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Human Vγ9Vδ2 T cell immune responses towards congenital *Toxoplasma gondii* infection and mistletoe extract drug stimulation

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Abstract

Vγ9Vδ2 T cells are the main circulating γδ T cells in human adult blood. They are known for their T cell receptor (TCR)-dependent recognition of microbe and endogenous-derived non-peptide pyrophosphate antigens (phosphoantigens, PAg). With the intrinsically biased type 1 immune responses, Vγ9Vδ2 T cells are an important force in the defense of infections and tumors. However, the immune responses of Vγ9Vδ2 T cells in early life infections and in immunotherapies are not clear yet. In this thesis, we explored Vγ9Vδ2 T cell immune responses in both aspects.

Vγ9Vδ2 T cells are abundant in human fetal peripheral blood, but compared to their adult counterparts they have a distinct developmental origin, are hyporesponsive towards *in vitro* phosphoantigen exposure and they do not possess a cytotoxic effector phenotype. In order to obtain insight into the role of Vγ9Vδ2 T cells in the human fetus, we investigated in the first part of this thesis their responses upon *in utero* infection with the phosphoantigen-producing parasite *Toxoplasma gondii* (*T. gondii*). Most congenital infections are caused by viruses, *T. gondii* is one of the exceptions. The organelle apicoplast present in *T. gondii* can generate the most potent Vγ9Vδ2 T cell activator. Thus infection *in utero* with *T. gondii* makes it a good model to observe Vγ9Vδ2 T cell immune responses in early life. By comparing to age-matched controls, we found that fetal Vγ9Vδ2 T cells were highly expanded in congenital *T. gondii* infected newborns, and these expanded cells were highly differentiated towards potent cytotoxic effector cells. While the impact of congenital infection on Vγ9Vδ2 T cell expansion and function waned after birth, the Vγ9Vδ2 TCR repertoire of infected infants possessed a clear fetal footprint with public clonotypes, reflecting the Vγ9Vδ2 T cell response *in utero*. Indeed, verification of the antigen recognition related complementarity-determining region 3 (CDR3) of the TCR for γ and δ chain by high-throughput sequencing revealed the enrichment of three Vδ2 sequences in congenitally-infected infants that are already generated at 8 weeks of gestation.

Vγ9Vδ2 T cells possess several characteristics, including MHC-independent recognition of tumor cells and potent killing potential, that make them attractive candidates for cancer immunotherapeutic approaches. In the second part of this thesis we investigated Vγ9Vδ2 T cell responses towards two kinds of hemiparasite plant *Viscum album* L. (European mistletoe) extract drugs *in vitro*. Mistletoe therapy is the most used complementary cancer therapy in European countries. Mistletoe extract drugs are considered to benefit for increasing the quality of life of cancer patients and modulate immune cells, but the mechanism of action is not clear. Here, we investigated in-depth the *in vitro* response of human T cells towards mistletoe extract drugs by analyzing their functional and TCR responses using flow cytometry and high-throughput sequencing respectively. Non-fermented mistletoe-extract drugs (AbnobaViscum), but not their fermented counterparts (Iscador), induced specific expansion of Vγ9Vδ2 T cells among T cells. Furthermore, AbnobaViscum rapidly induced the release of cytotoxic granules and the production of the cytokines IFNγ and TNFα in Vγ9Vδ2 T cells. This stimulation of anti-cancer Vγ9Vδ2 T cells was mediated by the butyrophilin BTN3A, did not depend on the accumulation of endogenous phosphoantigens and involved the same Vγ9Vδ2 TCR repertoire as those of phosphoantigen-reactive Vγ9Vδ2 T cells.

In summary, in the first part of this thesis we showed that the human fetus intrinsically possesses a group of Vγ9Vδ2 T cells that are responding to congenital parasite infections that provide potential protective effects to the fetus. In the second part, we provided insight into the *in vitro* responses of Vγ9Vδ2 T cells towards mistletoe extract drugs, indicating that Vγ9Vδ2 T cells can be an important target in mistletoe therapy.

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Content

Abbreviations

A	
ADCC	antibody-dependent cell-mediated cytotoxicity
ALCAM	activated leukocyte cell adhesion molecule
AM	AbnobaViscum Mali
AP	AbnobaViscum Pini
APC	antigen presenting cell
ATG	autophagy-related protein
B	
BTN	butyrophilin
C	
C	constant gene segment
CDR	complementarity-determining regions
CI	credible interval
CNS	central nervous system
CTB	cytotrophoblast
CTL	cytotoxic lymphocyte
D	
D	diversity gene segment
DC	dendritic cell
DN	double negative stage
DMAPP	dimethylallyl pyrophosphate
D50	percentage of unique clonotypes required to account for 50% of the total repertoire
DNAM-1/CD226	DNAX accessory molecule-1
E	
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EVT	extravillous trophoblast
F	
FasL	Fas ligand
FPS/FPPS	farnesyl pyrophosphate synthase (EC 2.5.1.10)
FRET	Förster resonance energy transfer
G	
GBP	guanylate-binding protein
GDP	gross domestic product
GIS	geographic information system
GM-CSF	granulocyte/macrophage colony-stimulating factor
GzmA	granzyme A
GzmB	granzyme B
H	
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HMG-CoA	hydroxymethylglutaryl CoA
HMBPP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
HMPC	Committee on Herbal Medicinal Products
hsp65	65000MW mycobacterial heat shock protein
I	
ICAM-1	intercellular adhesion molecule-1
ICOS	inducible T-cell costimulator
IFNγ	interferon γ
IFNγR	IFNγ receptor
Ig	immunoglobulin
IL	interleukin

IM	Iscador Malus
IP	Iscador Pinus
IPP	Isopentenyl pyrophosphate
IRG	immunity-related GTPase
ITAM	immunoreceptor tyrosine-based activation motif
IVS	intervillous space
J	
J	joining gene segment
K	
KIR	killer cell immunoglobulin-like receptor
KLR	killer cell lectin-like receptor
L	
LC3	microtubule-associated protein 1 Light Chain 3, ATG8
LFA	lymphocyte function-associated antigen
LRC	leukocyte receptor complex
M	
MCP-1	monocyte chemotactic protein-1
MEP	2-C-methyl-D-erythritol 4-phosphate
MHC	major histocompatibility complex protein
MICA/MICB	MHC-I-like molecules
ML	mistletoe lectin
MVA	mevalonate
N	
NA	North America
N-BP	aminobisphosphonate
NCR	natural cytotoxicity receptors
NK cell	natural killer cell
NKC	natural killer complex
NKG2	natural killer group 2
NO	nitric oxide
P	
PAg	pyrophosphate antigens; phosphoantigens
PAM	pamidronate
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCA	principal-components analysis
PD-1	programmed cell death protein 1
PD-L1	(programmed death-ligand 1
PHA	phytohaemagglutinin
Poly(I:C)	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
PRS	proline-rich sequence
PV	parasitophorous vacuole
R	
RAG-1/2	Recombination activating gene 1/2
ROS	reactive oxygen species
S	
SA	South America
SBA	sec-butylamine
SCID	severe combined immunodeficiency
SIV	simian immunodeficiency virus
STAT1	signal transducer and activator of transcription 1
SYN	syncytiotrophoblast

T

TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
Tfh	follicular T helper
TGFβ	transforming growth factor β
T. gondii	*Toxoplasma gondii*
Th	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TRAIL	tumor-necrosis factor-related apoptosis-inducing ligand
Treg	regulatory T cell
t-SNE	t-distributed stochastic neighbor embedding

U

ULBP	unique long-16-binding protein

V

V	variable gene segment
VA	*Viscum album* L.
Vγ9/TRGV9	γ chain variable region 9
Vδ2/TRDV2	δ chain variable region 2

W

WHO	World Health Organization

Z

ZOL	zoledronate

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1. Introduction

Immunity is about protecting the organisms from invaders and betrayers. It involves many layers of defense: the physical and chemical barriers from mucosal surfaces, the innate immunity, and the adaptive immunity. Innate immunity evolved with early invertebrate, and characterized by pattern recognition and fast responding; while adaptive immunity appeared with the emergence of vertebrate, and the main feature is that it engages with gene rearrangement of the antigen receptors[1]. A typical immune response includes firstly recognition of the pathogen or malignant cells, then the involved immune cells make responses to the recognition by becoming effector cells or stay calm and ignore it. In the adaptive immune system, memory cells record the event so that next time when the same antigen appears, the body will respond faster and more efficiently.

1.1 Human Vγ9Vδ2 T cells

In human blood, there are three cellular elements derived from hematopoietic stem cells of the bone marrow: the oxygen transporter red blood cells, the clots forming platelets, and the immune defender white blood cells. T cells along with B cells and natural killer (NK) cells are coming from the lymphoid lineage of white blood cells. Depending on the receptors expressed on T cell membrane, there are two types of T cells: αβ T cells and γδ T cells (Figure 1); the former contains a heterodimer of α chain and β chain and is the major subset of T cells; the latter contains a γ and δ heterodimer, and represent an average of 4% of human peripheral blood[2]. Among this 4%, those T cell receptor (TCR) γ chain expressing variable region 9 (Vγ9/TRGV9) and paired with a δ chain expressing variable region 2 (Vδ2/TRDV2) are Vγ9Vδ2 T cells. They dominate in adult peripheral blood and mid-gestation of the fetal blood[3] and exhibit innate-like immune features.

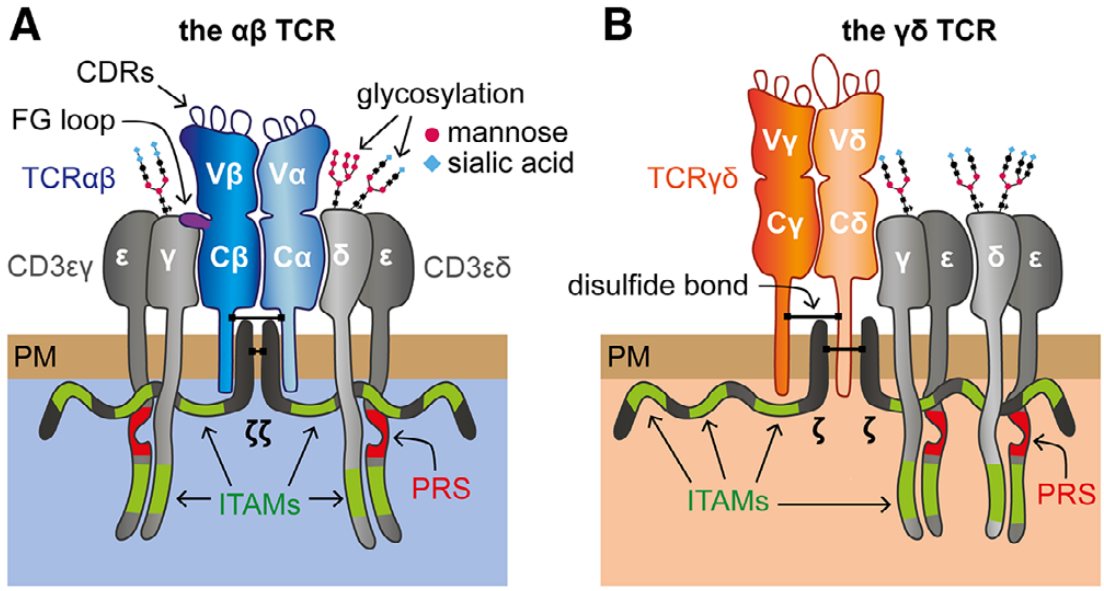


Figure 1 Structure of human αβ and γδ TCRs.[4]

(A) The αβ TCR. The antigen binding heterodimer consisting of TCR-α/TCR-β (TCRαβ) is shown in blue and the CD3 complex in grey. TCRαβ is divided into the variable (V) region with the CDRs that form the antigen binding site and the constant (C) region. The FG loop in TCR-β that communicates with CD3 is shown in violet. CD3 glycosylation patterns and intracellular motifs, such as the immunoreceptor tyrosine-based activation motif (ITAMs, green) and the proline-rich sequence (PRS, red) in CD3ε, are also indicated. **(B)** The γδ TCR. The antigen binding TCR-γ/TCR-δ heterodimer (TCRγδ) is drawn in orange. The CD3 subunits are arranged differently, the FG loop is much shorter, the disulfide-bond between the ligand binding subunits is placed differently and CD3 glycosylation is different. PM, plasma membrane.

1.1.1 Generation of T cell receptor and γδ T cell development

The first and most important step in T cell immunity is the recognition of antigens, this job relies on the unique receptor on T cell membrane. For ‘conventional’ αβ T cells, they receive messages from the antigen presenting protein called major histocompatibility complex proteins (MHC), ‘messages’ from the cell are cut into short peptide and displayed by MHC on cell membrane. There are two classes of MHC molecules, MHC-I and MHC-II, the former one exists on most of the cells and reflects the information of what’s going on inside the cells; MHC-II exists on professional antigen presenting cells (APC), which reflect the information of the ‘problem’ they engulfed. Co-receptors on T cells decide which MHC molecule the T cell will react to. CD8 co-receptor recognizes MHC-I molecule and ring the bell of ‘kill’ to the T cells; T

helper cells which express CD4 co-receptor recognize MHC-II molecule and commit to activate other cells[5].

