [226]. Conversely, pVC is resistant to auto-oxidation in culture medium and releases VC once it is inside the cells via hydrolysis mediated by alkaline phosphatase present on the plasma membrane [225]. In line, our results provided evidence that pVC (i.e. L-ascorbic acid 2-phosphate) was not toxic and therefore, was used in the subsequent investigations.

In addition to the studies on the modulation of $\gamma\delta$ T-cell proliferation during primary *in vitro* activation (Fig. 4), we also investigated the effect of pVC in a different setting where ZOL-expanded $V\gamma9V\delta2$ T cells were re-stimulated with BrHPP in the absence of presence of pVC. It is well known that in the presence of IL-2, preactivated $\gamma\delta$ T cells rapidly undergo activation-induced cell death (AICD) upon TCR/CD3 engagement [192,231]. In line with previous studies, we observed that BrHPP-re-stimulation induced nearly 80% apoptosis in $\gamma\delta$ T cells that rapidly progressed to loss of membrane integrity and cell death, as evidenced by Annexin V/PI co-staining. Interestingly, we observed that the addition of pVC drastically increased the number of proliferating $\gamma\delta$ T cells within BrHPP-re-stimulated $\gamma\delta$ T-cell cultures (Fig. 5b). Surprisingly, however, pVC treatment did not prevent or reduce the BrHPP-induced apoptosis of $\gamma\delta$ T-cell cultures (Fig. 7a, b), in contrast to previous studies in other cell systems. As an example, pVC in combination with the antioxidant N-Acetylcysteine (NAC) has been reported to exert a synergistic protection of human mesenchymal stem cells against different forms of cell death [206]. Similarly, VC has been found to enhance the *in vitro* proliferation of mouse $\alpha\beta$ T cells by acting as a potent inhibitor of various forms of cell death [172]. In the current thesis, I observed that the AICD of ZOL-expanded $V\gamma9V\delta2$ T cells upon BrHPP-re-stimulation was not prevented by pVC (Fig. 7a, b), although pVC exerted antioxidant activity as it reduced the spontaneous and BrHPP-induced ROS production (Fig. 14). Rather than inhibiting cell death, we found that pVC enhanced the expansion of the BrHPP-restimulated ZOL-expanded $V\gamma9V\delta2$ T cells by stimulating their cell cycle progression. The cell cycle comprises the four stages (G_1), (S), (G_2), and (M) [232]. G_1/S and G_2/M phases have been considered as specific checkpoints that monitor the entire cell division [233]. We observed a significant enrichment of the BrHPP-re-stimulated ZOL-expanded $V\gamma9V\delta2$ T cells in the G_2/M phase in the presence of pVC (Fig. 8a, b). In line, $\gamma\delta$ T cells treated with pVC also showed an increased expression of Ki-67 (Fig. 8c, d) which is a marker of cycling cells (S/ G_2/M) [207]. Taken together, these data indicate that pVC induces the expansion of re-stimulated ZOL-expanded $V\gamma9V\delta2$ T cells not by inhibiting AICD but rather by promoting cell cycle progression. However, it cannot be excluded that other factors like the reduced ROS production upon BrHPP re-stimulation in the presence of pVC could also play some role. ROS are chemically reactive free radicals with one unpaired electron in their outer orbit. Although ROS were long considered as harmful metabolic by-products that cause cellular damage, it has been appreciated more recently that ROS are crucial for T-cell functions [234]. While excessive production of ROS is known to accelerate cell death, low to moderate amounts of ROS are emerging as positive contributors to normal TCR signaling pathways involved in T-cell activation and growth. For example, TCR-induced discrete generation of ROS modulates the phosphorylation of the extracellular signal-regulated

kinases (Erk)1/2 [235,236]. In addition, ROS play a role upstream of the activation of I κ B kinase complex (IKK) which frees NF- κ B from its inhibitor (I κ B) and thus its activation [237].

Further experiments were also conducted to determine the optimal time point for adding pVC to the BrHPP-re-stimulated ZOL-expanded V γ 9V δ 2 T cells (Fig. 6). These results clearly showed that pVC had to be present at the initiation of the re-stimulation with BrHPP to exert its growth-promoting effect, which may suggest, together with the pVC-mediated ROS buffering, a possible interaction with molecules implicated in the TCR signaling, thereby modulating the $\gamma\delta$ T-cell proliferation.

4.1.2. pVC sustains the polarization of activated $\gamma\delta$ T cells towards Th1+Th2-like phenotype

Depending on the polarizing cytokine signals during activation by antigen, naive CD4 T cells can initiate various differentiation programs resulting in the commitment into Th cell lineages with distinct functions [238]. The developmental programs are each characterized by the enhanced expression of a key transcription factor that acts as a lineage-specifying “master regulator” coordinating the accessibility and expression of defined subsets of cytokine genes and other genes. For instance, Th1 cell differentiation is governed by the transcription factor T-bet [239] and is induced by IFN- γ - and IL-12-signaling via the Signal Transducer and Activator of Transcription (STAT)1 and 4, respectively [240,241], whereas the transcription factor GATA-3 directs the Th2 cell differentiation driven by IL-4-signalling via STAT6 [242-244]. Th1 are characterized by the production of IFN- γ [245] while Th2 are characterized by the secretion of IL-4, IL-13, and IL-5 [244,246]. Like $\alpha\beta$ T cells, murine as well as human $\gamma\delta$ T cells, depending on the cytokine priming condition can be polarized towards Th1 or Th2-like phenotype. Murine $\gamma\delta$ T cells can produce IFN- γ and IL-4 *in vivo* in response to Th1 or Th2 stimulating pathogens [247]. Peripheral human $\gamma\delta$ T cells promote a Th1 immune response after pAg activation by producing large amounts of IFN- γ and TNF- α [248,249]. Moreover, resting V γ 9V δ 2 T cells upon activation and priming under Th1 conditions induced a Th1 profile characterized by increased secretion of IFN- γ and TNF- α , while under Th2 conditions, activated V γ 9V δ 2 T cells secreted IL-4 (Th2 profile) [84]. Examining the effect of pVC on the functional differentiation of human $\gamma\delta$ T cells, we observed that $\gamma\delta$ T cells isolated from the human peripheral blood and stimulated with BrHPP or A/E beads in the presence of IL-2 (non-polarizing condition) can be differentiated into Th1+2-like phenotype characterized by the intracellular co-expression of T-bet and GATA-3 (Fig. 9a, b). Interestingly, this Th1+Th2 bias was more sustained in the presence of pVC (i.e. increased number of T-bet⁺GATA-3⁺ $\gamma\delta$ T cells). We also observed the production of cytokines such as IFN- γ , IL-13 and to a lesser

extent IL-5 by the IL-2-expanded $\gamma\delta$ T cells. Strikingly, in the presence of pVC, the production of IFN- γ and IL-13 was further increased significantly (Fig. 10).

Given the distinct expression patterns of key transcription factors and cytokines in the Th cell lineages differentiation and reciprocal inhibition, existing models for Th1 and Th2 differentiations have postulated a mutually exclusive expression of the key transcription factors T-bet and GATA-3 [250,251]. However, the plasticity of Th cell lineages and their functional relevance have been reevaluated based on the observation that both human and murine T cells can co-express Th1 and Th2 transcription factors as well as the related cytokines [252-255], thus revealing an unappreciated flexibility of the classic Th cell lineages.

T cells isolated from VC-treated mice when activated *in vitro* secrete elevated levels of Th1-related cytokines (IL-2, IFN- γ and TNF- α) [184]. With respect to our findings, the mechanisms underlying the simultaneous Th1 and Th2 differentiation observed in activated human $\gamma\delta$ T cells primed under non-Th1 (or Th2) polarizing conditions and the stabilizing effect of pVC need to be further investigated. Th1 and Th2 differentiation share a common requirement for signals emanating from the TCR. Based on that, we suggested as possible mechanism that during initial activation, cytokine loci become rapidly activated in a manner linked with TCR/CD28 stimulation and independent of cytokine-regulated STAT1 and STAT6 modulation. Although STAT factors were reported to be crucial for the optimal induction of T-bet and GATA-3 [256], it is possible that the initial activation further induced STAT-independent T-bet [257] and GATA-3 [258] induction. In addition to activating specific transcription factors that act directly on cytokine loci, TCR signaling may trigger epigenetic changes leading to a sustained gene expression in Th cells. For example, the basal cytokine gene methylation patterns are disrupted in DNMT-1-deficient murine T cells, and activation of these cells elicits overexpression of IFN- γ , IL-4, IL-13 and IL-2 [144]. Although signature cytokine genes, such as *IFNG* and *IL4*, mainly carry the active histone mark H3K4me3 in the lineage where there are expressed and the repressive mark H3K27me3 in all other lineages, it was described that some key transcription factor genes can be poised by bivalent modifications [259]. *TBX21* (encoding T-bet) showed only activating H3K4me3 at the promoter in Th1 cells but bivalent modifications (H3K4me3 and H3K27me3) in all other investigated subsets. Likewise, *GATA3* showed only H3K4me3 in Th2 cells but bivalent modifications in the other subsets, suggesting that these transcription factors are not stably repressed and thus, could potentially be activated in other lineages. This could significantly contribute to generation of the aforementioned Th1+2 hybrid phenotype in activated $\gamma\delta$ T cells, which potentially creates a unique gene-regulatory landscape by inducing both GATA-3 and T-bet along with the Th1 and Th2 signature cytokines. With respect to the well-known

role of VC in the epigenetic reprogramming of gene expression, our data suggest that the pVC-mediated sustained polarization of activated $\gamma\delta$ T cells towards hybrid Th1+2-like cells may involve epigenetic reprogramming modulated by pVC treatment.