Each T cell possesses tens of thousands copies of the same TCR with a unique antigen-binding site which determines the antigen it can recognize. Billions of T cells make up a receptor pool, it bears the capacity of recognizing basically every substance that an organism would encounter. To generate such a great amount of diversity, the organisms use the strategy of permutation, by which receptor genes lie on the genome in the form of gene segments (Figure 2A-B). For α chain and γ chain, each is encoded by three sets of gene segments: variable (V), joining (J), and constant (C); while for β and δ chain, there is also a diversity (D) gene segment[5]. The antigen-binding site of TCR is constructed by three hypervariable loops called complementarity-determining regions (CDR) (Figure 2C), CDR1 and CDR2 are generated from germline V gene segments of each chain, and are considered to be more conserved since they mainly contact the relatively less variable MHC component of the ligand. CDR3 is formed by V(D)J junctions and modified by random deletion and addition of P- (template) or N- (non-template) nucleotides to each V-D or D-J joint, this makes it the most diverse region in the TCR. γδ T cells have less gene segment choices than αβ T cells, but they compensate with more N diversity regions. TRDD gene segments can be translated in any reading frame and even allow D-D joint, it dramatically increases the junctional diversity[6]. Recombination activating gene 1 (RAG-1) and RAG-2 complex, which is the lymphoid-specific components of the V(D)J recombinase and is unique to jawed vertebrates[7], initiates the splicing of gene segments; the enzyme terminal deoxynucleotidyl transferase (TdT) is responsible for the N-nucleotides addition to the single-strand ends, and together with other proteins in the recombinase complex carry out the complete somatic V(D)J recombination process[5].

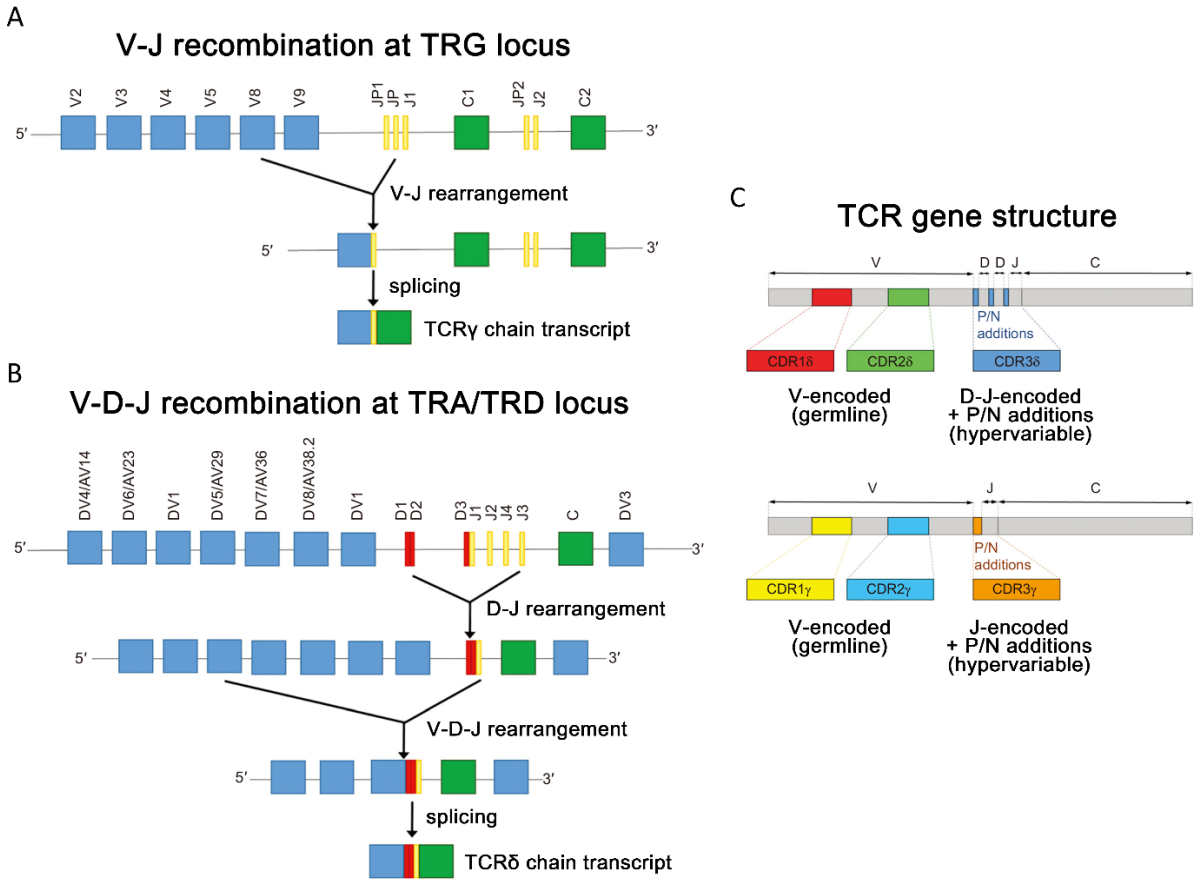


Figure 2 V(D)J recombination and CDR loops.[8]

(A) TCRγ recombination. The TCR-γ chain is generated by a single V-J recombination. **(B)** TCRδ recombination. The TCR-δ chain undergoes two steps of gene segment rearrangement that can involve either 2 or 3 D segments. P/N additions exist in the junctional region of both γ and δ chain. Gene segments that can be used in TCR-δ chain rearrangement are indicated. Only the functional gene segments are shown in **(A-B)**. The organization of loci TCRγ and TCRα/TCRδ was adapted from IMGT database. **(C)** γ and δ chain mRNA architecture. The CDR loops are colour-coded.

V(D)J recombination of T cells happens in the thymus. T cell progenitors from bone marrow (in the fetus, progenitors also come from fetal liver[9]) migrate to thymus and mature there. These developing T cells are also called thymocytes. Thymocytes undergo a series of stages to become 'qualified' T cells, and these stages are characterized by different surface molecules. T cell progenitors can be detected as early as in the 8th week of gestation in the human fetal thymus[10,11], they lack the CD3:TCR complex and the co-receptors CD4 or CD8, and are facing the choice of either becoming αβ T cells or γδ T cells. This is the first stage called double negative (DN, with no CD4 and CD8 expression) stage. There is a competition going on at the

beginning of this stage. TCR-β, TCR-γ, and TCR-δ chain start to undergo recombination at the same time, and the fate of the cell is determined by the first receptor expressed on the membrane. If the cell expresses a functional γδ receptor, then it leaves the thymus without expressing CD4 and CD8 and later become a γδ T cell. Interestingly, different from the more thymus-dependent αβ T cells maturation, γδ T cells have extra-thymus origins, and Vγ9Vδ2 T cells are found in pre-thymic fetal liver and intestine at around 5-7.5 weeks of gestation[12,13], and in fetal thymus at as early as 8 weeks of gestation[13].

1.1.2 Activation of Vγ9Vδ2 T cells

1.1.2.1 Vγ9Vδ2 TCR repertoire

Human TCR-γ and TCR-δ gene locus lie on chromosome 7 and chromosome 14 respectively[14,15]. As mentioned earlier, γ chain is formed by V-J rearrangement while δ chain is generated from V-D-J rearrangement. In general, the γ CDR3 chain is shorter and more restricted than the δ CDR3 chain[16,2]. Circulating γδ TCR is skewed to be formed by Vγ9 chain paired with a Vδ2 chain[17]. Among the five J gene segments, within Vγ9Vδ2 T cells, Vγ9 is often rearranged with JγP (Vγ9-JγP/TRGV9-TRGJP) (Figure 2A)[18,17,19,20]. Further sequencing studies have found a highly repeated germline (no N- or P- addition) Vγ9-JγP clonotype: CALWEVQELGKKIKVF, which presents in both fetal and adult peripheral blood and irrespective of age, sex, and race[21,22,3,23]. Comparing to Vγ9 chain, Vδ2 chain is highly diverse and shows a difference in the preferential usage of J gene segments in adult and fetal. Jδ1 is predominant in adult circulating Vγ9Vδ2 T cells, and Jδ3 is more used in fetal Vγ9Vδ2 T cells[19,20,23,24]. Also, N nucleotides insertion is higher in adult Vγ9Vδ2 T cells in both Vγ9 chain and Vδ2 chain[23]. In addition, certain clonotypes (CACDVLGDTD, CACDTGGYTD) are abundant and shared in pre-thymic Vγ9Vδ2 T cells from fetal liver and fetal intestine[12,13]. Further, an N-addition generated hydrophobic amino acid (normally Val/Leu/Ile) is typically found at position 97 (position 5 of CDR3) of

peripheral Vδ2 sequences[20,25,26,23,27].

1.1.2.2 TCR-dependent stimulation

Unlike the 'conventional' αβ TCR which recognize processed peptide antigens presented by MHC molecules, Vγ9Vδ2 TCR recognize non-peptide pyrophosphate antigens (phosphoantigens, PAg) in an MHC-independent manner. The prevalent Vγ9-JγP rearrangement and the pairing with Vδ2 gene segment are important for PAg recognition[28]. Based on structural and mutation studies, the preserved lysine (Lys, K) residues (KKIK, start at position 108, the first two Lys residues are found in most primates and they are associated with PAg activation, especially Lys108; the last Lys is preserved universally in all types of J region of both primate and rodent and is related to CD3:TCR formation) in JγP and the hydrophobic residue at position 97 in Vδ2 joint region are crucial for PAg activation[29,30,25,26].

The crystal structure of Vγ9Vδ2 TCR indicates a positively-charged pocket which is related to PAg binding[30,31]. Also, the rougher surface of the binding area suggesting the ligand is rather a small molecule antigen and if any a different presenting molecule other than the MHC complex[31]. Indeed the need for all CDR loops to be involved in activation suggest the binding region is larger than just for binding of IPP or HMBPP[26]. These fit with the description of PAg molecule (which is negatively charged and with low molecular weight) accompanied with the butyrophilin-family related antigen recognition.

1.1.2.2.1 PAg generated from the MVA and MEP pathway

The search for Vγ9Vδ2 T cell activators can be traced back to early 1980s, CD4CD8 double negative subset of T cells was first found in response to *Mycobacterium tuberculosis* patients[32], and this triggered the later studies to look at γδ T cell (which is normally CD4-CD8-) responses towards mycobacteria[33,34]. Human γδ T cells were found to react to mycobacteria-derived antigens with a small molecular weight and

resistant to protease digestion[34,35], and later on these mycobacteria reacting γδ T cells were pinpoint to the Vγ9Vδ2 subset with TCR-dependent recognition and the ligands were found to be phosphorylated molecules[36-38]. The first natural PAg of Vγ9Vδ2 T cells was identified from the mycobacteria extracts called isopentenyl pyrophosphate (IPP) (Figure 4)[39].

IPP along with its isomer dimethylallyl pyrophosphate (DMAPP) are the two crucial building blocks of all isoprenoids[40]. Isoprenoids are a huge class of natural compounds which exist in all living organisms. They are important metabolites in many different biological activities, such as sterols (stabilizing plasma membrane for most eukaryotes and precursors of hormones in vertebrates), carotenoids (photosynthetic apparatus constituents in plants), and quinones (electron carriers in electron transport chain)[41,40].

There are two pathways for the biosynthesis of IPP and DMAPP: the first pathway was discovered in the 1950s in yeasts and cholesterol synthesis in liver tissues which named the mevalonate (MVA) pathway (Figure 3, left); the second pathway was discovered in the 1990s in bacteria, green algae, and plants and named after the first biosynthetic intermediate 2-C-methyl-D-erythritol 4-phosphate (MEP) (Figure 3, right)[42,41,43,40]. MEP pathway is absent from archaebacteria, fungi, and mammals but is the only one present in most eubacteria, green algae, cyanobacteria, and Apicomplexan parasites; while plants utilize both pathways with the MVA pathway occurs in cytosol and mitochondria and the MEP pathway in chloroplasts[43-46].

At the time that IPP was identified as Vγ9Vδ2 T cells natural stimulant, the last step was still missing for the MEP pathway (Figure 3, right). Studies observed that those bacteria with MEP pathway can induce higher Vγ9Vδ2 T cells proliferation and it was not due to the IPP from the lysates, since IPP level either under the detecting threshold or lower than the minimum concentration for Vγ9Vδ2 T cells activation[47,48]. And at the time for the last two genes from the MEP pathway uncovered[49,50], gene

mutation studies demonstrated the unknown Vγ9Vδ2 T cell antigen is generated from the last steps of MEP pathway[51,52] and finally identified as (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate(HMBPP) (Figure 4)[53], which is at least 10,000 times more potent than IPP as an activator.

Thus, both MVA and MEP pathways can generate IPP while only MEP pathway generates HMBPP. And, since the MEP pathway does not exist in mammals and Vγ9Vδ2 T cells are more sensitive to HMBPP, it becomes a potent indicator for pathogen invasion.

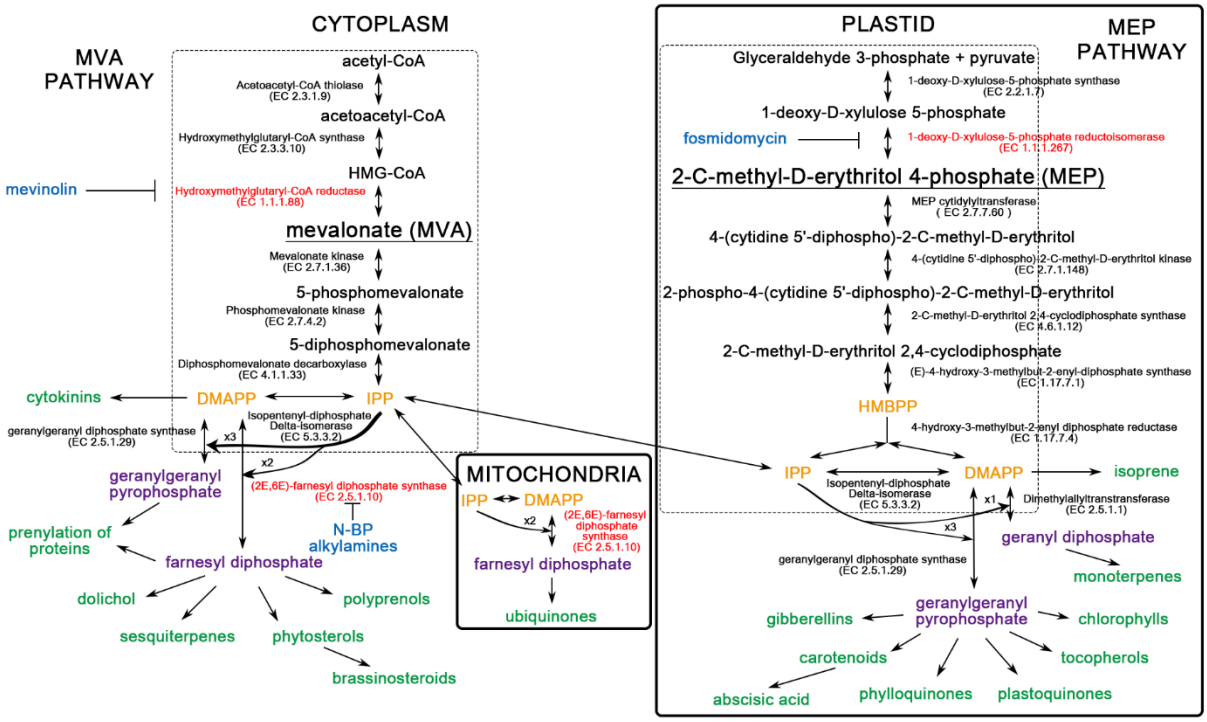


Figure 3 Isoprenoid biosynthesis pathways in the plant cell.[43]

The first intermediate specific to each pathway is underlined. Enzymes are indicated with enzyme commission number. The steps specifically inhibited by mevinolin, N-BP, alkylamines, and fosmidomycin are indicated in red. Steps downstream of MVA and MEP pathway are indicated with purple and green.