In functional terms, Th1 differentiation program and the associated IFN- γ production are required for the efficient immune responses against intracellular pathogens [239]. Th2 cell population and the related cytokines are critical for the immunity against helminths and other extracellular pathogens [244]. However, differentiated Th cells along with their cytokines can also contribute to undesired, disadvantageous immune responses. Particularly in allergy and asthma, the persistence of Th2 cells and Th2 cytokine production (e.g. IL-13) are associated with exacerbation of disease [260]. Interestingly, the development of hybrid Th1+2 cells in the context of parasites (helminth) and virus (lymphocytic choriomeningitis virus) may have a functional role in dampening the exacerbated immune responses and establishing a protective antiviral response, thus preventing fatal immunopathology and viral persistence [254,255,261]. Moreover, a recent study showed a crucial effect of IL-13-producing murine V γ 5 $\gamma\delta$ T cells in maintaining skin integrity and therefore protecting against inflammation-driven carcinogenesis *in vivo* [262]. Although the mechanism by which $\gamma\delta$ T cell-derived IL-13 protects against the carcinogenesis was not formally established, this study showed that IL-13 activates keratinocytes via the IL-13 receptor (IL-13R α 1) to produce various cytokines and mediates the migration of keratinocytes through the epidermis. Thus, the pVC-induced IL-13-production in activated human $\gamma\delta$ T cells has to be considered in the context of $\gamma\delta$ T cell-based cancer immunotherapy.

4.1.3. pVC enhances the cytotoxic effector function of activated $\gamma\delta$ T cells against pancreatic cancer cells

Pancreatic cancer is an extremely aggressive malignancy with poor prognosis. More than 90% of pancreatic cancer have ductal morphology and is classified as pancreatic ductal adenocarcinoma (PDAC) [263]. Pancreatic intraepithelial neoplasia (PanINs) are the most important type of PDAC precursors. Tumorigenesis is believed to be a step-wise progression from low-grade PanINs to high-grade PanINs and then to invasive adenocarcinoma [264]. Standard treatment for PDAC includes surgical resection and chemotherapy, but occasional resistance of PDAC to chemotherapy demanded alternative therapeutic approaches [265,266]. An increasing number of novel therapeutic strategies, including targeted therapy or immunotherapy alone or in combination with chemotherapy, have been described. While some strategies still failed to considerably improve survival times of the patients [267,268], others interestingly showed promising outcome [269]. Of note, $\gamma\delta$ T cell-based immunotherapy for both hematological and solid tumors is of particular interest since $\gamma\delta$ T

cells can distinguish tumor cells from normal cells and then kill them directly, without MHC restriction but via TCR $\gamma\delta$ or BTN3A1-mediated activation [270,271]. Clinical trials involving adoptive transfer of *in vitro*-expanded $\gamma\delta$ T cells to control tumors have been already carried out, and they have shown some promise regarding efficacy and safety of the cellular product [272,273]. However, the efficacy of $\gamma\delta$ T-cell-based immunotherapy is still limited largely because of the difficulty of obtaining sufficient numbers of viable $\gamma\delta$ T cells and the limited efficacy upon adoptive transfer. Expanding $\gamma\delta$ T cells *in vitro* using a variety of mitogenic stimuli including anti-CD3 or anti-TCR $\gamma\delta$ antibodies or pAgs, is a widely used strategy to increase their yield. However, human $\gamma\delta$ T cells rapidly undergo apoptosis or AICD upon TCR engagement in the presence of IL-2 [192,231]. Therefore, there is strong rationale for identifying the optimal combination treatment strategy for an optimal efficacy. In a previous study the tumor cell killing activity of $\gamma\delta$ T cells against PDAC could be enhanced by bispecific antibodies [269]. Other agents such as epigenetic drugs have been recognized as useful agents in cancer immunotherapy, including $\gamma\delta$ T-cell-based immunotherapy [274,275]. For instance, decitabine has been shown to have a direct anti-tumor activity, but also was reported to enhance the *in vitro* V γ 9V δ 2 T cell-mediated cytotoxicity effect [275]. Moreover, VC deficiency has been widely reported in cancer patients and has been associated with the cancer progression in disease models [276,277]. Therapies involving high-dose VC administration are currently gaining attraction in the treatment of many cancers. Study by Serrano et al. reported that pharmacological VC has an antitumor effect against melanoma cell lines and in murine models [278]. In the same line, other studies also demonstrated the antitumor effect of VC against tumor entities such as colon cancer and liver cancer cells [209,210,279]. Mechanistically, this VC-effect may be due to several factors, including decreased concentrations of antioxidant defenses (catalase and superoxide dismutase) in tumor cells [280,281], increased glycolytic metabolism with subsequent increase of glucose transporters, which facilitates the entry of DHA inside the tumor cells [280], and higher sensitivity of the mitochondria of tumor cells to ROS which inactivate glyceraldehyde-3-phosphate dehydrogenase [210,224]. Although the exact mechanism of the VC-mediated cytotoxicity remains unsolved, a previous study demonstrated that VC promotes the proliferation of NK-cell populations and increases their cytotoxicity against leukemic cell lines [183], suggesting that the combined effect of VC and immune cells may be considerable in adoptive transfer. However, the synergistic potential of VC and human $\gamma\delta$ T cells has not been so far explored. We showed in this thesis that pVC (the phosphorylated form of VC) supplementation is a potent strategy to improve $\gamma\delta$ T-cell expansion resulting in higher cell numbers as required for adoptive immunotherapy. A previous study from our group has shown that V γ 9V δ 2 T cells efficiently kill PDAC upon *in vitro* pAg-activation as well as *in vivo*

after repetitive adoptive transfer of $\gamma\delta$ T cells together with aminobisphosphonate and low dose IL-2 in SCID mice [282]. In the present study, we observed that pVC further significantly increased the pAg-reactivated V γ 9V δ 2 T-cell cytotoxicity against Panc89 tumor cells *in vitro* (Fig. 11b). We also investigated whether pretreatment of the Panc89 cells would render them more susceptible to killing by the activated ZOL-expanded V γ 9V δ 2 T cells. A previous study investigating the effects of VC on the viability of some pancreatic cancer cells showed that pharmacological (millimolar) VC application induced death in pancreatic cancer cell lines both *in vitro* and *in vivo* [283]. In further investigations the study demonstrated that VC-induced cytotoxicity was due to the generation of H₂O₂ through the ascorbate radical. This notwithstanding, we observed in our study that treatment of Panc89 cells with 173 μ M pVC (physiological concentration) did not impair their viability. However, pVC-treated target cells were slightly (but not significantly) more susceptible to $\gamma\delta$ T-cell killing (Fig. 11d).

The antitumor effect of $\gamma\delta$ T cells is induced by the interaction of $\gamma\delta$ TCR with tumor cells [284]. Additionally, other activating receptors, notably NKG2D can recognize NKG2D ligands (NKG2DLs), which can also trigger the antitumor function of $\gamma\delta$ T cells. In fact, the engagement of NKG2D activates cytolytic responses in human $\gamma\delta$ T cells [285], which are mediated by the granule exocytosis pathway through secretion of the pore-forming molecule perforin and the pro-apoptotic protease granzyme B [105,269,286]. Our data showed that NKG2D receptor was highly expressed on expanded as well as on BrHPP-re-stimulated V γ 9V δ 2 T cells and remained unchanged in the presence of pVC (Fig. 12c).

The pVC-enhanced $\gamma\delta$ T-cell cytotoxicity prompted us to analyze the exocytosis of cytolytic granules with the CD107a degranulation assay. The co-culture of Panc89 with ZOL-expanded V γ 9V δ 2 T cells in the absence of BrHPP resulted in a weak induction of CD107a surface expression. In the presence of BrHPP, the induction of CD107a was significantly increased, which could be correlated with the enhanced $\gamma\delta$ T-cell-mediated cytotoxicity observed in the same condition. In line, enhanced production of granzyme B and perforin were also detected after stimulation with BrHPP. However, CD107a induction, as well as granzyme B and perforin release remained unchanged in the presence of pVC (Fig. 13a-c).