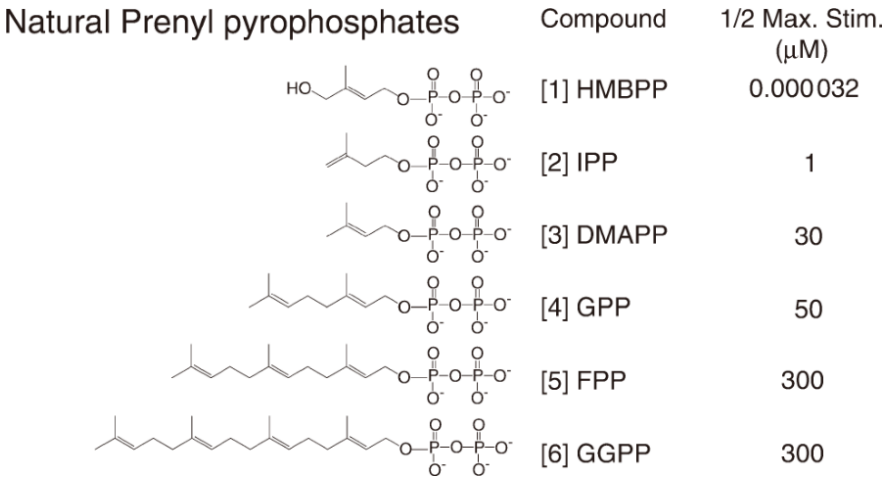


Figure 4 Structure and activity of prenyl pyrophosphate antigens and analogs.[44]
Structure and biological activity of various prenyl pyrophosphates is shown. Values are the concentration required for half-maximum stimulation of proliferation for the Vg2Vd2 T-cell clone 12G12.

1.1.2.2.2 Other TCR-dependent activators

In addition to PAg, aminobisphosphonates (N-BP) and alkylamines indirectly activate Vγ9Vδ2 T cells in a TCR-dependent manner. N-BP, such as pamidronate (PAM) and zoledronate (ZOL), are a class of drugs used for treating bone resorption diseases, they are synthetic analogues of endogenous pyrophosphate[54,55]. Not all alkylamines can induce Vγ9Vδ2 T cells activation, those antigenic alkylamines can be found in bacteria, plants (e.g. apple), wines, and human body fluids (such as urine from tea drinkers, breast milk, amniotic fluid, vaginal secretions, with a much higher concentration than activation threshold)[56]. Both N-BP and antigenic alkylamines work as inhibitors of farnesyl pyrophosphate synthase (FPS, or in some paper termed as FPPS) (Figure 3, left), an enzyme downstream of IPP synthesis[57-61], and this leads to the accumulation of endogenous IPP and then the activation of Vγ9Vδ2 T cells.

1.1.2.2.3 The BTN family

PAg can induce proliferation, cytokine production, and cytotoxic effects of Vγ9Vδ2 T cell clones directly without other accessory cells (e.g. APC), but cell-cell contact is essential and APC (such as monocytes) enhances the activation[62]. Since PAg and N-BP work differently, earlier studies found N-BP pulsed tumor cells and APC can

activate Vγ9Vδ2 T cells while PAg pulsed cells cannot. Also, these accessory cells must have a human origin, and this species specificity was also true for continuous stimulation with PAg[63,64]. This indicates that the activation is not by PAg directly binding to the TCR but rather associated with human-derived molecules to initiate the activation. This putative 'presenting' molecule is different from the ones commonly recognized by αβ T cells[62]. The structural study of Vγ9Vδ2 TCR also supports the possible presenting molecule does not belong to MHC class molecules[30].

As it is well known for conventional T cells, in order to sufficiently activate the naive T cells, co-stimulatory receptors are needed (Figure 5). The best understood co-stimulatory receptor is CD28, which recognizes B7 family (B7.1 as CD80, B7.2 as CD86) co-stimulatory ligands that are commonly expressed on APC and are upregulated after the APC encountering with pathogens (Figure 5-6)[65]. Thus in the search of B7 family related molecules, studies identified the human butyrophilin (BTN) family, which clustered in the extended MHC-I region in chromosome 6 and found to be regulator of immune responses[66,67].

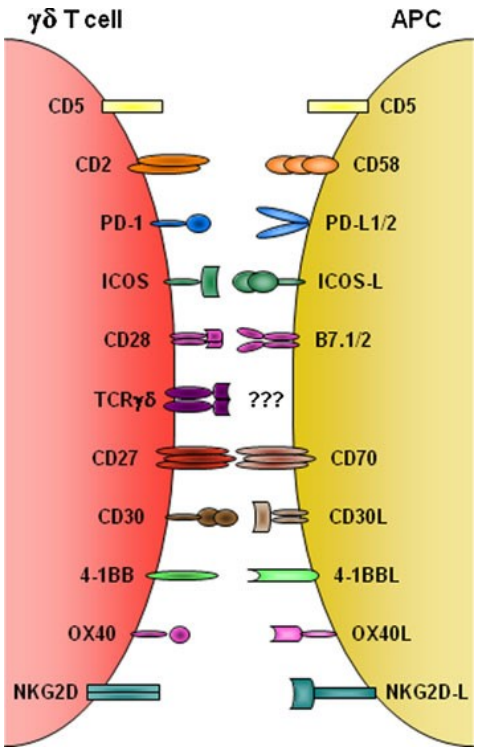


Figure 5 Costimulatory receptors on γδ T cells and corresponding ligands on antigen-presenting cells.[68]

The main costimulatory receptors expressed on human and/or mouse γδ T cells are indicated. Except PD-1 and CD5, the other coreceptors provide positive signals that enhance (TCR-driven) γδ T cell proliferation and/or cytokine production. The ligand of each coreceptor is expressed on a variety of possible ‘‘antigen presenting cells’’ (APC), such as dendritic cells, epithelial cells or activated lymphocytes.

Figure 6 Structural organization of human BTN and B7 family.[69,67]

TM, transmembrane.

BTN and BTN-like molecules belong to the immunoglobulin (Ig) superfamily, which is typically characterized by two Ig-like extracellular domains (IgV, IgC), a transmembrane domain and for most members also an intracellular B30.2 domain

(Figure 6)[69,67]. In humans, there are seven BTN genes that can be grouped into three subfamilies: *btn1* (*btn1a1*), *btn2* (*btn2a1, btn2a2, btn2a3*, with *btn2a3* is a pseudogene) and *btn3* (*btn3a1, btn3a2, btn3a3*) (Figure 6); among these genes, *btn1a1* is considered to be ubiquitously present in placental mammals, *btn2* and *btn3* subgroups are more exclusively to primates, and for rodent lineage, *btn2* only have one gene, which is the ortholog for human *btn2a2* [69-71].

Together knockdown the three isoforms of BTN3 (CD277) on target cells abolish Vγ9Vδ2 T cells activation towards the N-BP pretreated target cells. Re-expression of each isoform on target cells indicates BTN3A1 is crucial for Vγ9Vδ2 T cells activation, while only present BTN3A2 or BTN3A3 on target cells does not restore the activation[72,73]. One possible model could be that BTN3A1 acts like a classic presenting molecule to present PAg through the more conserved extracellular IgV domain to Vγ9Vδ2 TCR. However, the binding affinity is low, and the crystal structure shows the possible PAg binding site is shallower than the classic MHC:peptide binding site[74]. On the other hand, a BTN3 mAb (monoclonal antibody, clone 20.1) was found to indirectly induce Vγ9Vδ2 T cells activation through all the three isoforms without accumulation of IPP in the target cells, truncate the intracellular domain B30.2 of BTN3A1 on target cells diminishes the N-BP induced activation (IPP accumulate inside the target cells) of Vγ9Vδ2 T cells, but not by 20.1 mAb; and N-BP pretreated target cells expressing chimeric CD277 with the extracellular of BTN3A3 and B30.2 of BTN3A1 also activates Vγ9Vδ2 T cells. These prompt another model in which PAg is sensed by the intracellular B30.2 domain of BTN3A1 causing conformational changes that mimicked by 20.1 mAb[72]. Later studies supported this model. B30.2 has a deep and highly basic pocket on the surface formed by three arginines (Arg412, Arg418 and Arg469), two histidines (His351 and His378) and one lysine (Lys393), which is highly attractive to the negatively charged pyrophosphate group[75]. Mutations to this pocket abrogate the binding of PAg and the activation of Vγ9Vδ2 T cells. A single mutation from Arg351 to His351 gives BTN3A3 (which also has a B30.2 domain but

with a ~70 amino acid tail) the ability to bind PAg and activate Vγ9Vδ2 T cells.

There is little evidence for BTN3A1 directly interacting with Vγ9Vδ2 TCR. No direct binding has been detected by surface plasmon resonance test and BTN3A1-transfected murine cell lines do not activate Vγ9Vδ2 T cells in the presence of PAg or 20.1 mAb[75,74]. These indicate that BTN3A1 is crucial but insufficient for Vγ9Vδ2 T cell activation. Complete activation might involve other molecules lay in the human chromosome 6[76]. Recently, by using Vγ9Vδ2 TCR tetramers (with high affinity and specificity to recognize receptor-ligand interaction, in this context, a Vγ9Vδ2 TCR tetramer recognizes the unknown BTN-PAg complex) generated from PAg-reactive Vγ9Vδ2 T cells, BTN2A1 was found to directly interact with the Vγ9Vδ2 TCR[77]. Thus, BTN2A1 and BTN3A1 are both crucial to Vγ9Vδ2 T cells activation. Together transfect BTN2A1 and BTN3A1 to murine cells obtains the ability to activate Vγ9Vδ2 T cells towards PAg, while BTN3A2 enhances the activation but not necessarily needed. In addition, Förster resonance energy transfer (FRET) shows BTN2A1 and BTN3A1 are close to each other both on the cell membrane and intracellular domains[77]. From the opposite perspective, BTN2A1 tetramers bind to all Vγ9+ cells in regardless of δ chain pairing (both Vδ1+ and Vδ2+). Further tests indicate BTN2A1 binds to the side of Vγ9 chain (Arg20, Glu70 and His85), distal to δ chain and CDR loops, via germline-encoded regions[77,78]. As to PAg reactivity, except the residues crucial for BTN2A1 binding, another two closely neared residues, Lys108 (one of the conserved Lys residues found in germline Vγ9) from CDR3 loop of Vγ9 and Arg51 from CDR2 loop of Vδ2 are also important. Mutations from these residues abrogate Vγ9Vδ2 T cells activation by PAg and this indicates a second binding site beyond BTN2A1[77,78].

BTN3 is coevolved with Vγ9Vδ2 T cells[70]. It is broadly expressed on human tissues including immune cells and also expressed by many tumor cells (https://www.proteinatlas.org/ENSG00000026950-BTN3A1)[79]. This greatly enlarges the range of immune surveillance by Vγ9Vδ2 T cells.

1.1.2.3 Co-receptors and their ligands

As an 'unconventional' T cell, except activation through TCR, γδ T cells also express receptors that typically belong to innate immune cells[80-82]. Once a pathogen breaks the epithelial barrier, innate immune cells, including the phagocytic macrophages, granulocytes, dendritic cells, are the first immune cells to react. Unlike T cells and B cells, innate immune cells use invariant pattern recognition receptors (PRR) to distinguish the 'non-self', which recognize unique patterns (such as lipoteichoic acids on Gram-positive bacteria, lipopolysaccharide on Gram-negative bacteria) exhibited on common pathogens. These recognized molecules usually termed as pathogen-associated molecular patterns (PAMP). In response to this pattern recognition, innate immune cells produce chemokines to recruit more immune cells to the infection site; produce cytokines to upregulate adhesion molecules which are important for the migration process and cell-cell contact, as well as cytokines that can activate NK cells or innate-like lymphocytes and upregulate B7 co-stimulatory ligands to prepare for adaptive immunity. Some of the important innate receptors used by Vγ9Vδ2 T cells including typical PRR (such as Toll-like receptors (TLR)), NK receptors (NKG2D), adhesion molecules, and Fc receptors are described in more detail below.