To identify other possible mechanisms underlying the pVC-mediated increased $\gamma\delta$ T-cell cytotoxicity, we mined published data that mTOR inhibition can increase the yield and cytotoxic function of $\gamma\delta$ T cells. The inhibition of mTOR signaling using rapamycin increases the cytotoxicity of *ex-vivo*-expanded mouse V γ 4 T cells to various tumor cell lines via upregulating NKG2D and TNF- α [42]. Earlier study focusing on human $\gamma\delta$ T cells also

revealed that mTOR signaling inhibition in the *in vitro* expanded human V γ 9V δ 2 T cells increased their yield and their cytotoxicity [43]. In the present study we investigated whether pVC could increase the V γ 9V δ 2 T-cell-mediated cytotoxicity by counteracting mTOR signaling. The results showed that total mTOR expression and its phosphorylation were reduced in the presence of pVC in both unstimulated and BrHPP-re-stimulated V γ 9V δ 2 T cells (Fig. 15). The regulatory role of mTOR signaling in the tumor-killing ability of $\gamma\delta$ T cells might be unique among cytolytic cells. A previous study showed that inhibiting mTOR signaling during the antigen-specific maturation of CD8 T cells reduced their cytotoxic activity with decreased IFN- γ and granzyme B [287]. Further investigation revealed that mTOR inhibition hindered the sustained expression of T-bet but enhanced Eomesodermin (transcription factor associated with memory T cell differentiation during the maturation of CD8 T cells, which led to the conversion from effector into memory precursors. An inhibiting effect of rapamycin treatment on granzyme B production and cytotoxicity of NK cells was also reported [288]. However, it is also known that the PI3K/mTOR inhibitors have an important immunomodulatory impact on the tumor microenvironment and angiogenesis (reviewed in [289]). The modulation in the number and/or function of cells in the tumor microenvironment involved in tumor progression is often associated with a better outcome in cancer therapy, and for this reason, the selective inhibition of the PI3K/mTOR can improve the immunotherapy against leukemia and different solid tumors [290,291]. Thus, mTOR signaling might have context-dependent roles in different cell subsets or under distinct physiological conditions.

The cytotoxicity of V γ 9V δ 2 T cells against tumor cells can also be mediated through secretion of proinflammatory cytokines such as TNF- α and IFN- γ [292]. Interestingly, one recent study has shown that mTOR signaling inhibition in murine V γ 4 T cells increases their cytotoxicity via an enhanced expression of TNF- α [42]. Our data showed that the BrHPP-induced IFN- γ -production in V γ 9V δ 2 T cells (associated with their cytotoxicity) was significantly enhanced in the presence of pVC (Fig. 13d). IFN- γ is a crucial cytokine in the $\gamma\delta$ T-cell-mediated anti-tumor responses [293]. Its multiple anti-tumor effects include direct inhibition of tumor growth, stimulation of macrophages and blocking of angiogenesis [294,295].

The present study unveiled an unprecedented role of pVC in modulating mTOR kinase. However, whether these effects of pVC on mTOR kinase inhibition and IFN- γ production explain the observed enhanced cytotoxic activity is not entirely clear and has yet to be formally established.

4.2. pVC supports conversion of human $\gamma\delta$ T cells into Foxp3-expressing regulatory cells

4.2.1. pVC augments the expression of Foxp3

Foxp3 is a winged-helix family transcription factor, which acts as a master controller of gene expression in $\alpha\beta$ Tregs [92]. Mutations within the Foxp3 gene or deletion of Foxp3 in mouse models results in the development of fatal autoimmunity [92,296]. Foxp3 is a multi-domain protein with an N-terminal repressor, a zinc-finger, a leucine-zipper, and a C-terminal forkhead DNA-binding domains [297]. Already very early after its initial discovery in 2003 as lineage-specification factor for $\alpha\beta$ Tregs, it could be shown that the transcriptional program of $\alpha\beta$ Tregs is critically dependent on its expression and its ability to bind to DNA [298]. It has been suggested that Foxp3 can act in concert with other transcription factors, including Ikaros family zinc finger 4 (Eos) and GATA-3, to regulate gene expression in $\alpha\beta$ Tregs through binding to promoter regions thereby influencing gene transcription [299]. Foxp3 expression is required throughout the lifespan of animals as selective depletion of $\alpha\beta$ Tregs or disruption of Foxp3 expression in adult mice induces a fatal lymphoproliferative disorder [300]. Three Treg-cell types have been described, Thymus-derived (t)Tregs comprise the vast majority of Tregs generated already during thymic development and it is assumed that tTreg development relies on factors such as a high-affinity TCR binding to antigens presented on thymic APC and a specific cytokine milieu in combination with co-stimulation by thymic APC [301]. In addition to tTregs, Tregs can also be generated in the periphery from naïve CD4⁺ $\alpha\beta$ T cells [302]. These peripherally-induced Tregs (pTregs) are important for the acquisition of oral, mucosal and feto-maternal tolerance [93,303,304]. In addition to tTregs and pTregs, induced Tregs (iTregs) can be generated *in vitro* from naïve CD4⁺ $\alpha\beta$ T cells stimulated in the presence of IL-2 and TGF- β [305]. All three Treg-cell types rely on proper Foxp3 expression for their function. Like $\alpha\beta$ iTregs, there is now compelling evidence indicating that $\gamma\delta$ T cells can also be converted into Foxp3⁺-expressing $\gamma\delta$ Tregs upon TCR stimulation together with IL-2/IL-15 and TGF- β [98,99]. The Foxp3-induction in Tregs, but more importantly its stability, is a prerequisite for the regulatory function of the expressing cells. Seminal works by several groups have identified VC, as a natural compound that enhances and stabilizes Foxp3 expression *in vitro* during iTreg differentiation [189,190]. In the present study, we therefore analyzed in detail the effects of a combined exposure to TGF- β and pVC on V γ 9V δ 2 T cells. We observed that addition of pVC significantly increased the Foxp3 protein-expression in purified V γ 9V δ 2 T cells activated with pAg BrHPP or with A/E-beads in the presence (but not absence) of TGF- β (Fig. 17a, b). We also provided evidence that when pVC was added together with the TCR stimulation (and not at later time

point), the Foxp3 protein-expression was more pronounced as measured on day eight following stimulation (Fig. 18a, b). This may suggest that pVC initially facilitates the TGF- β -induced Foxp3 induction via modulation of the TCR-induced signaling implicated in the process of Foxp3 induction. Additional investigations are required to identify the target(s) of pVC in the TCR signaling involved in the induction of Foxp3. This might represent an additional mode of action of VC apart from the direct modulation of *FOXP3* CNS2 demethylation [189,190]. Concomitant pVC-mediated *FOXP3* CNS2 demethylation may further stabilize the induced Foxp3 expression. Along this line, the effect of pVC on the DNA methylation status of the *FOXP3* TSDR is discussed in section 4.4.2.

Moreover, we observed that, after removal of the remaining TGF- β and pVC on day eight, $\gamma\delta$ T cells initially stimulated with A/E-beads (but not BrHPP) and pVC maintained their Foxp3 protein-expression for at least another six days (Fig. 19a, b). This may suggest an important role for CD28 co-stimulation in the pVC-mediated maintenance of Foxp3 in TGF- β -expanded, independently of the induction of IL-2, since V δ 2 T cells are poor producers of IL-2. Such a role for CD28 co-stimulation has been reported for TGF- β -induced tTregs [306]. Altogether, these results indicate that pVC supports the maintenance of Foxp3 expression in TGF- β -expanded human $\gamma\delta$ T cells. We also investigated the expression of other additional markers which are used to characterize regulatory T cells and the findings of these results are described in the following section.

4.2.2. Modulation of the surface expression of TIGIT and CD39/CD73 by pVC

To further investigate the role of pVC in inducing a regulatory phenotype in human $\gamma\delta$ T cells, we extended our analyses to surface proteins known to be associated with Treg function. Surface expression levels of “regulatory-related” molecules including TIGIT, CD39 and CD73 were then measured. TIGIT is a novel co-inhibitory receptor that, together with CD226 (DNAM 1), comprises a pathway that closely parallels the CD28/CTLA-4 pathways [96]. The protein of human TIGIT bears a single extracellular Ig-like domain, a type I transmembrane region and cytoplasmic tail bearing two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [96]. Engagement of CD226 enhances T-cell activation [307], whereas engagement of TIGIT was reported to inhibit T-cell response [96,308]. TIGIT executes its function by binding to poliovirus receptor (PVR, also known as CD155) and PVRL2 (nectin2, also known as CD112) [307]. TIGIT is expressed in natural killer (NK) cells [309], activated CD4 T cells [308], CD8 T cells [310], V δ 1 $\gamma\delta$ T cells [311] and Tregs [312]. Our data showed that TIGIT expression on BrHPP or A/E beads-activated V γ 9V δ 2 T cells is significantly enhanced in the presence of pVC (Fig. 21a). Although this expression is irrespective of cytokine culture

condition, we observed that TIGIT is induced to higher levels in IL-2- than in [IL-2 + TGF- β]-culture condition.