1.1.2.3.1 Toll-like receptors

Humans have 10 functional *TLR* genes, with TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 expressed on the cell surface and TLR3, TLR7, TLR8, TLR9 located on intracellular endosomes of innate immune cells (such as dendritic cells, monocytes, NK cells) and epithelial cells[5]. The TLR monitors threats from extracellular bacterial lipids to virus nucleic acids (single-stranded RNA, double-stranded RNA or DNA of unmethylated CpG) already invaded inside the cells[83,84]. Some TLR are also expressed on T cells and showed co-stimulatory effects. Resting Vδ2 T cells (the majority of Vγ9+ or Vδ2+ T cells are Vγ9Vδ2 T cells, but to be precise, will only indicate the antibody mentioned in references unless both antibodies were included) express TLR1, 2, 3, 6, 7 at RNA

level and TLR2, 3, 6, 8 are detected by flow cytometry at the protein level[85]. TLR2 agonist (Pam_3Cys) enhances Vγ9Vδ2 T cells IFNγ production and upregulates CD107a (lysosome-associated membrane protein, an indicator for degranulation) expression in conjunction with anti-TCR stimulation[86]. Peripheral isolated γδ T cells (presumably mainly Vγ9Vδ2 T cells) were reported to express TLR3 intracellularly and stimulation with TLR3 ligand (synthetic polyinosinic-polycytidylic acid (Poly(I:C))) upregulated surface expression of TLR3 and enhanced IFNγ secretion during TCR stimulation[87,88]. Pretreated tumor cells with Poly(I:C) or TLR7 surrogate ligand (imiquimod) increases Vγ9Vδ2 T cell cytolysis to the tumor cells, and this is further enhanced by pretreating Vγ9Vδ2 T cells with PAg[89]. TLR8 surrogate ligand TL8-506 and TLR7/8 surrogate ligand resiquimod induce Vγ9Vδ2 T cell (tested on peripheral blood mononuclear cells (PBMC), not on purified Vγ9Vδ2 T cells) IFNγ production in the absence of PAg 6 hours after stimulation and synergized in the presence of PAg after 12 hours stimulation, but inhibit purified Vγ9Vδ2 T cell expansion in the presence of purified monocytes and ZOL or PAg when added at the beginning of coculture. The inhibition might be due to increased monocyte cell death after stimulating with TL8-506 or resiquimod thus failed to provide accessory functions and also upregulate monocyte PD-L1 (programmed death-ligand 1) expression to induce T cell exhaustion[90]. Moreover, although TLR4 expression is not detected on Vγ9Vδ2 T cells[85], these cells produce significant and exclusive IFNγ after co-culture with short-term TLR4 ligand (LPS)-stimulated immature DC[91].

1.1.2.3.2 NK receptors

NK cells come from the same progenitor cells as T and B lymphocytes. They are armed with cytotoxic granules, monitor the level of MHC-I molecules on target cells, and produce cytokines to regulate adaptive immunity[5]. The ‘kill or not to kill’ decision of NK cells depend on the overall signaling coming from germline-encoded activating receptors and inhibiting receptors. Structurally there are two classes of NK receptors: Killer Ig-like receptors (KIR, encoded from leukocyte receptor complex (LRC) gene

cluster), killer lectin-like receptors (KLR, encoded from natural killer complex (NKC) gene cluster)[92]. The earliest detected and exclusively expressed on NK cells are the natural cytotoxicity receptors (NCR, Ig-like receptors), including NKp30, NKp44, and NKp46, which all belong to activating receptors[93]. NKp44 is found expressed on cytokine-induced activated γδ T cells (majority is Vγ9Vδ2 T cells) after 7 days of culture and influences the cytolysis capacity to myeloma cell lines[94]. KIR contain both activating and inhibitory receptors. The inhibiting KIR2DL1 (CD158a) was significantly less on IFNγ producing Vγ9Vδ2 T cells after *in vitro* malaria parasite infection[95]. The most studied NK receptor in γδ T cells is NKG2D, which belongs to natural killer group 2 (NKG2) and is a member of KLR. NKG2D is expressed on NK cells, CD8 T cells, and γδ T cells[96]. Different from other NKG2 members which form a heterodimer with CD94 on the membrane and bind to MHC-I molecule, NKG2D recognizes stress-induced MHC-I-like molecules (MICA, MICB) and unique long-16-binding proteins (ULBP). NKG2D works as a co-stimulatory receptor to enhance Vγ9Vδ2 T cell cytolysis and IL-2 production when coculture with MICA expressed tumor cells[97]. ULBP4 induces tumor-infiltrating Vδ2 T cells proliferation, type-I cytokine production, and tumor cytolysis in TCR and NKG2D dependent manner[98]. There was a report suggesting NKG2D can independently activate Vγ9Vδ2 T cells[99]. Moreover, NK receptors such as NKG2A, NKG2C, NKG2D, and CD158 are highly expressed on mid-gestation fetal γδ T cells compared to αβ T cells; also, NKp30 was detected in RNA level in fetal peripheral Vγ9Vδ2 T cells, which was not found expressed on adult blood (in protein level) but could be induced on Vδ1 γδ T cells after phytohaemagglutinin (PHA) stimulation[3,100].

1.1.2.3.3 Adhesion molecules

Cell-cell contact is needed for activating Vγ9Vδ2 T cells and is vital for performing cytotoxic activity. Cytokines produced from the innate immune cells at the site of inflammation upregulate adhesion molecules on naïve T cells and enhance their ability to interact with APC or the target cells[101]. Typical adhesion molecules include

lymphocyte function-associated antigen (LFA) from integrin family and Ig superfamily. LFA-1, CD2 (LFA-2), and LFA-3 (ligand of CD2) are all found to be expressed on Vγ9Vδ2 T cells[102]. Antibody blocking tests showed that LFA-1 is important for the cytotoxicity of Vγ9Vδ2 T cells, while CD2:LFA-3 influence Vγ9Vδ2 T cell proliferation and TNFα production but not cytolysis of tumor cells[102]. LFA-1 ligand such as intercellular adhesion molecule-1 (ICAM-1) is highly expressed on certain tumor cell lines. N-BP pulsed tumor cells conjugate with Vγ9Vδ2 T cells. Blocking LFA-1 of Vγ9Vδ2 T cells or ICAM-1 of tumor cells inhibit conjugation, IFNγ production, and cytotoxicity, but LFA-1:ICAM-1 interaction is not sufficient for Vγ9Vδ2 T cell activation[63,103-105]. Also, CD6 (a member of the scavenger receptor family) is highly expressed on Vγ9Vδ2 T cells[106]; its ligand CD166 (ALCAM, activated leukocyte-cell adhesion molecule) is broadly expressed on human tumor cell lines[106]. CD6:CD166 is related to synaptic conjugation and colocalized with CD3:γδTCR complex after presenting N-BP pretreated tumor cells to Vγ9Vδ2 T cells[106]. In addition, DNAX accessory molecule-1 (DNAM-1/CD226), an adhesion glycoprotein, is constitutively expressed on resting and activated Vγ9Vδ2 T cells, and is associated with increased effector functions to tumors (such as hepatocellular carcinoma) express Nectin and Nectin-like molecules[107].

1.1.2.3.4 Fc receptors

Fc receptors bind to the constant region (also known as fragment crystallizable (Fc) region) of an antibody. They are typically expressed on a variety of innate immune cells, such as the phagocytic macrophages and dendritic cells, and nonphagocytic NK cells. Fc receptors recognize antibody neutralized pathogens and enhance the uptake of pathogens by phagocytic cells and trigger the secretion of cytokines and cytotoxic molecules (antibody-dependent cell-mediated cytotoxicity, ADCC) by NK cells. CD16 (FcγRIII, a low affinity type 3 receptor) is expressed on NK cells and stimulated Vγ9Vδ2 T cells. After activation through Vγ9Vδ2 TCR, CD16 expression is gradually upregulated; cross-linking CD16 through immobilized anti-CD16 mAb or IgG-coated

cells induce a higher level of TNFα production[107]. Terminally differentiated CD27-CD45+CD16+ Vγ9Vδ2 T cells decrease the response to TCR stimulation but react through CD16 ligation for IFNγ and TNFα production and acquire higher cytotoxicity[108]. CD16 expression on Vγ9Vδ2 T cells is related to better cytotoxic effects to infected cells and tumor cells[109-112].

1.1.3 Vγ9Vδ2 T cell immune responses

Vγ9Vδ2 T cells sense the trace from HMBPP-generating invaders and monitor the level of IPP within transformed cells. These make the major circulating γδ T cell an important immune effector in controlling both infectious diseases and tumors. Beyond these, Vγ9Vδ2 T cells also have influences on other aspects, such as the regulation of other immune cells[113].

1.1.3.1 Vγ9Vδ2 T cell immune responses in adults

1.1.3.1.1 Activation, expansion and differentiation

It seems that Vγ9Vδ2 T cells are designed to react to intracellular bacteria and parasite infections, as these pathogens normally produce the most potent Vγ9Vδ2 stimulant: HMBPP. Once the cells have been hijacked to become the 'replication factory', the endogenous MVA pathway has also been influenced by some of the pathogens[114,115], this increases the susceptibility of the infected cells to be sensed by Vγ9Vδ2 T cells. As mentioned previously, γδ T cells constitute around 4% of peripheral blood T cells. However, when the human body encounters certain bacteria or apicomplexan parasites, Vγ9Vδ2 T cells can expand to a very high level and become the major T cells in some individuals[44]. In contrast, the expansion dynamics of Vγ9Vδ2 T cells are different in viruses infections. Reports show that Vγ9Vδ2 T cells from rhesus monkeys experience a short proliferation after the initial simian immunodeficiency virus (SIV) infection *in vivo*[116]; in humans, Vγ9Vδ2 T cells were found to proliferate towards human immunodeficiency viruses (HIV)-infected cells *in*

vitro[117,118]. Vγ9Vδ2 T cells are gradually decreased in chronic infection stages[117,116] and their frequency is associated with the disease progress. For example, higher Vγ9Vδ2 frequency in the blood is found in asymptomatic hepatitis B virus (HBV) infected patients and people recovered from coronavirus infections[119-121]. *In vitro* study also observed proliferation toward Epstein-Barr virus infected cells[122].

Vγ9Vδ2 T cells have been found activated in both acute and chronic infections by upregulating activation markers such as CD69, HLA-DR, CD25, CD38[123-125]. Also, Vγ9Vδ2 T cells are differentiated, expressing higher CD45RO[126], exhibiting effector phenotype(CD27-CD45RA+) during infections[127,123,128] and effector memory phenotype(CD27-CD45RA-) in cancer patients[129,130].

1.1.3.1.2 Cytotoxicity

Vγ9Vδ2 T cells use different mechanisms to lyse pathogen-infected cells and tumor cells. One common way is through granule exocytosis, which is associated with upregulated CD107a, perforin, and granzymes upon infection and stimulation with tumor cells[109,124,129,131,130]. Vγ9Vδ2 T cells from cancer patients have reduced reactivity to stimulant compare to healthy controls[129]. In addition, Vγ9Vδ2 T cells express a high level of Fas (CD95, containing a death domain in its cytoplasmic region, which is essential for the induction of apoptosis) and low level of Fas ligand (FasL)[132]. Blockade of FasL reduces the cytotoxicity of Vγ9Vδ2 T cells against viruses (such as influenza A) infected macrophages and lung alveolar epithelial cells or IFNγ pretreated tumor cells, indicating that the Fas-FasL pathway is involved in this cytotoxic process[133-135]. Also, tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) is involved in the lysis of influenza A virus infected lung alveolar epithelial cells and Epstein-Barr virus-transformed lymphoblastoid B cells by Vγ9Vδ2 T cells[134,136]. Further, as infected cells and tumor cells often express NK receptor ligands such as MICA/B and ULBPs, Vγ9Vδ2 lysis capacity is also associated with NK receptors, blockade of NKG2D leads to reduced cytotoxicity to infected and abnormal

cells[122,133,137,138,136].

1.1.3.1.3 Secretions

1.1.3.1.3.1 Cytokines

Vγ9Vδ2 T cells can differentiate into different types of functioning cells according to the stimulation environment, including Th1-like, Th2-like, follicular T helper cells (Tfh)-like, Th17-like, or regulatory T cells (Treg)-like characteristics[113].

Upon infections and tumor stimulations, the intrinsic reaction of Vγ9Vδ2 T cells is to produce Th1 cytokines such as IFNγ and TNFα[127,139-142,138,103]. But this reaction is reduced in patients with chronic infections and tumors during ex vivo stimulation[119,140,129].

Vγ9Vδ2 T cells can be primed into Th2-like cells by IPP or Daudi cell stimulations within an IL-4 and anti-IL-12 condition, and this reaction is characterized by increased IL-4 production and weak IFNγ, TNFα production after long time culture[143].

Studies have found upon PAg stimulation, peripheral CXCR5+CD27+ Vγ9Vδ2 T cells gradually upregulate B cell co-stimulatory markers such as ICOS (Inducible T-cell COStimulator) and CD40 ligand, produce more IL-4 and IL-10 compared to CXCR5- counterpart, and CXCR5+ Vγ9Vδ2 T cells are highly enriched in inflammation site. These Tfh-like cells provide help to both plasma B cells and naïve B cells to produce IgG, IgA, and IgM[144,145].

IL-17+ producing Vγ9Vδ2 T cells are at a very low level in peripheral blood, but increased in infections and autoimmune diseases. For example, in the blood and cerebrospinal fluid of children with bacterial meningitis, IL-17+ Vγ9Vδ2 T cells are significantly higher than the healthy controls, and antibacterial treatment reverse this change[146,147]. *In vitro*, IPP-induced effector memory (CD27-CD45RA-) Vγ9Vδ2 T cells increase IL-17 secretion in long term culture[147].

Also, upon IPP stimulation and the presence of TGFβ and IL-15, Vγ9Vδ2 T cells can be polarized to Treg-like cells characterized by FOXP3 expression, TGFβ production, and inhibiting PBMC proliferation *in vitro*[148].

1.1.3.1.3.2 Chemokines

Chemokines are important for cell migration, differentiation, lymphoid organ development, neuronal activities, and they are also involved in pathology of diseases, such as tumor development and metastasis, infections control, and autoimmune diseases in the central nervous system[149].