Yu et al. initially indicated that TIGIT inhibits T-cell responses indirectly by triggering CD155 in dendritic cells (DC), thereby preventing DC maturation and inducing production of the immunosuppressive cytokine IL-10 [96]. TIGIT-expressing NK cells exhibit potent suppression of IFN- γ production through engagement with CD155 [309]. Joller et al. demonstrated that TIGIT has T-cell intrinsic effects. The study reported that loss of TIGIT in murine T cells results in hyperproliferative T-cell responses and increased susceptibility to autoimmune disorders [308]. Indeed, the two ITIMs in TIGIT have been shown to mediate recruitment of the phosphatase SHIP-1 [309], thus providing a mechanism by which TIGIT can act cell intrinsically to dampen activating signals. In CD8 T cells, the upregulation of TIGIT is correlated with CD8 T-cell exhaustion [310]. TIGIT expressed on Tregs can enhance their function specifically by suppressing proinflammatory Th1 and Th17 (but not Th2) responses [312]. TIGIT is a direct target of *FOXP3* [313]. In Tregs, TIGIT expression correlates with markers for Tregs (Foxp3, CD25 and CTLA-4) and TIGIT⁺ Tregs show enhanced demethylation in *FOXP3* CNS2 compared to their TIGIT⁻ Tregs counterparts, leading to higher lineage stability [312]. All of these evidences support the role of TIGIT in directing regulatory function. As a consequence, the function of TIGIT was initially investigated in models of autoimmunity and tolerance. Indeed, the absence of TIGIT has been shown to exacerbate experimental autoimmune encephalomyelitis (EAE) marked by elevated levels of proinflammatory cytokines and signs of neurologic dysfunction reminiscent of Th17-driven disease [308,314]. In addition to EAE, TIGIT engagement has been shown to ameliorate collagen-induced arthritis (CIA) and graft-versus-host disease (GvHD) [315]. In both models, blocking of TIGIT results in an exacerbation of the disease driven by enhanced proinflammatory T-cell responses.

On the other hand, inflammation-mediated tissue damage induces the release of ATP into the extracellular compartment [316]. Here, ATP acts as positive regulator of the immune response [317]. Upon the release of ATP, it is converted in a stepwise manner into adenosine diphosphate (ADP) and adenosine mono-phosphate (AMP) by ecto-ATPase (CD39), and finally by the the ecto-5'-nucleotidase (CD73) into adenosine [318]. Adenosine acts as negative regulator of the immune response. Therefore, cells co-expressing CD39 and CD73 may exhibit immunosuppressive activity through the generation of adenosine [97]. Multiple lines of evidence have shown that $\gamma\delta$ T cells express CD39 only after TCR stimulation, while CD73 is constitutively expressed on a population of freshly isolated T cells and is down-modulated after activation [319,320]. We confirmed in our culture system that in

human $\gamma\delta$ T cells, independently of the cytokine milieu and the presence of pVC, CD39 is upregulated after activation (Fig. 21b). In line with previous findings [319,321], we observed that CD73 expression on freshly isolated $\gamma\delta$ T cells drastically decreased upon TCR stimulation and was not affected by pVC addition. Interestingly, in A/E beads stimulated cells, IL-2-expanded $\gamma\delta$ T cells as well as [IL-2 + TGF- β]-expanded $\gamma\delta$ T cells co-expressed CD39 and CD73; this co-expression was further enhanced in the presence of pVC (Fig. 21b). Notably, the pVC-induced co-expression of CD39 and CD73 was higher in IL-2-expanded $\gamma\delta$ T cells than in the [IL-2 + TGF- β]-expanded group. These findings indicate that, depending on the initial stimulation and the cytokine milieu, pVC can enhance the expression of CD39 and CD73, suggesting a role of pVC in modulating the entire degradation of ATP into adenosine.

Taken together, the pVC-enhanced expression of Foxp3, TIGIT and CD39/CD73 may have important implications for the generation of stable regulatory human $\gamma\delta$ T cells.

4.2.3. pVC facilitates the *in vitro* suppressive activity of regulatory $\gamma\delta$ T cells

Foxp3-expressing Tregs have an essential role in maintaining homeostasis of the immune system and in preventing the autoimmune reactivity of self-reactive T cells [322]. Because of their clinical relevance in different pathological settings, such as autoimmune diseases (e.g., lupus, ulcerative colitis, rheumatoid arthritis, diabetes, etc.), allergy and solid organ transplant rejection, different studies have tested the ability of small molecules to enhance the generation, proliferation and function of Tregs [323,324]. Due to their relative infrequency in peripheral blood, current protocols for clinical application of human Tregs infusion are based on the isolation and the *in vitro* expansion of the patient's tTregs using anti-CD3/CD28-coated microbeads and IL-2 with or without rapamycin [324,325]. However, for reasons which have yet to be elucidated, tTregs cultured *in vitro* undergo apoptosis [326], which may render their use difficult in therapeutic settings. On the other hand, although several murine studies successfully established a protocol to improve the generation of Tregs from naive CD4 T cells, using TGF- β , retinoic acid, and/or rapamycin [327,328], its translation to human Tregs has not been effective enough, as this acquired Tregs phenotype is unstable and the cells fail to retain Foxp3 expression and to suppress GvHD [329]. Stable Foxp3 expression is clearly a prerequisite for the maintenance of suppressive properties in Tregs. Given the potential for these cells in a therapeutic context, it is essential that the factors governing the expression of this lineage-specification factor be defined more precisely.

TIGIT, and more importantly Foxp3 expression became more stable in pVC-treated TGF- β -expanded $\gamma\delta$ T cells, which might suggest an enhanced immunosuppressive function of these cells. With the hypothesis that stable expression of Foxp3 sustains suppressive function of Tregs, we investigated the influence of pVC in modulating the suppressive activity of TGF- β -expanded $\gamma\delta$ T cells. In these experiments, we observed that $\gamma\delta$ T cells cultured with pVC (particularly those initially activated with A/E-beads) inhibited more potently the proliferation of CD4 responder T cells in co-cultures (Fig. 16a, b). Our findings are in line with recent studies pointing to a role of VC in the generation of stable iTregs. These studies demonstrated that VC increases the stability and the suppressive function of both murine and human iTregs [189,190], suggesting that manipulation of culture conditions using VC might be a useful strategy for stabilizing iTregs function in the clinic. The importance of alloantigen-specific Tregs in the context of clinical transplant tolerance was recently highlighted. A superior immunosuppressive capacity of these cells was reported [330]. Interestingly, a previous study demonstrated that the addition of VC to alloantigen-specific $\alpha\beta$ Tregs cultures led to the generation of a stable Treg population (epigenetically akin to naturally occurring Tregs) with enhanced ability to promote skin allograft acceptance [191]. Although some studies have highlighted the potential involvement of regulatory $\gamma\delta$ T cells in the pathogenesis of some autoimmune diseases [331,332], others provided evidence that $\gamma\delta$ T cells could exert regulatory functions resulting in the prevention of autoimmune disorders [103,333]. In line, it was also found that, regulatory $\gamma\delta$ T cells induced in the presence of decitabine maintained potent immunosuppressive functions against GvHD *in vivo* [334]. Moreover, GATA-3 can be expressed by Tregs and is required for their suppression of Th2 responses [220,221]. In addition, suppressive TGF- β -expanded V δ 2 T cells expressed high levels of GATA-3, which could be correlated with their suppressive function [99]. In line, we observed GATA-3 protein-expression in a proportion of TGF- β -expanded $\gamma\delta$ T cells, which was significantly enhanced in the presence of pVC (Fig. 20).

Altogether, our study demonstrated that the addition of pVC to TGF- β -stimulated $\gamma\delta$ T-cell cultures facilitates the induction of immunosuppressive cells. It remains to investigate if the Foxp3-positive $\gamma\delta$ T cells generated by combined TGF- β and VC treatment, are superior suppressive cells in comparison to conventional $\alpha\beta$ Tregs in certain situations, due to their homogeneous expression of the pAg-reactive V γ 9V δ 2 TCR.

4.3. Modulation of the gene expression profile of activated $\gamma\delta$ T cells by pVC

Genome-wide effects of VC on gene expression in different cell types have been previously described [182,335,336]. We performed gene expression profiling in order to identify genes whose expression in expanded V δ 2 T cells was altered by exposure to pVC. pVC had a modest effect on the transcriptome of V δ 2 T cells expanded in the presence of IL-2 only. However, other cell types such as stromal cells, primary human dermal fibroblasts, or mouse and human induced pluripotent stem cells, display a higher sensitivity toward VC as revealed by pronounced alterations in gene expression upon exposure to VC [182,335,336]. These findings indicate that VC is capable of inducing global changes at the transcriptional level. We suggest that the overall low pVC-induced changes in gene expression in the expanded V δ 2 T cells observed in this study might be related to the time point of analysis which was day eight following stimulation. Gene expression is a dynamic process and it may be that more significant changes might have occurred at earlier time points. Supporting this idea, the pronounced VC-induced gene expression changes reported in the above studies were observed at 24h and 72h following stimulation in the presence of VC. This notwithstanding, pVC-regulated genes were further grouped according to the KEGG pathway analysis. The most striking effect of pVC on the transcriptome of the IL-2 expanded V δ 2 T cells was the modulation of the expression of a large set of genes that are involved in cytokine-cytokine receptor interaction (Table 1). Gene network and KEGG pathway analyses identified *MAPK8* clustered with *CDKN1A* and *DUSP1* as the most pVC-affected genes due to their central position in the network (Fig. 22a) and the fact that they appeared across multiple pathways (Table 1). *MAPK8*, which is also known as c-JUN N-terminal kinase (JNK), is a member of the MAPK family [337], involved in various processes such as cell differentiation, proliferation, migration, transformation and programmed cell death ([338,339]. Activation of the TCR is required, but it is not clear what signals regulate the enhanced expression and activity of JNK proteins. Interestingly, although CD28 costimulation appears to maximize JNK activity [340,341], it is not essential for increased expression of JNK mRNA [340]. A previous study indicated that dietary factors involved in inflammation and oxidative stress interacted with MAPK signaling genes [342]. Of note, the study evaluating the association between diet and the MAPK-signaling genes, identified calcium to interact with *MAPK8* to alter the risk of colon and rectal cancer. Although this study did not provide details regarding the nature of the interaction, in further investigations they could find another *MAPK* gene, the *MAP3K7* to be associated with VC to alter the risk of colorectal cancer.

In the present study, we observed that *MAPK8* was upregulated by pVC, in contrast to *DUSP1* and *CDKN1A* which were downmodulated (Fig. 22a, b). These findings are in line

with a previous study showing that VC at physiological concentration was able to amplify the activation-dependent induction of phospho-p38 and phospho-JNK in macrophage lineage [179]. *DUSP1* encodes a protein phosphatase which can dephosphorylate both threonine and tyrosine residues of MAPKs. This dephosphorylation negatively regulates the intensity and the duration of MAPK activation [343,344]. *CDKN1A*-encoded protein p21 is a member of cyclin-dependent kinase (CDK) inhibitors family [345]. In general, p21 is an important regulator of cell proliferation, induced in response to various intra- and extracellular stimuli to arrest the cell cycle and thus inhibit cell proliferation directly through binding to CDK [345]. Additionally, we observed that *TNFRSF10B* encoding TRAIL-R2, whose activation can induce apoptosis [346], was downmodulated, while *TIMP1*, whose encoded protein, the metalloproteinase inhibitor 1 is able to promote cell proliferation, was upregulated in a pVC-dependent manner. Recent studies reported that high expression of *TIMP1* resulted in activation of pro-survival and anti-apoptotic signaling through Erk and Bcl-2 [347,348]. Taken together, our data suggest that pVC promotes the proliferation of the IL-2-expanded $\gamma\delta$ T cells at least partially by regulating the expression of the genes mentioned afore. However, results were obtained from only two donors and remain to be further investigated in larger sample size and earlier time points.

The stimulatory effect of pVC on the transcription of genes like *TIGIT*, *GATA3* and *IL13* also correlated with enhanced protein expression (Fig. 22c). In contrast, the pVC-dependent induction of T-bet protein-expression was not transcriptionally regulated, which could suggest that pVC modulates T-bet expression at post-translational level.

On the other hand, we also investigated the influence of pVC on gene expression in TGF- β -expanded $\gamma\delta$ T cells. The transcriptome data revealed only minor differences between the pVC-treated- and the untreated group (Fig. 23a, b), although KEGG pathway analysis identified genes involved in cytokine-cytokine receptor interaction to be the most abundantly regulated by pVC (Table 2). CD103 was recently described by our group to be essential for upregulated cytotoxic function of TGF- β -expanded V δ 2 T cells [349]. Here, we confirmed the TGF- β -dependent upregulation of CD103 at transcriptional level (*ITGAE*), which was further increased upon pVC-treatment. Interestingly, we observed that *FOXP3* and *TIGIT*, in line with the protein-expression, were upregulated in the presence of pVC (Fig. 23c), indicating that pVC can modulate $\gamma\delta$ T-cell differentiation both at protein and transcriptional level. Our findings on pVC-mediated *FOXP3* expression were in line with previous data on polyclonal-iTregs and alloantigen-specific Tregs, where VC was described to increase both the Foxp3 protein and gene expression [189,191]. But rather than indiscriminately activating many loci, pVC appeared selective in that it mainly affected *FOXP3* among the Tregs signature genes.

This selective effect led us to hypothesize that the functional difference (suppressor activity) along with the Foxp3 expression between the two differentially expanded cells (i.e. TGF- β vs TGF- β + pVC) were dependent on pVC-induced epigenetic reprogramming.

4.4. Epigenetic regulation of gene expression by pVC

4.4.1. Genome-wide DNA methylation

To further evaluate whether VC regulates gene expression by modulating epigenetic modifications, we took a genome-wide approach to investigate DNA methylation changes in BrHPP-activated V δ 2 T cells cultured in IL-2-containing medium in the presence or absence of TGF- β . Previous studies have analyzed the pVC effect on DNA methylation as a global event [180,182] or only in a gene-specific context [189,190]. The present study used the power of RRBS to find changes in DNA methylation at single nucleotide resolution, which provides (i) a coverage of the most part of the genome at high resolution and (ii) quantification of methylation changes at nucleotide resolution. The analysis performed by Qiwei Sun (BGI Genomics Institute, Shenzhen, China) showed that pVC induced subtle changes in DNA methylation. The changes seen in CpG context were not discernible at global (Fig. 24a) or even at chromosome scale (Fig. 24b) but were only seen once individual loci (e.g. *FOXP3*) was analyzed with the integrative genomic viewer by zooming into the RRBS data (Fig. 25a). These findings are in contrast with a previous study investigating the effect of VC on the DNA methylation profile of human embryonic stem cells (hESC) revealing that VC (used at 50 μ g/mL) causes a widespread DNA demethylation of 1,847 genes in *in vitro* cultured hESC, with a bias toward demethylation not in the classical CGI, but in sequences up to 2kb distant termed “CpG shores” [180]. Their data further showed that the VC-induced global demethylation at CpG shores was associated with altered expression of the demethylated genes. We also extended our investigations to the CpG shores and shelves, which however, showed similar methylation patterns in the expanded cells, irrespective of VC supplementation (data not shown).

In mammals, DNA methylation is often restricted to CpG context within the genome. However, to a lesser extent, this epigenetic mark also occurs at non-CpG sequences, CHG and CHH where H equals A, C or T [350,351]. We then analyzed the average global changes to CHG and CHH methylation contexts across the promoter and CGI. The analysis revealed that pVC promoted minor demethylation at CHG and CHH across the promoter and the CGI (Fig. 24d, e). This pVC-induced low demethylation was however more pronounced in IL-2-expanded cells than in the TGF- β expanded population. The overall low changes in genome-wide methylation in pVC-exposed $\gamma\delta$ T cells led us to speculate that our observations might

be related to the time point of the analysis. DNA methylation/demethylation is a dynamic process, and it is conceivable that more significant changes in DNA methylation might have occurred at earlier time points. However, we cannot also rule out the possibility that pVC exposure might affect other epigenetic marks. Previous studies reported significant crosstalk between DNA methylation and histone PTM [136,137]. Specifically, there is considerable evidence for crosstalk between H3K27me3 and DNA methylation [352,353]. Furthermore, a previous study showed that during VC-mediated T-cell maturation, both DNA methylation and histone H3K9me2 marks are co-regulated [182]. In this study, they demonstrated that VC induced DNA demethylation and loss of H3K9me2 marks, whereby promoting T-cell maturation. A more recent study also reported that VC may also act in DNA demethylation-independent manner. Rather, VC reduced H3K9me3 marks in the regulatory elements of the *IL17* locus, thereby increasing IL-17A expression in *in vitro* generated murine Th17 cells [186]. Further investigations in this study showed that VC induced the specific loss of H3K9me3 by increasing the activity of Jumonji-C domain-containing protein 2 (Jmjd2) which catalyzed histone demethylation. Although, the effect of pVC on histone PTM was not studied in this thesis, the deposition of specific histone modifications during pVC exposure has to be considered. Taken together, our data suggest that modulation of DNA methylation by pVC during $\gamma\delta$ T-cell differentiation may be qualitative (i.e. locus-specific) rather than quantitative and hence requires further investigations.