PAg or live *Helicobacter pylori*-stimulated Vγ9Vδ2 T cells produce CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES). Blockade of these chemokines reduces the inhibition of HIV replication *in vitro*[118,141]. Intralesional Vγ9Vδ2 T cells produce CXCL8, CCL3, and CCL5 upon ex vivo stimulation[150]. Also, differential chemokine production has been seen in avian and human seasonal influenza A viruses by Vγ9Vδ2 T cells[151].

Chemokine receptors, such as CCR1, CCR5, and CXCR5, on activated Vγ9Vδ2 T cells are also upregulated upon influenza A virus infection; blocking CCR5 inhibits Vγ9Vδ2 T cells migrate to the infection site[151]. Also, peripheral and tumor-infiltrating Vγ9Vδ2 T cells exhibit different chemokine profiles. Renal cell carcinoma patients have less CXCR3 and increased CXCR1, CXCR4 on circulating Vγ9Vδ2 T cells, blockade of CXCR3 and CXCR4 inhibits tumor-infiltrating Vγ9Vδ2 T cell migrate to tumor cells[130].

1.1.3.1.3.3 Antimicrobial peptide

Antimicrobial peptides are one of the most ancient forms of defense against infections[5]. These peptides can be expressed either constitutively or induced in response to pathogens. There are mainly two classes: defensins and cathelicidins. A study found that *in vitro*, the supernatant of activated Vγ9Vδ2 T cells contains α-defensins and LL-37 (cathelicidin) that are involved in reducing extracellular

Brucella numbers. And LL-37 nonfunctional precursor hCAP18 is stored both in resting and activated Vγ9Vδ2 T cells[152].

1.1.3.1.4 Interactions with other immune cells

1.1.3.1.4.1 Monocytes and Dendritic cells

Vγ9Vδ2 is synergized with monocytes in the early defense of infections. PAg activated Vγ9Vδ2 T cells increase monocytes' survival, induce monocytes to undergo morphology and phenotype changes within 18 hours of coculture and differentiating into functional antigen presenting dendritic cells (DCs), and this process is mediated both by cell-cell contact and cytokines such as IFNγ, TNFα, IL-4, and granulocyte/macrophage colony-stimulating factor (GM-CSF) secreted by Vγ9Vδ2 T cells[153,154].

In vitro studies show that Vγ9Vδ2 T cells can upregulate HLA-DR, CD86, CD83 on DCs which indicate DCs undergo certain maturation. IFNγ and TNFα released from Vγ9Vδ2 T cells are involved in the induction of IL-12 by DC[155-157]. DC maturation, production of IL-12 and antigen presenting activity are abrogated during certain infections. Vγ9Vδ2 T cells using both contact-dependent mechanism and soluble factors can fully restore DC maturation, induce IL-12 production thus indirectly prime naive CD4 T cells to proliferate[158,156].

1.1.3.1.4.2 B cells

As mentioned above, Vγ9Vδ2 T cells are also involved in B cell activities. *In vitro*, both allogenic and autologous Vγ9Vδ2 T cells induce APC markers such as CD40, CD86, and HLA-DR expression, IL-4, TNFα production on B cells, and induce resting T cells activation and proliferation[157]. Vγ9Vδ2 T cells are found in the T zone and germinal centers as opposed to the mantle zone of B-cell follicles within secondary lymphoid tissues[159]. IL-21 enhances Vγ9Vδ2 T cells to acquire follicular helper T cell features to provide help to B cells[160,145].

1.1.3.1.4.3 Antigen-presenting to αβ T cells

Professional antigen presenting cells (APC) are characterized by tissue homing capability, phagocytosis, processing and presenting antigens to induce T cell activation and differentiation. Except for the above mentioned APC features of Vγ9Vδ2 T cells, such as upregulated APC associated markers and migration to lymph nodes after activation[157,159], activated Vγ9Vδ2 T cells can become professional phagocytic cells to uptake extracellular bacteria and form phagolysosome in a TCR dependent manner[161,162]. In addition, Vγ9Vδ2 T cells readily exist in healthy human intestinal mucosa[163]. Circulating Vγ9Vδ2 T cells express tissue-homing receptors which can let them migrate to intestine and skin, and PAg stimulation upregulates the gut-homing receptor. *In vitro*, IFNγ producing CD103- Vγ9Vδ2 T cells polarize autologous αβ T cells toward Th1 phenotype[163]. Vγ9Vδ2 T cells can uptake and process soluble proteins to induce both CD4+ and CD8+ αβ T cells proliferation and cytokine production, and are more efficient than DCs through a different antigen processing mechanism[164-166]. *In vivo*, APC-like Vγ9Vδ2 T cells are significantly increased in the synovial fluid of rheumatoid arthritis patients and associated with proinflammatory effects[147].

1.1.3.2 Vγ9Vδ2 T cells immune responses in early life

In general, neonate immune cells have weaker responses than their adult counterparts[167,168]. However, this does not mean the immune system of early life is quiet. In contrast, newborns face many major challenges within a relatively short period of time, as they just developed from a semi-allogeneic sterile environment and suddenly came into a world full of microbes, their immune systems undergo dramatic transitions and this takes time. Neonates are susceptible to infections and non-infectious diseases as they possess more naïve phenotype immune cells. Also, neonatal T cells tend to exhibit Th2 phenotype, with or without stimulation[169,168], and they have more regulatory T cells to suppress effector T cell functions than adults in

blood and tissues[170].

As mentioned earlier, Vγ9Vδ2 T cell is the predominant γδ T cells in mid-gestation fetal peripheral blood and they are armed with effector molecules such as Granzyme A and K, express proinflammatory receptors and readily produce IFNγ after short ex vivo stimulation[3,171]. However, compared to adult Vγ9Vδ2 T cells, *in vitro* stimulate fetal or neonatal Vγ9Vδ2 T cells with much higher concentrations of HMBPP or IPP results in lower IFNγ production; and addition of extra cytokines other than IL-2 are needed to augment the production[172,3]. Interestingly, stimulation of neonatal Vγ9Vδ2 T cells with N-BP drugs induce significantly higher proliferation compared to stimulation with HMBPP or IPP, and the expanded cells are highly activated, differentiate into effector memory (CD27-CD45RA-) T cells, and produce a sufficient amount IFNγ and TNFα but low perforin after re-stimulation[173,174]. Addition of IL-23 (IL-12 family member, easier to be induced in neonates after TLR stimulation) enhances the production of cytotoxic molecules such as perforin, granzymes, and granulysin[174]. To note, IL-17 and Th2 cytokines like IL-4, IL-13, IL-10 are observed in *in vitro* stimulation under different stimulation and culture conditions[174,171].

Under infectious circumstances, Vγ9Vδ2 T cells from children with tuberculosis have higher proliferation and lower IFNγ and granulysin production upon *in vitro* restimulation with IPP compared to healthy age-matched controls. Treatment successfully restores IFNγ and granulysin production[175]. *In vitro*, neonatal Vγ9Vδ2 T cells produce less perforin and cytokines (IFNγ, TNFα) in response to influenza virus stimulation compared to adult counterpart, while preterm newborns produce even less than full-term newborns. Neonatal Vγ9Vδ2 T cell performance towards *in vitro* virus stimulation is positively correlated with gestation week and birth weight[176]. Also, in a longitudinal study, Vγ9Vδ2 T cells were gradually decreased in HIV-1 infected children and adolescents treated with antiretroviral therapy compared to age-matched controls, and negatively correlated with virus load in the blood[177]. The public Vγ9 sequence is also decreased in infected children compared to controls[177].

1.2 Immune responses to *Toxoplasma gondii* infection

1.2.1 The parasite – *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*) was first described in 1908 in North Africa and Brazil[178]. It is an obligate intracellular protozoan parasite which can infect nucleated cells from basically all warm-blooded animals (such as mammals and birds). *T. gondii* has a bow-shaped look and belongs to the Apicomplexa phylum, which is characterized by a cytoskeletal structure called conoid within the apical part of the parasites and secretory organelles such as rhoptries, micronemes, and dense granules (Figure 7). Interestingly, *T. gondii* possess three sets of genomes: the nuclear genome, the mitochondrial genome, and an algal plastid genome from the organelle called apicoplast[179]. Among all the genotypes detected, it can be classified into four groups, with three closely related to the conventional clonal lineages (Type I-III). The predominant genotype of *T. gondii* is different according to geographical regions. Dominant genotypes are more commonly found in Africa, Europe, and the Middle East, while in South America hundreds of genotypes coexist with no clear dominance[180,181].

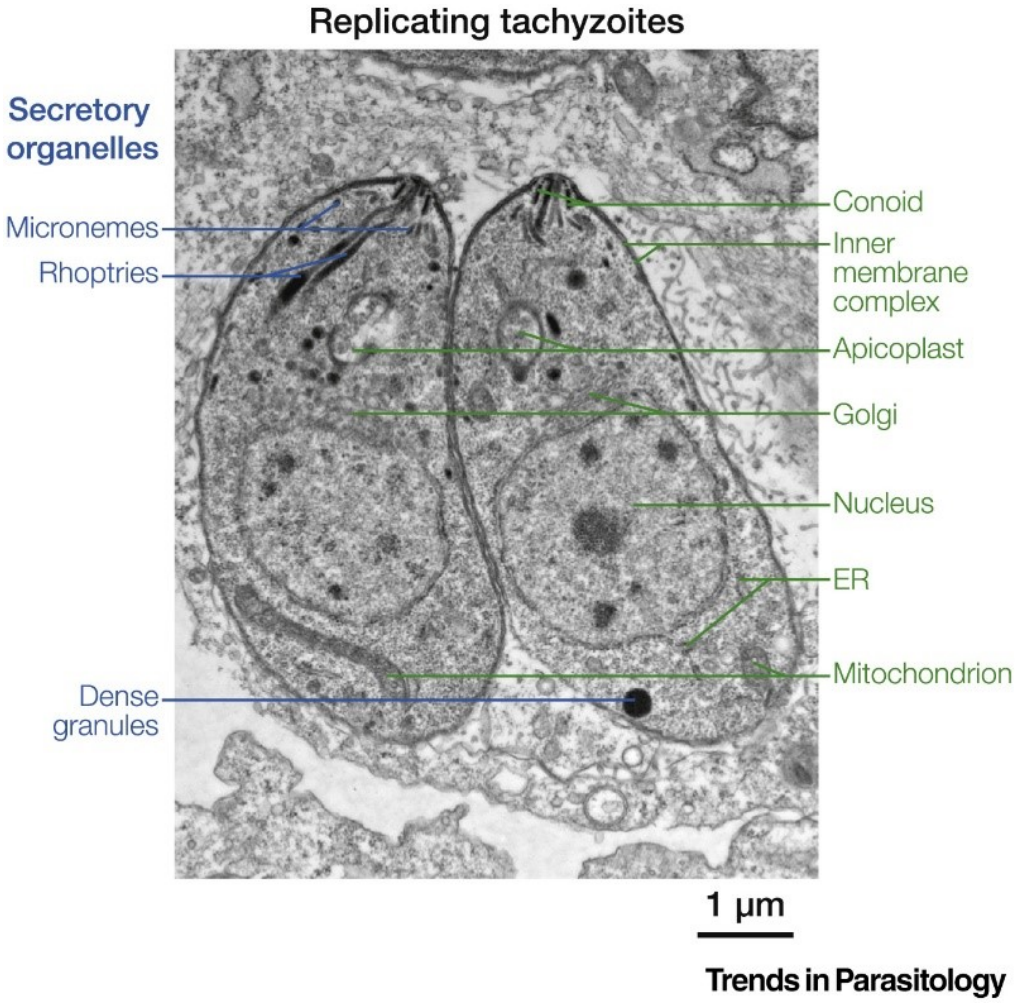


Figure 7 Subcellular structure of *T. gondii* tachyzoites.[182]

The life cycle of *T. gondii* contains two phases: one is the sexual replication restricted to their definitive feline hosts; the other is the asexual replication within their nonfeline intermediate hosts[183]. Details see Figure 8.