4.4.2. pVC promotes the demethylation of *FOXP3* locus

Three intronic enhancers, designated conserved noncoding sequences (CNS) 1, 2, and 3, in addition to a promoter, have been identified at the *FOXP3* locus, and were revealed to play important roles in Foxp3⁺ Tregs differentiation [148,298,354]. Of these, CNS2 (also known as Treg cell-specific demethylated region, TSDR; [148]) is unusual in that it controls the stability of Foxp3 expression in a manner linked to the Tet-mediated DNA modification status of CNS2 [189,190]. CNS2 is a CpG-rich element that lies in the first intron, roughly 4 kb downstream of the promoter [299]. CpG sites in the *FOXP3* CNS2 element are predominantly unmethylated in tTregs expressing Foxp3 stably, but it is uniformly methylated in activated T cells or iTregs expressing Foxp3 transiently [148,188,355]. In cell proliferation and adoptive transfer experiments, Foxp3 expression was lost more rapidly in iTregs with methylated CNS2 compared with tTregs bearing unmethylated CNS2 [188,356], supporting the idea that the DNA methylation status of CNS2 correlates with the stability of Foxp3 expression [148,188,298]. Given the established connection between CNS2 methylation status and Treg stability, previously published studies have shown that VC, by providing iron substrate to Tet enzymes, facilitates a concomitant *FOXP3* CNS2 demethylation and a stable Foxp3 expression in iTregs [189,190].

In line with these studies, when investigating the molecular mechanisms underlying the pVC-mediated stable Foxp3 protein-expression in activated V γ 9V δ 2 T cells, we could clearly demonstrate that pVC induced changes in the methylation status of CpG motifs in the CNS2, as revealed by analyzing Foxp3⁺ and Foxp3⁻ cell populations sorted from $\gamma\delta$ T-cell cultures supplemented with TGF- β and pVC. Remarkably, a pronounced *FOXP3* CNS2 demethylation was only observed in Foxp3⁺ $\gamma\delta$ T cells sorted from TGF- β plus pVC supplemented cultures and not in Foxp3⁺ sorted $\gamma\delta$ T cells derived from cultures with TGF- β but without pVC (Fig. 26d, e). Importantly, Foxp3⁻ cells derived from the very same cultures did not show any signs of a significant CNS2 demethylation irrespective of pVC supplementation (Fig. 26d, f), suggesting that pVC may be specifically acting on transcriptionally active genes as previously described [189,191].

4.5. Combining Vitamin C and $\gamma\delta$ T cells for immunotherapy

In the past years, the host's immune system has become a target for the development of new therapeutic strategies, such as immunomodulatory drugs or monoclonal antibodies, and cancer immunotherapy has achieved remarkable clinical efficacy in the treatment of many cancer patients, by promoting the antitumor activity of the immune system [357]. Adoptive immunotherapy is accomplished by expanding immune effector cells *in vitro* and transferring the activated immune cells into the patients, thus targeting them against tumor cells or to stimulate immune response to eliminate tumor cells. In this respect, $\gamma\delta$ T cells are a population of effector cells with potent and MHC-independent anti-tumor efficacy. Activated human V γ 9V δ 2 T cells exert a strong cytotoxicity against a broad spectrum of leukemias, lymphomas and solid tumors [358,359]. Moreover, small clinical phase I/II studies have been performed with adoptively transferred activated human V γ 9V δ 2 T cells in patients with various solid tumors, so far however with limited efficacy [360]. In addition, and in contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells can be safely applied in an allogeneic setting without need for MHC compatibility with limited side effects [361]. Because of this anti-tumor potential of $\gamma\delta$ T cells, there is an increased necessity for new strategies that can improve their differentiation and effector function in relevant clinical settings. In this regard, VC as a natural compound abundant in fruits and vegetables, has been reported to stimulate cell proliferation, maturation process, differentiation and function of immune cells [182,183,203]. Moreover, in patients with glioblastoma, small pre-clinical and clinical case studies have shown that the combination of conventional treatments with intravenous high-dose VC therapy improved quality of life, progression-free and overall survival [362,363]. In the present study, we observed that pVC significantly enhanced the proliferation and the cytokine production as well as the cytotoxic activity in human V γ 9V δ 2 T cells expanded in the absence of TGF- β . To

apply adoptive V γ 9V δ 2 T-cell transfer into cancer patients as an immunotherapeutic approach, large cell numbers are required. Therefore, we suggest that the addition of pVC to cell cultures for large scale expansion requiring occasional re-stimulation will greatly improve the generation of sufficiently large V γ 9V δ 2 T-cell numbers. Moreover, pVC-treated V γ 9V δ 2 T cells produce larger amounts of IFN- γ and IL-13 which can support anti-tumor immunity [262,293], and thus can be considered as an additional benefit.

Furthermore, the importance of alloantigen-specific Tregs with superior immunosuppressive capacity in both experimental models and patients was recently highlighted [330,364]. Accordingly, it has been demonstrated that alloantigen-specific Tregs in the presence of VC displayed a superior immunosuppressive capacity conferring protection against skin allograft rejection [191]. By analogy, this thesis showed that pVC together with TGF- β can induce the conversion of human $\gamma\delta$ T cells in Foxp3-expressing Tregs which can exhibit potent suppressive activity. Hence, pVC treatment acts in a similar fashion on iTregs and $\gamma\delta$ Tregs. Additional studies are however required, to determine if pVC-treated allogeneic $\gamma\delta$ Tregs might play a role in the context of clinical transplant tolerance and autoimmune disorders.

4.6. Concluding remarks

In conclusion, this thesis explored the influence of pVC on human $\gamma\delta$ T-cell functional plasticity. Within the underlying themes of this study, we demonstrated that pVC increased the *in vitro* proliferation, the cytokine production and the cytotoxic effector function of cultured V γ 9V δ 2 T cells. Possible mechanisms underlying the pVC-enhanced $\gamma\delta$ T-cell proliferation and cytotoxicity could involve the impairment of ROS production, the inhibition of mTOR kinase expression/activity and the increased IFN- γ production which has been described to regulate $\gamma\delta$ T-cell-mediated cytotoxicity. We demonstrated that pVC induces a hybrid Th1+2-like phenotype in IL-2-exposed $\gamma\delta$ T cells characterized by the co-expression of T-bet and GATA-3 as well as the related cytokines IFN- γ and IL-13. From another angle, we also observed that pVC selectively influenced the transcriptome of TGF- β -expanded human $\gamma\delta$ T cells. However, TGF- β -exposed human $\gamma\delta$ T cells treated with pVC showed a more pronounced demethylation of *FOXP3* CNS2 accompanied by an increased stability of Foxp3 protein and gene expression. Importantly, the stabilization of the Foxp3 expression directly translates into higher suppressive capacity, as the TGF- β -exposed $\gamma\delta$ T cells treated with pVC showed a superior suppressive function. Finally, whole transcriptome and genome-wide DNA methylation analyses revealed a minor effect of pVC on gene expression and methylation. As analyzed after 8 days of *in vitro* expansion, depending on the additional signals (e.g. TGF- β) and on the “clinical target” (i.e. cancer immunotherapy versus

autoimmune diseases or organ transplantation), pVC can have beneficial/detrimental effect: (i) adoptive transfer in cancer, without TGF- β , pVC increases effector functions of $\gamma\delta$ T cells; (ii) autoimmunity/transplantation, together with TGF- β , pVC increases Foxp3 expression and suppressive activity.

Altogether, the data accumulated in this thesis, identified pVC as a factor enhancing cellular differentiation processes during human $\gamma\delta$ T-cell differentiation and thus, should be considered as a safer formulation in conditioning human $\gamma\delta$ T cells for immunotherapy. A schematic summary of the effects of pVC on human $\gamma\delta$ T-cell differentiation and function is proposed below (Fig. 27).