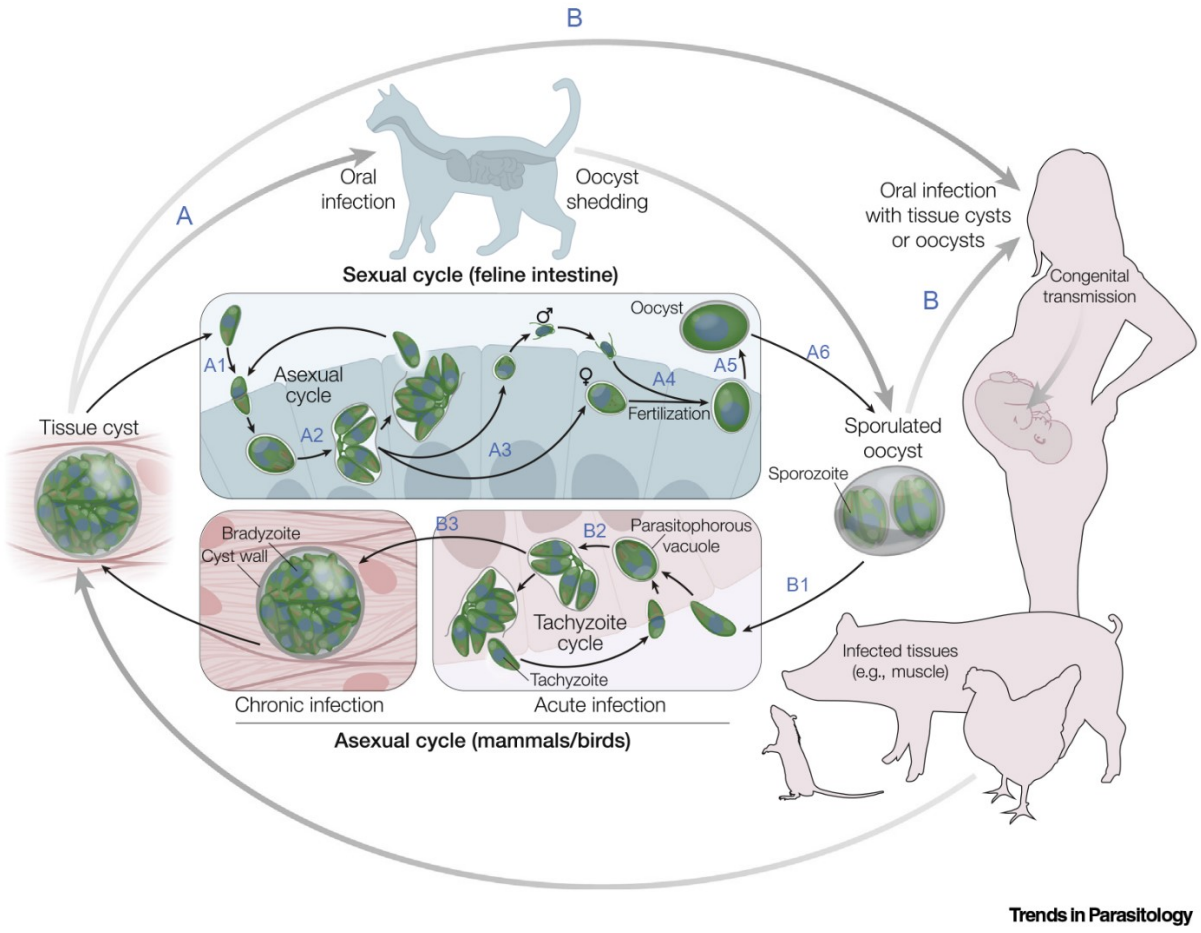


Figure 8 Life Cycle of *T. gondii*.[182]

The sexual stage (**A**) starts when the definitive hosts ingest tissue cysts(**A1**) from *T. gondii* infected intermediate hosts or oocysts from water (though less efficient). Within the intestinal cells, bradyzoites undergo a transient asexual replication to become merozoites (**A2**). Then, the sexual development begins, male and female gametes are formed within the feline enterocytes (**A3**). After fertilization (**A4**), unsporulated oocysts are formed and released after cell rupture, then shed in feces to the outer environment (**A5**). After a few days in the external environment, a process called sporogony (**A6**) begins within the oocysts and forms eight haploid sporozoites within each oocyst. These oocysts can keep the sporozoites survive for more than one year in moist environment. The asexual stage (**B**) begins when an intermediate host ingest the oocysts or tissue cysts from row meat (**B1**). Subsequently, the sporozoites are released from oocysts and infect intestinal epithelial cells where they differentiate into tachyzoites (**B2**). *In vitro*, replication time for tachyzoites is about 6-8 hours and released to infect other cells. They disseminate throughout the body, while under the pressure of the host immune system, tachyzoites differentiated into bradyzoites (**B3**) after 7-10 days after initial infection. Bradyzoites replicate very slowly and form cysts in tissues, especially in central nervous system, eyes and in muscle. In certain cases (maybe even periodically[184]), tissue cysts are reactivated and transform into tachyzoites and may lead to severe outcomes[178,183].

1.2.2 Basics about toxoplasmosis

1.2.2.1 Transmission and Prevalence

Since around 35% and 59% of domestic cats and wild felids respectively are seropositive for *T. gondii* [185], and this is not including all the other infected animals, the risk of exposure to *T. gondii* is quite high globally. Transmission is influenced by environmental conditions (e.g. latitude, humidity, sanitation), gross domestic product (GDP) per capita, cultural and eating habits, and felids distribution[186]. In early years, ingestion of cysts from meat or meat derivatives and through milk contaminated with tachyzoites were more common for toxoplasmosis outbreaks. In the past two decades, oocysts from water, sand, soil, row fruits, and vegetables become the major reason for outbreaks of toxoplasmosis[187]. In addition, transplant patients can acquire *T. gondii* infection by receiving organs from seropositive donors. Heart transplant patients are at the highest risk of being infected[183].

More than one-third of the human world population is *T. gondii* infected and this makes it one of the most prevalent infectious diseases in humans. The seroprevalence of *T. gondii* infection is different through geographic areas (Figure 9). The prevalence of latent toxoplasmosis in pregnant women is estimated to be 33.8% worldwide. The highest infection rate happens in South America with 56.2%, followed by Africa with a seroprevalence of 48.7%, and the lowest prevalence is in the Western Pacific countries with 11.8% (Figure 9)[188]. To be noticed, most of the worldwide toxoplasmosis seroprevalence data are only available from pregnant women. Some meta-analysis also include women of childbearing age but very rarely focused on the general population[189,186].

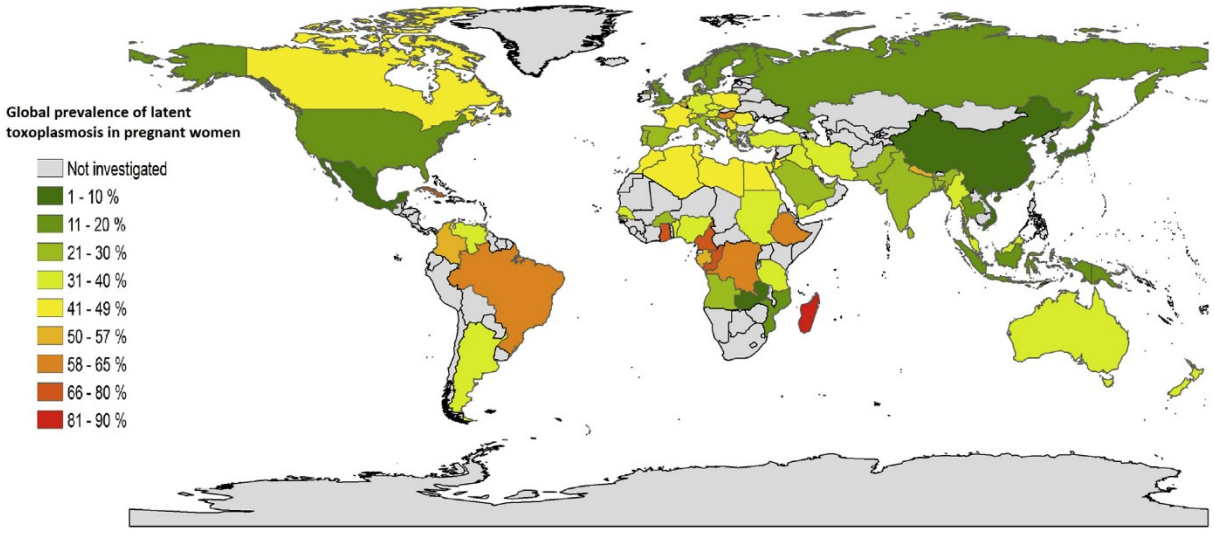


Figure 9 Prevalence of latent toxoplasmosis in pregnant women in different countries using geographic information system (GIS).[188]

1.2.2.2 Symptoms, diagnosis and treatments

In immunocompetent people, *T. gondii* infection is mostly asymptomatic or only causes subclinical flu-like symptoms during acute infection. The manifestations of symptomatic primary infection are influenced by parasite strains and infection sites. The most common systematic symptoms are fever, lymphadenopathy, headache, fatigue, or muscle pain. Sometimes, chorioretinitis can also appear during postnatal acute infection or reactivation[190,191,183]. Once infected, the tissue cysts can last for lifelong without causing clinical symptoms. This is normally considered as the latent toxoplasmosis which happens in most cases; while chronic toxoplasmosis is associated with continuous or recurrent clinical symptoms[186].

In immunocompromised people, such as HIV infected people and people under immunosuppressive therapies, the situations are more dangerous. About 35.8% HIV-infected patients co-infect *T. gondii* worldwide[192]; encephalitis is the most common symptom in these patients[190,193,183]. Other most frequently involved organs include lungs, eyes, heart, but *T. gondii* can also be found in liver, pancreas, bladder, kidney, lymph nodes, bone marrow, spleen, and skin[183].

Diagnose of *T. gondii* infection in immunocompetent people normally involved with indirect serological tests (e.g. *T. gondii* specific IgG or IgM); while in immunocompromised patients, direct detection by PCR for *T. gondii* DNA and histopathologic examination are used[183,191].

Common drugs used for treating toxoplasmosis are pyrimethamine and sulfadiazine. Pyrimethamine is a synthetic medicine that inhibits the enzyme (dihydrofolate reductase) that is essential for producing the cofactor (tetrahydrofolate) of DNA and proteins synthesis[194]. Sulfadiazine is an antibiotic that inhibits bacterial folic acid synthesis[195]. Combination of pyrimethamine and sulfadiazine for 4-6 weeks is the most common prescription for anti-*T. gondii* treatment. Together with folinic acid is recommended to prevent hematological side effects associated with the use of pyrimethamine[193,191]. Current treatment is effective to acute infection but not for eliminating cysts[183].

Next, congenital toxoplasmosis which is the disease investigated in this thesis will be described in more details below.

1.2.2.3 Congenital toxoplasmosis

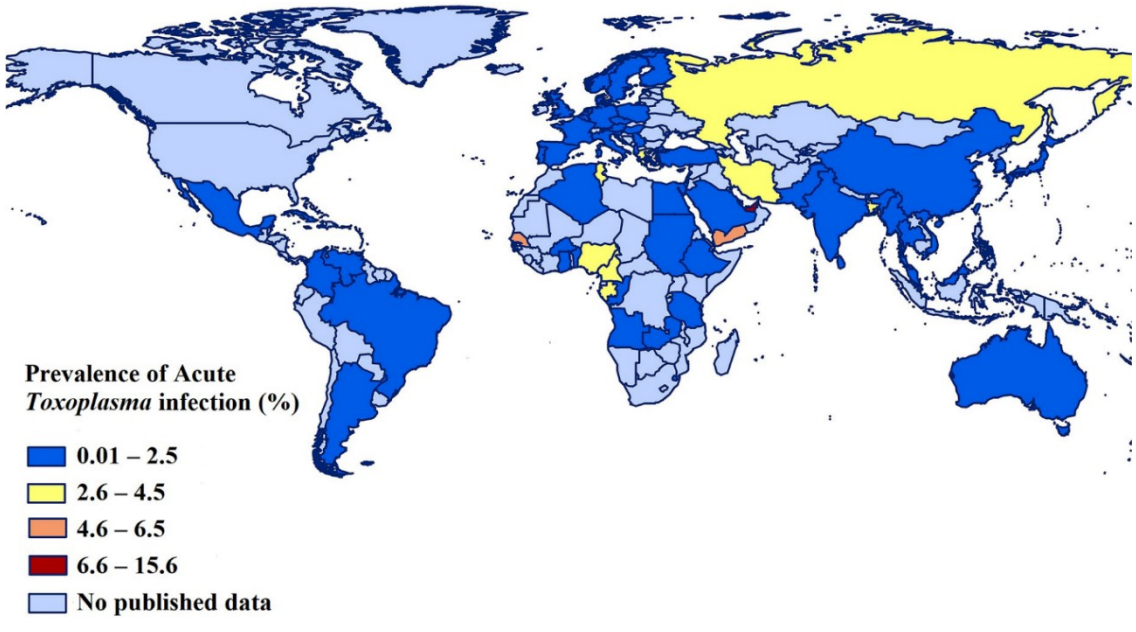


Figure 10 Prevalence of acute *T. gondii* infection in pregnant women in different countries using

(continued from previous page) GIS.[196]

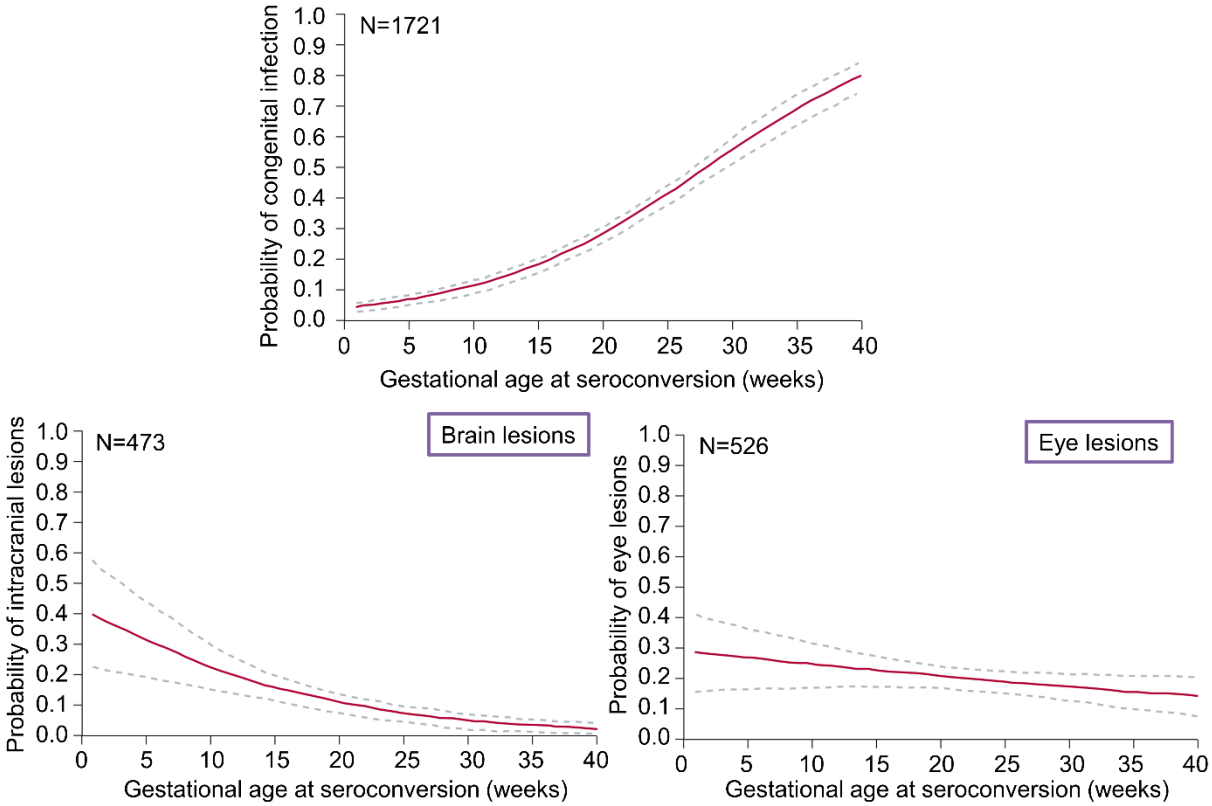


Figure 11 Risks of *T. gondii* vertical transmission and clinical manifestations.[197]

Risk of mother-to-child transmission of T gondii (top) and risk of clinical manifestations (bottom) in children infected by *T. gondii* by gestational age at maternal seroconversion. Dotted lines are bounds of 95% CI.

The first recorded case of congenital toxoplasmosis is an 11 months infant with tissue cysts of *T. gondii* in the retina and diagnosed with congenital hydrocephalus and microphthalmia in 1923[184]. *T. gondii* can enter placenta and infect fetus during acute infection of the mothers. The overall worldwide prevalence of acute *T. gondii* infection during pregnancy is 1.1% (Figure 10)[196], of which an average around 30% of these acute maternal infections would lead to congenital *T. gondii* infection[184]. The global estimated incidence rate of congenital toxoplasmosis is 1.5 cases per 1000 live births (with a range from 0.4 - 4.1 in different areas)[198]. Vertical transmission rate during the three trimesters of pregnancy is gradually increasing from 14% in the first trimester, 30% in the second to 59% in the third trimester (Figure 11, top)[184,197]. But the risk of showing symptoms and the severity of the symptoms are decreasing through

gestation (Figure 11, bottom)[184,197]. The risk of symptomatic toxoplasmosis and severity is also linked to the genotypes of *T. gondii*. For example, the dominant Type II parasite in European countries is less virulent than the atypical genotypes prevalent in South American countries. This may partially explain the different symptomatic toxoplasmosis rates between these two regions[198]. It is not easy to detect abnormalities during prenatal ultrasound[191], and 75% of newborns with congenital toxoplasmosis do not have symptoms[199]. The most common symptoms of congenital toxoplasmosis appear in the eyes and the brain, leading to the classic triad including chorioretinitis, intracranial calcifications, and hydrocephalus, but all symptoms happen in the same time is rare and none of them is specific for *T. gondii* infection[191,199]. To be noticed, although most newborns are asymptomatic, they are under high risk of developing symptomatic toxoplasmosis later in life (Table 1).

Syndrome	***Incidence a (95% CI)***
Fetal loss (>24 weeks gestation)	2.4 (2.3-6.3)
Neonatal death	0.7 (0.4-1.2)
Chorioretinitis in first year of life	13 (12-15)
Chorioretinitis later in life	16 (5-52)
Chorioretinitis in first year of life (NA and SA)	80 (70-90) b
Chorioretinitis later in life (NA and SA)	10 (5-15)b
Intracranial calcification	11 (7.9-12)b
Hydrocephalus	2.0 (1.0-3.0)
CNS abnormalities	2.9 (1.0-6.0)

Table 1. Estimated incidence of sequelae associated with congenital toxoplasmosis.[198]
CNS, central nervous system; CI, credible interval; NA, North America; SA, South America.
[a], Per 100 cases.
[b], To avoid having the incidence of sequelae being greater than the total incidence, in cases from South America these incidences were applied to the residual incidence once sequelae with higher disability weights had been extracted.

Once the mother is diagnosed with acute *T. gondii* infection, prenatal diagnosis can be done by PCR for detecting *T. gondii* DNA in the amniotic fluid; this method is more safe after 16-18 weeks of gestation[183,200]. If prenatal diagnosis is not feasible or the

acute maternal infection happens in late pregnancy, postnatal diagnosis is recommended for placenta examination, serology tests which including using enzyme-linked immunosorbent assays (ELISA) for detecting *T. gondii* specific IgG, IgM, IgA in the newborn, and western blot for detecting *T. gondii* specific IgG or IgM from mother-newborn paired serums. Paired examination excludes maternal IgG that is transferred to the newborns and reduces false results[183].

Managements of diagnosed or highly suspected maternal *T. gondii* infection include treatment of spiramycin (a macrolide antibiotic and antiparasitic[201]) as prevention before the diagnosis of fetal infection[202]. Once the fetus is diagnosed and also after 20 weeks of gestation, treatment should be switched to pyrimethamine and sulfadiazine[193,183]. Pyrimethamine, sulfadiazine, and folinic acid for treating *T. gondii* infected newborns are recommended for the first year of life[183].

1.2.3 Immune responses to toxoplasmosis

1.2.3.1 Innate immunity to *T. gondii* infection

After ingestion of *T. gondii*, the first site of infection takes place in the intestinal mucosa. The released parasites cross the intestinal epithelial barrier and enter lamina propria. From there they transform to the more active tachyzoites and use innate immune cells to disseminate through blood flow and cross biological barriers[183,203].

The intestinal mucosa consists of two parts: a single layer of epithelial cells lines the luminal surface; under it is the connective tissue of lamina propria. The single mucus layer covering the epithelium[204,205], the tight junctions between enterocytes[206], and the antimicrobial peptides secreted by the Paneth cells[207] build up the first physical and chemical barrier of host defense. There are several mechanisms proposed to explain how *T. gondii* crosses the barrier. One is involved with the ICAM-1 on epithelial cells and the gliding mobility of the parasite to cross the barrier without penetrating epithelial cell membrane; the other involves crossing through epithelial cells to reach

lamina propria[203]. Also, *T. gondii*-invaded neutrophils in lamina propria can take the parasites to cross the epithelium to the lumen and spread to other locations of the intestine[208,209,203]. Although *T. gondii* can penetrate the barrier, the efficacy of the intestinal mucosal defense is largely unknown. A shred of indirect evidence to consider the efficacy of this host defense might be through the differences of outcomes after infected by different types of *T. gondii*, since the most virulent Type I parasite has a greater ability to actively penetrate the barrier compares to the other types of parasites[210].

There are many types of immune cells resident in the lamina propria and once *T. gondii* enters this area, they start to actively invade all types of cells. At the early phase of infection, neutrophils are recruited to the replication foci, while DC do not show this tendency; thus monocytes and neutrophils are the most frequently infected immune cells in lamina propria, followed by lymphocytes (without considering γδ T cells) and macrophages[208,211]. Parasitized DC have the tendency to migrate to mesenteric lymph nodes and blood during early infection and disseminate to other organs[211,212].

It is well known that *T. gondii* infection elicits a strong IL-12/IFNγ mediated Th1 response (Figure 12)[213,214]. DC, macrophages, and neutrophils produce IL-12 after exposure to *T. gondii*, with DC being the main source of IL-12[215]. TLR in those first-line immune cells play an important role in sensing *T. gondii*. TLR11 and TLR12 are essential to sensing *T. gondii* profilin (key contributors to actin polymerization) and initiate IL-12 production in mice[216,213], while other TLR, such as TLR2 and TLR4 are found to engaged in DC and macrophages production of IL-12 after stimulating with parasite microneme proteins[217]; TLR9 is involved with the antimicrobial peptides α-defensins production in Paneth cells and inducing efficient Th1 response of innate immune cells[218,219]. Humans do not have TLR11 and TLR12, but still, human innate immune cells respond with IL-12 and TNFα production by phagocytosis (not invasion by) of *T. gondii* [220,221].

Rapid and sufficient secretion of IL-12 by DCs promote NK cells and T cells to produce IFNγ, the most essential mediator in *T. gondii* control[222,214]. In the absence of CD4 T cells, NK cells can prime CD8 immunity towards *T. gondii* under prolonged IL-12 circumstance[222]. However, both IFNγ production and the cytotoxicity of *T. gondii* infected NK cells are impaired[223].

T. gondii infection also induces other cytokines, such as IL-10, IL-6, IL-17, IL-22, IL-18, IL-15, IL-1β, IL-23, IL-27, they are involved in the homeostasis of the host defense and *T. gondii* infection[224-233].

Once inside host cells, *T. gondii* replicates within parasitophorous vacuole (PV) which is formed during the invasion by decorating with host membrane lipids. Two groups of interferon-inducible GTPases family proteins called guanylate-binding proteins (GBP) and immunity-related GTPases (IRG) are involved with the elimination of PV. IRGs are recruited to PV membrane and result in the rupture of the vacuole, thus lead to the death of *T. gondii* and the infected cell[234]. GBPs are stored in large units within healthy cells which allow fast migration to PV and form a huge complex to attack PV[235]. Also, *T. gondii* strains are influencing the host recognition by GBP[234,236]. In mice, GBPs work together with IRGs to mediate anti-intracellular infection; however, most IRGs are lost in higher primates, thus GBPs are the main GTPases involved in the clearance of PV in human[237,238].

Autophagy is also an important way of eliminating intracellular pathogens that integrate with cytokine signaling and PRRs in both mice and humans[239]. Formation of autophagosomes is the hallmark of autophagy and this process is involved with many autophagy-related proteins (ATGs). In *T. gondii* infection settings, ATGs are involved in the recruitment of GBPs and IRGs to *T. gondii* PV, however, even though the marker of autophagosome LC3 (ATG8, microtubule-associated protein 1 Light Chain 3, a ubiquitin-like protein) is shown to be recruited to PV membrane and many other ATGs are involved, the canonical autophagy process has not been found

here[234,240,237]. On the other hand, CD40-stimulated macrophages exhibit fusion of lysosome with *T. gondii* PV and lead to *T. gondii* death by autophagy[241,240].

Also, cell-autonomous immune responses such as the inhibition by reactive oxygen species (ROS)[242] and nitric oxide[243] during acute infection are contributing to the control of *T. gondii* infection, although the function of nitric oxide in human might be pro-infection[244].

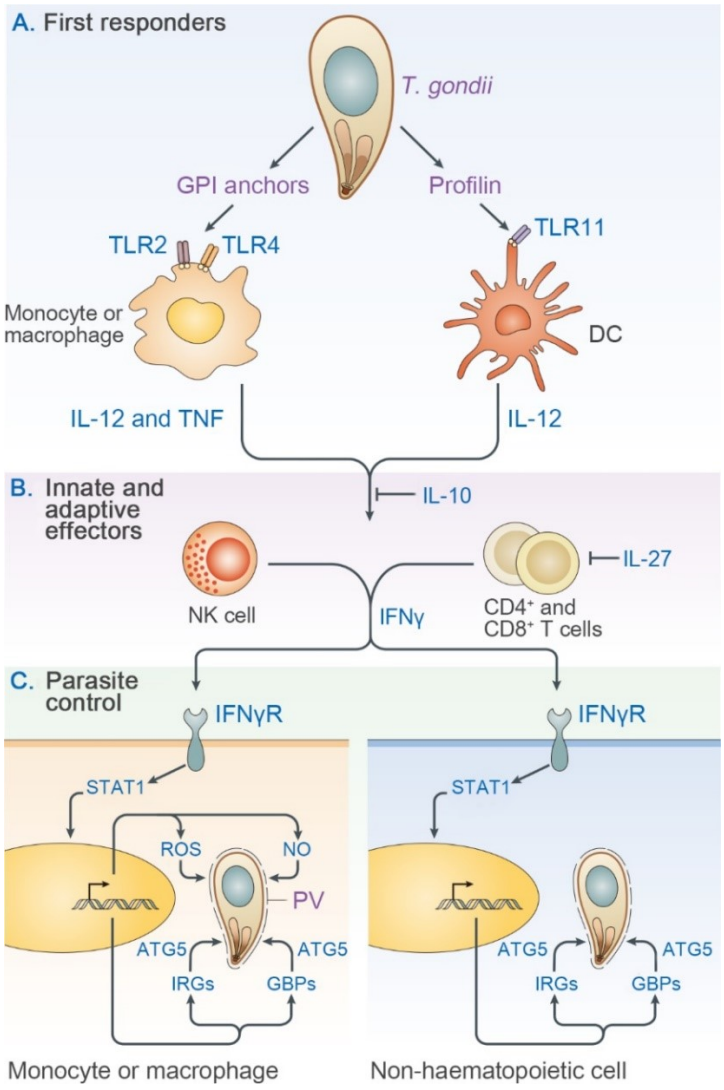


Figure 12 Innate immune responses to *T. gondii* during infection.[214]

(A) Early in infection, the first host cells to respond are dendritic cells (DCs), monocytes and macrophages. The interaction of *T. gondii* profilin with Toll-like receptor 11 (TLR11) on DCs is important for host production of interleukin−12 (IL−12). In addition to stimulating IL−12 production, macrophages also induce tumour necrosis factor (TNF), a cofactor in antimicrobial activity, in response to TLR2‑ and TLR4‑mediated detection of glycosylphosphatidylinositol (GPI)‑anchored parasite proteins. **(B)** The immune response results in the production of IFNγ from NK cells through the innate response and, eventually, from CD4+ and CD8+ T cells as the adaptive response ensues. IL−10 and IL−27 are key to

(continued from previous page) modulating these pathways and prevent the overproduction of Th1 type cytokines. **(C)** The production of IFNγ during the innate and adaptive phases is responsible for activating cells to control parasite infection. IFNγ propagates a signal through a surface IFNγ receptor (IFNγR) to activate signal transducer and activator of transcription 1 (STAT1), a nuclear transcription factor that controls the expression of many genes. In response to STAT1 activity, monocytes and macrophages upregulate their production of nitric oxide (NO) and reactive oxygen species (ROS), both of which contribute to the control of intracellular parasites. Both haematopoetic and non−haematopoetic cells upregulate two families of defence proteins called immunity-related GTPases (IRGs) and p67 guanylate-binding proteins (GBPs), which are recruited to the parasitophorous vacuole (PV) and are involved in parasite clearance. The function of IRGs and GBPs depends on autophagy protein 5 (ATG5).