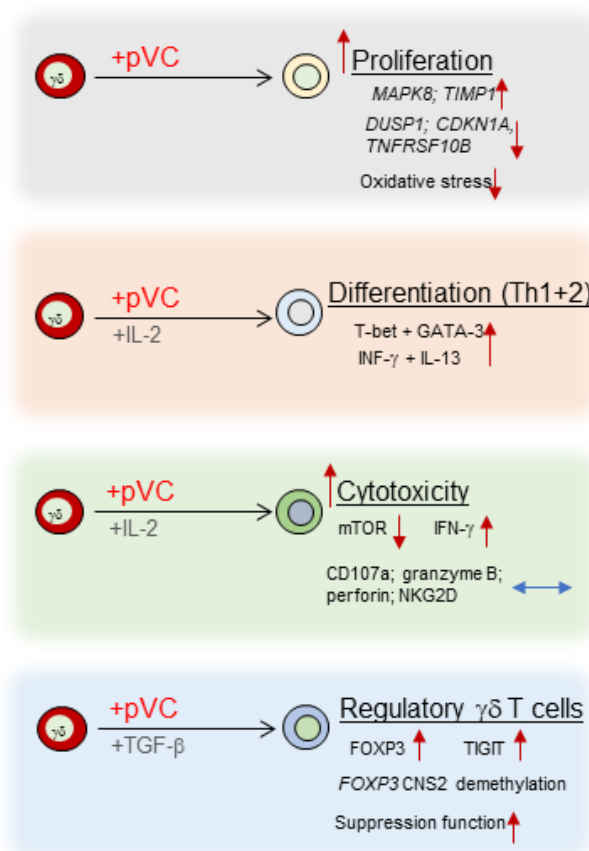


Figure 27. pVC modulates the human $\gamma\delta$ T-cell differentiation at protein, transcriptional and epigenetic levels.

The addition of pVC to human $\gamma\delta$ T-cell cultures augments their proliferation, the transcription factor expression and cytokine production as well as their cytotoxicity against tumor cells. Depending on the cytokine microenvironment, $\gamma\delta$ T cells cultured in the presence of pVC can be converted into Foxp3-expressing regulatory T cells which showed a more pronounced demethylation of *FOXP3* CNS2 accompanied by an increased stability of Foxp3 and a superior suppressive activity.

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APPENDICES

Appendix A : Kits

Human TCR $\gamma\delta$ microbeads kit	Miltenyi Biotec (Bergisch-Gladbach, Germany)
CD4 T Cell Isolation kit II	Miltenyi Biotec (Bergisch-Gladbach, Germany)
Dynabeads CD25 ⁺ Treg Isolation kit	Life Technologies (Carlsbad, USA)
Activation/ Expander T cell beads kit	Miltenyi Biotec (Bergisch-Gladbach, Germany)
Human IFN- γ DuoSet ELISA kit	R&D Systems (Wiesbaden, Germany)
Human IL-13 DuoSet ELISA kit	R&D Systems (Wiesbaden, Germany)
Human Granzyme B DuoSet ELISA kit	R&D Systems (Wiesbaden, Germany)
Human Perforin ELISA kit	Abcam (Cambridge, UK)
miRNeasy Mini kit	Qiagen (Hilden, Germany)
DNeasy® Blood & Tissue kit	Qiagen (Hilden, Germany)
EZ DNA Methylation-Gold and Lightning kit	Zymo Research (Irvine, USA)
CellROX™ Deep Red Flow Cytometry kit	ThermoFisher Scientific (Rockford, USA)
LEGENDplex™ human Th cytokine panel kit	BioLegend (London, UK)

Appendix B: Chemicals and reagents

Dimethylsulfoxid (DMSO)	Sigma-Aldrich (Darmstadt, Germany)
Ficoll-hypaque	Biochrom (Cambridge, UK)
Sodium azide (NaN ₃)	Sigma-Aldrich (Darmstadt, Germany)
Ethylendiamintetraacetate (EDTA)	Sigma-Aldrich (Darmstadt, Germany)
Fetal bovine serum (FBS)	Life Technologies (Carlsbad, USA)
Parafomladehyde (PFA)	Merck (Darmstadt, Germany)
Penicillin/ Streptomycin	ThermoFisher Scientific (Rockford, USA)
Dubelcco's Phosphate buffered saline (PBS)	Cell Concepts (Umkirch, Germany)
Propidium iodide (PI)	Serva (Heidelberg, Germany)
Sulfuric acid (H ₂ SO ₄)	Merck (Darmstadt, Germany)
Triton X-100	Sigma-Aldrich (Darmstadt, Germany)
Eosin	Biochrom (Cambridge, UK)
Trypsin/EDTA	Biochrom AG (Berlin, Germany)
FcR blocking reagent	Miltenyi Biotec (Bergisch-Gladbach, Germany)
Bovine serum albumin (BSA)	Serva (Heidelberg, Germany)
Ponceau S staining solution	Sigma-Aldrich (Darmstadt, Germany)

Tween 20	Merck (Darmstadt, Germany)
L-Ascorbic acid (VC) and L-ascorbic 2-phosphate acid (pVC)	Sigma-Aldrich (Darmstadt, Germany)
Sodium chloride (NaCl)	Merck (Darmstadt, Germany)
Protease and phosphatase inhibitors (sodium orthovanadate, sodium fluoride, sodium pyrophosphate, phenylmethylsulfonyl fluoride, and pepstatin A)	Sigma-Aldrich (Darmstadt, Germany)

Appendix C: Supplements and buffers

FBS

FBS used for medium supplementations was heated for 30 minutes at 56°C to inactivate the complement factors. Aliquots were then stored at -20°C until required.

Penicillin and Streptomycin

10000 IU/mL bottles were aliquoted into 5mL portions and frozen at -20°C until required. One 5mL aliquot was then added to 500mL of RPMI 1640 medium to provide 100 IU/mL penicillin and 100 µg/mL streptomycin

EDTA

EDTA was dissolved in MilliQ water and adjusted to pH 8.0 with NaOH. Solution was autoclaved and stored at RT.

4% (w/v) PFA stock solution

8 g of PFA was dissolved in 200 mL PBS by incubation at 60°C in the water-bath. pH was set to 7.4. Aliquots were stored at -20°C.

10% (w/v) Triton X-100 stock solution

1mL Triton X-100 (Sigma-Aldrich) was mixed with 9mL MilliQ water and stored at RT.

ELISA buffers

- Washing buffer

PBS containing 0.05% (v/v) Tween 20

- Blocking buffer

PBS containing 1% (w/v) BSA

- Reagent diluent

PBS containing 0.1% (v/v) BSA (and 0.05% Tween 20)

Flow cytometry buffers

- Staining buffer

PBS containing 1% (v/v) FCS stored at 4°C.

- MACS buffer

PBS containing 0.5% (v/v) BSA and 2 mM EDTA, stored at 4°C.

- Wash buffer

PBS containing 1% (v/v) BSA and 0.1% (v/v) NaN₃

- Dynal buffer

PBS containing 0.1% (v/v) BSA and 2 mM EDTA, stored at 4°C

- Cytotfix/Cytoperm buffer

1 mL Cytotfix/Cytoperm (eBioscience) was mixed in 3 mL Diluent (eBioscience) and stored at 4°C

- Fixation-Permeabilization buffer

1 mL (10x) Fixation-Permeabilization buffer (eBioscience) was mixed with 9 mL MilliQ and stored at 4 °C

- Annexin V binding buffer

MilliQ water containing 10 mM HEPES, 140 mM NaCl and 5 mM CaCl₂, stored at 4°C

Western blot buffers

- Transfer buffer

25 mM Tris, 192 mM Glycine, 20% (v/v) methanol, 0.015% (w/v) SDS in MilliQ water, stored at 4°C

- TBS (10x)

100 mM Tris, 1.5 M NaCl in ddH₂O

- Washing buffer (TBST)

10 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20 in MilliQ water, stored at RT

- Blocking buffer

5% (w/v) BSA in TBS and stored at 4°C

- Gel running buffer

50 mL (20x) NuPAGE MES SDS Running buffer (ThermoFisher Scientific) was mixed with 950 mL MilliQ water and stored at 4°C

- NP40 lysis buffer

1% (v/v) Nonidet[®] P40 (Sigma-Aldrich), 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 5 mM EDTA. Stored at 4°C

- 3x Sample loading buffer

200 mM Tris (pH 6.8), 6% (w/v) SDS, 30% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.015% Bromophenol blue stain

Appendix D: Standard cell dilution assay (SCDA)

Principle

SCDA is a rapid flow cytometry method to quantitatively determine the number of phenotypically definable lymphocyte subsets upon activation in heterogenous populations [193]. This method combines:

- (i) Specific detection of lymphocyte subsets by phycoerythrin-conjugated monoclonal antibodies,
- (ii) Exclusion of dead cells or cell debris by propidium-iodide staining and gating on forward vs. side scatter, respectively, and
- (iii) Admixture of a known number of standard cells immediately before analysis as a constant parameter to allow for calculation of cell quantity.

Preparation of fixed standard cells

The standard cells were purified CD4⁺ T cells that had been stained with APC-labeled anti-HLA class I mAb (clone w6/32) and anti-TCR $\alpha\beta$ mAb (clone BMA031) and then fixed in 1 % paraformaldehyde. Several steps have to be considered for the preparation of the standard cells:

- **CD4 T cell isolation**

- 1- From PBMC, proceed to CD4 isolation by MACS (negative isolation, human CD4 T cell isolation kit, Cat number: 11346D or 11352D, ThermoFisher Scientific)
- 2- Wash with 10-15 mL MACS Buffer (MP), spin down 1300 rpm, 5 min
- 3- Per 100 x 10⁶ PBMC, add 500 μ L MP + 200 μ L heat inactivated FCS + 200 μ L anti-cocktail Ab; incubate 20 min, 4°C
- 4- Wash with 10-15 mL MACS Buffer (MP), spin down 1400 rpm, 5 min
- 5- Homogenize thoroughly the tube containing the Dynabeads
- 6- Wash the beads: add 1 mL beads/100 x 10⁶ cells into 5 mL MP and with the 10 mL pipet resuspend at least 10x
- 7- Add to the cell pellet, 1 mL of the washed beads + 2 mL MP
- 8- Place the tube on a rotator for 15 min at room temperature
- 9- Resuspend slowly with 10mL pipet
- 10- Place the tube in the MACS Flowtube magnet for 2 min
- 11- Carefully collect the supernatant (=CD4) and transfer it into a new 15 mL tube
- 12- Place the tube containing the supernatant into the magnet for another 2 min
- 13- Count the cells

- **Control staining**

After MACS, proceed to control staining in order to check the purity of the isolated CD4 T cells. Prepare in 96-V bottom plate, 4 cell aliquots (each 20-50 μ L) and stain with the following fluorochrome-labeled mAbs:

- (a) α CD3/ α CD4
- (b) α CD3/ α CD8
- (c) α CD3/ α CD56
- (d) α CD14/ α CD19

- **Staining**

- 1- Add to the rest of the isolated CD4 T cells, 5 mL wash Buffer (WP) and spin down 1300 rpm, 5 min

- 2- Resuspend the pellet in 130 μ L WP
- 3- Add the following mAbs
 - (a) anti-HLA class I mAb (W6/32b): 10 μ L (final concentration 25 μ g/mL)
 - (b) anti-CD2 mAb (OKT11b): 20 μ L (30 μ g/mL)
 - (c) anti-CD4 mAb (OKT4b): 10 μ L (20 μ g/mL)
 - (d) anti-CD3 mAb OKT3b): 30 μ L (44.7 μ g/mL)
- 4- Incubate 30 min, 4°C
- 5- Wash 2x with 5 mL WP, 1300 rpm, 5 min
- 6- Add to the pellet, 10 μ L biotinylated goat anti-mouse (final 100 μ g/mL) + 90 μ L WP
- 7- Incubate 20 min, 4°C
- 8- Repeat step 5-
- 9- Add to the pellet, 30 μ L Streptavidin-APC + 170 μ L WP
- 10- Repeat step 7-
- 11- Repeat step 5-
- 12- Pellet in 5 mL PFA 1%
- 13- The next day, wash 1x with WP and adjust to 1×10^7 cells/mL in WP.
- **Staining of the different populations**
 - 1- Perform cell culture in 96- well U-bottom plates. Each condition in triplicate
 - 2- Stain aliquots of the cell mixtures to be analyzed in 96-well V-bottom plate
 - 3- With the multi-channel pipet, remove 100 μ L of the supernatant to the trash
 - 4- With the same pipet, resuspend carefully (3x) the remaining aliquots (100 μ L) and transfer them into 96-V bottom plate
 - 5- With a second multi-channel, rinse the cell culture plate with 100 μ L PBS
 - 6- Use the 1st multichannel pipet to resuspend and transfer into the corresponding wells in the 96-well V-bottom plate
 - 7- Spin down 1200 rpm, 5 min
 - 8- Block with 10 μ L FcR Blocking solution (Miltenyi Biotec) and vortex
 - 9- Without washing, stain with the mastermix of the mAbs specific for the target populations (each 5 μ L per Ab)
 - 10- Vortex and Incubate 30 min, 4°C
 - 11- Wash with 200 μ L WP, spin down 1200 rpm, 5 min
 - 12- Add 100 μ L of the mix of standard cells + propidium iodide prepared in WP as follow
Standard cells: Stock 1×10^7 cells/mL; Final concentration: 1×10^5 cells/mL
Propidium iodide: Stock 1 mg/mL; Final concentration: 0.2 μ g/mL
 - 13- Resuspend with the multichannel pipet and transfer into FACS tube

14- Measure samples immediately on the flow cytometer. Collect at least 4000-6000 events per sample

- **Determination of the absolute cell number**

To determine the absolute number (N) of viable cells, the following calculation can be performed:

$$N = \frac{\text{Proportion of stained target cells}}{\text{Proportion of APC-labelled standard cells}} \times 10^4$$

The absolute cell number is determined by multiplying the T-cell subset ratio with the number of standard cell per sample, i.e. 10^4 per $100 \mu\text{L}$

Appendix E: Publications and scientific contributions to conferencesParts of this thesis have been published

- **Kouakanou L**, Xu Y, Peters C, He J, Wu Y, Yin Z, Kabelitz D. Vitamin C promotes the proliferation and effector functions of human Gamma Delta T cells. *Cell Mol Immunol* (2019); <https://doi.org/10.1038/s41423-019-0247-8>

Parts of this thesis have been submitted

- **Kouakanou L**, Peters C, Sun Q, Floess S, Bhat J, Huehn J, Kabelitz D. Vitamin C supports conversion of human $\gamma\delta$ T cells into FoxP3-expressing regulatory T cells. (*submitted for publication in Scientific Reports*)

Contributions to other publications

- Peters C, Meyer, **Kouakanou L**, Feder J, Schrickler T, Lettau M, Janssen O, Wesch D, Kabelitz D. TGF- β enhances the cytotoxic activity of V δ 2 T cells. *OncolImmunology* (2019), 8:1
- Bhat J, **Kouakanou L**, Peters C, Yin Z, Kabelitz D. Immunotherapy with human Gamma Delta T cells-Synergistic potential of epigenetic drugs? *Front Immunol* (2018); 9:512
- Bhat J, Helmuth J, Chitadze G, **Kouakanou L**, Peters C, Vingron M, Ammerpohl O, Kabelitz D. Stochastics of cellular differentiation explained by epigenetics: The case of T-cell differentiation and functional plasticity. *Scand J Immunol* (2017); 86(4): 184-195
- Xu Y[#], Xiang Z[#], Alnaggar M[#], Li J[#], **Kouakanou L**[#], Yang J, Hu Y, Chen Y, He J, Lin L, Hao J, Li J, Chen J, Li M, Wu Q, Zhou Q, Li J, Peters C, Liang Y, Wang X, Han B, Ma M, Kabelitz D^{*}, Xu K^{*}, Tu W^{*}, Wu Y^{*}, Yin Z^{*}. Allogeneic V γ 9V δ 2 T-cell immunotherapy helps to prolong the survival of patients with late-stage lung and liver cancer, (*submitted for publication in Clinical Cancer Research*)
([#], ^{*}) equal contribution

Scientific contributions to conferences

- Oral presentation: Phospho-Vitamin C (L-ascorbic acid 2-phosphate) promotes the proliferation and effector functions of human gamma delta T cells. 22nd Joint Meeting of German Signal Transduction Society, November 5-7, 2018, Weimar, Germany
- Poster presentation: Vitamin C increases the potential for cancer immunotherapy. $\gamma\delta$ FOR 2799 Workshop Receiving and Translating Signals via the $\gamma\delta$ TCR, June 12-14, 2019, Erlangen, Germany
- Poster presentation: Increased stability of Foxp3 and superior suppressive capacity of TGF- β -expanded human gamma delta T cells in the presence of Vitamin C. 8th International Gamma Delta T Cell Conference, June 7-10, 2018, Bordeaux, France
- Poster presentation: Modulation of V γ 9V δ 2 T-cell activation by Vitamin C. 47th Annual Meeting of German Society for Immunology (DGfI), September 12-15, 2017, Erlangen, Germany

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit (abgesehen von der Beratung des Betreuers) eigenständig und nur mit der angegebenen Hilfe angefertigt habe. Die Arbeit wurde noch keiner anderen Stelle zur Prüfung vorgelegt. Teile der Arbeit sind bereits in einer Fachzeitschrift veröffentlicht (Kouakanou *et al*, Cell Mol Immunol; 2019 <https://doi.org/10.1038/s41423-019-0247-8>) bzw. in einem weiteren Manuskript eingereicht (Kouakanou *et al*, Sci Rep). Ferner bestätige ich, dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist und dass mir kein akademischer Grad entzogen worden ist.

Kiel, den.....

Léonce Kouakanou

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