1.2.3.2 Adaptive immunity to *T. gondii* infection

Although innate immunity has many ways to defense host against *T. gondii* infection, T cells are crucial in both acute phase resistance and long-term control of the parasite (Figure 13)[245]. Early studies demonstrated the crucial role of CD4 and CD8 T cells (especially CD8 T cells) in the resistance to *T. gondii* infection in an immunized mice model. Adoptive transfer of blocking antibodies (anti-CD4 or anti-CD8) treated immunized spleen cells to naïve mice reduce their resistance to *T. gondii* infection[246]. Also, IFNγ is demonstrated to be the key mediator in *T. gondii* immune defense[247]. Later studies showed that antigen specific CD8 T cells along with IFNγ are crucial in the protection against acute *T. gondii* infection in mice[248]. In addition, CD8 T cells can recruit microglia cells and macrophages to eliminate the cysts and also directly remove cysts via perforin mediated cytotoxicity from chronically infected mice brain[249]. Series blocking tests showed that IFNγ and CD4/CD8 T cells network are in the center of inhibiting the reactivation of the parasite from chronic stage[250].

CD8 T cells from circulation and tissues become exhausted during long time *T. gondii* infection, with an upregulated PD-1 (programmed cell death protein 1) expression and a decreased cell number and function[251]. Blockade of PD-1/PD-L1 recognition rescues the dysfunction of CD8 T cells by increasing cell number and cytotoxic function and accompanies with decreased *T. gondii* reactivation[251]. The mechanism within this rescue is involved with the costimulatory CD40[252]. In addition, memory CD8

T cells tend to have the most PD-1 expression thus are more susceptible to apoptosis and dysfunction during *T. gondii* infection[253].

CD4 T cells provide the major source of IFNγ after both acute and chronic *T. gondii* infection which support the cytotoxic immunity of CD8 T cells[245]. Compared to CD8 T cells, CD4 T cells exhibit stronger upregulation of inhibitory markers during chronic *T. gondii* infection, especially on central memory cells. Fully functioned *T. gondii* specific CD4 T cells can rescue exhausted CD8 T cells[254].

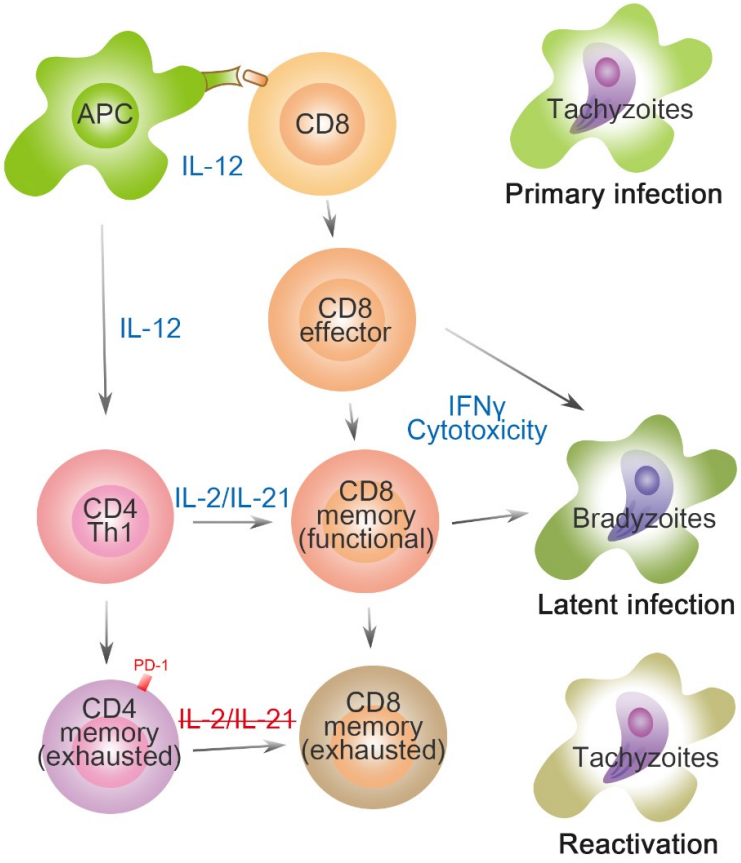


Figure 13 CD4 and CD8 T cells in *T. gondii* infection.[245]

T. gondii infection typically induces IL-12 production by APC that polarizes CD4 and CD8 T cells to Th1 cells. Effector CD8 T cells are an important source of IFNγ and also exhibit cytotoxic activity against infected targets, both important mechanisms for controlling the infection. CD4 T cells produce IL-2 and IL-21 to help the maintenance of long-term CD8 T cell immunity (memory response). During chronic infection, antigen-specific CD4 T cells upregulate inhibitory receptors such as PD-1 and gradually get exhausted. The vital help needed for CD8 T cells is deprived, thus lead to the reactivation of *T. gondii*.

1.2.4 Immune responses to congenital toxoplasmosis

Most *T. gondii* studies are focused on acquired toxoplasmosis, though vertical transmission of *T. gondii* is observed in humans and other animals such as pigs, sheep, and murine animals. *T. gondii* strains, genotypes of common laboratory animals, infection methods and doses all influence the vertical transmission rate and outcomes[255-258], which build up the difficulties for laboratory studies. In the meantime, the differences in placentation among species remind the gap between humans and laboratory animals.

The maternal-fetal interface makes the most important barrier for congenital pathogen infections. Briefly, the maternal-fetal interface consists of a maternally derived decidua (specialized uterine epithelium) and a fetally derived placenta (the sole place for gas, nutrient, and waste exchange between the fetus and mother) (Figure 14)[259]. Only after the first trimester, the maternal blood can directly contact the placenta. Around 40% of the cells in decidua are leukocytes, of which NK cells take up to 70%, followed by macrophages (20-25%) and T cells (3-10%)[259]. Decidua NK cells are different from their circulating counterpart; also, macrophages there exhibit an anti-inflammatory M2-like phenotype. Both decidua NK cells and macrophages are involved with the remodeling of maternal-fetal interface and their numbers are decreased in late gestation[259]. Human maternal-fetal interface with the unique structure, actively introducing maternal IgG into fetus, and the autonomous defense through TLR signaling and high rate of basal autophagy by placental trophoblast all contribute to the protection within *utero*[259].

Human placenta consists of two types of villi, the anchoring villi are attached to maternal decidual tissue by extravillous trophoblasts (EVTs). It is suggested that *T. gondii* first infects the decidua, and since the EVTs are in deep communication with decidua, *T. gondii* takes the chance to invade EVTs, from there the parasite meets the fetal vasculature and eventually leads to the infection of the fetus (Figure 14)[260].

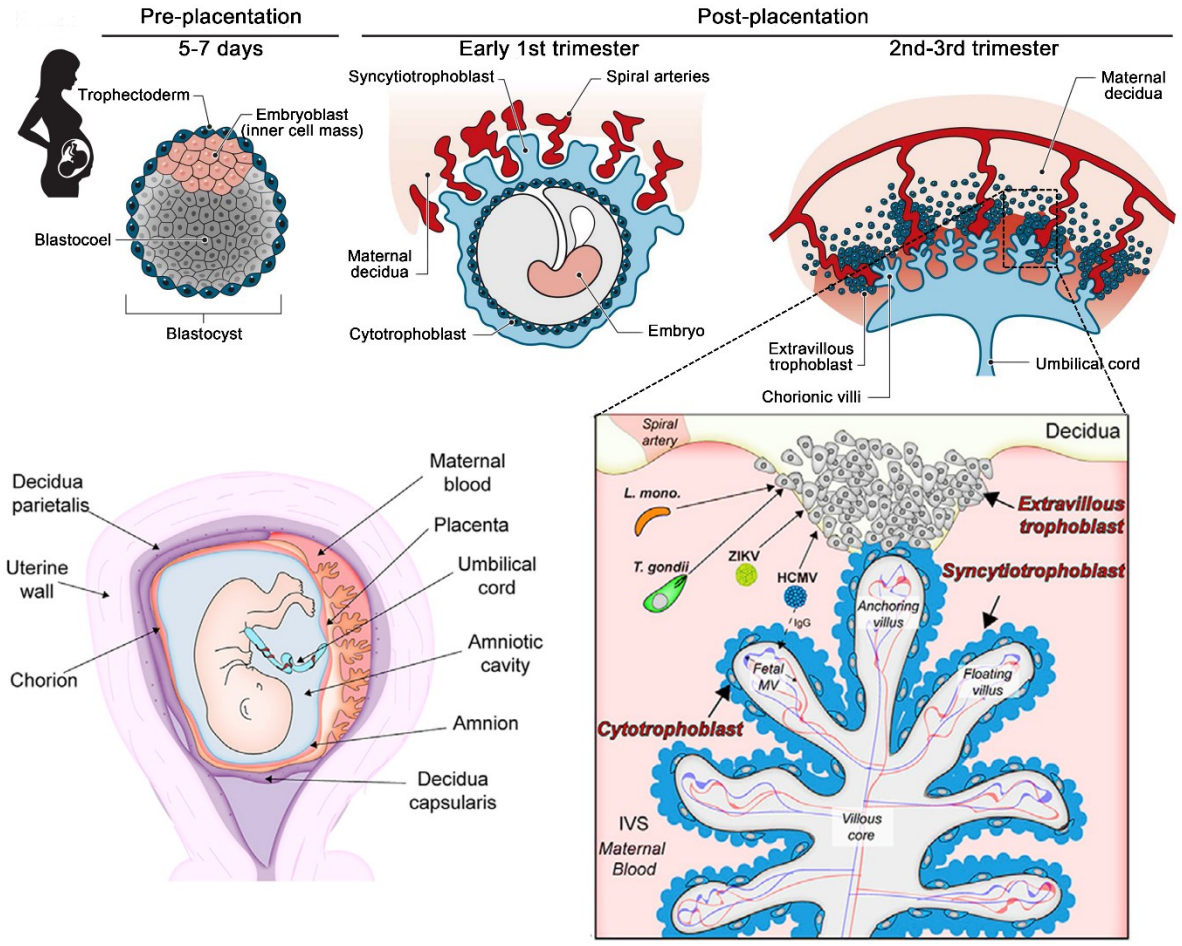


Figure 14 Human placentation and structural/cellular composition of the placenta.[259,260]
Before placentation, the blastocysts of humans and mice are similar (**top left**). However, upon implantation, placental development progresses differently (**top middle and right**). After blastocyst implantation, the human syncytiotrophoblast (SYN) layer burrows into the maternal decidua. By the third week of gestation, the definitive human placenta is formed and is composed of villous trees. However, at this stage of human pregnancy, the fetal-derived placenta does not directly contact maternal blood (**top middle**). Extravillous trophoblasts (EVTs) anchor the villi to the decidua and are involved in the remodeling of the spiral arteries to flood the intervillous space (IVS) with maternal blood toward the end of the first trimester of pregnancy. The surface of the villi is covered by the SYN layer, which directly contacts the maternal blood and facilitates the transport of nutrients, gases, and waste across the placental barrier. Underlying the SYN layer are mononucleated cytotrophoblasts (CTBs) that can either fuse to replenish the syncytial layer or differentiate into EVTs (**top right**). Several pathogens, including Listeria monocytogenes, *Toxoplasma gondii*, human CMV, and Zika virus, are thought to access the villous core following replication in EVTs (**lower right**). A overall schematic of the uterine cavity during pregnancy is shown in the **lower left**.

As mentioned earlier, *T. gondii* infection elicits a clear pro-inflammatory response, however, a strong and persistent Th1 response is not favored during pregnancy[259]. The immune system faces the balance between eliminating pathogens and

successfully conducting pregnancy. Reports showed that in mice TLR4 is more expressed in term placenta than in the first trimester[261], and *T. gondii* load is higher in late pregnancy[262]. This correlates with the time course observation in human susceptibility to congenital *T. gondii* infection. In addition, IFNγ knockout mice have higher *T. gondii* load *in utero* and placenta and lower weight of the newborns, while wild type infected mice have higher abortion rate[258]. In humans, pregnant women who transmit *T. gondii* to newborns normally have stronger humoral and cellular immune responses than non-transmit women[263]. Besides, *in vitro*, *T. gondii* infected human decidual DCs produce IL-12 and promote decidual NK cells' IFNγ production and NKG2D expression which lead to increased cytotoxicity[264]. These indicate that when encountering *T. gondii*, the maternal-fetal interface fights back, but with increased risks of a failed pregnancy.

PBMC of adults with congenital ocular toxoplasmosis have lower proliferation and IFNγ, IL-2 responses toward *T. gondii* lysate *in vitro* compared to acquired ocular toxoplasmosis, which suggests congenital infection may lead to tolerance in later life[265].

1.2.5 The role of γδ T cells in *T. gondii* infection

The majority of the studies regarding the role of γδ T cells in *T. gondii* infection were conducted during the 1990s. The earliest report was in 1992, peripheral Vγ9Vδ2 T cells with upregulated CD45RO were found to be elevated in acute primary infected adults[266]. In vitro, γδ T cells from patients significantly proliferated towards PHA but not *T. gondii* antigens[266]. At the same time, expansion of Vγ9Vδ2 T cells was also found in acute toxoplasmosis children, and this expansion disappeared after one month of onset[267].

Peripheral blood γδ T cells from BCG vaccinated people are reported to respond to a 65000MW mycobacterial heat shock protein (hsp65) *in vitro*[268]. This lead to a series of studies in mice focused on γδ T cells in inducing hsp65 after *T. gondii* stimulation[269-273].

Heat shock proteins are a bunch of highly conserved chaperones that can be induced upon stress; they help to reduce the damage or degradation of other proteins after stressful challenges as well as normal protein folding, and they are involved in infection and cancer events[274,275]. γδ T cells as well as hsp65 expression and nitric oxide production in macrophages are increased in peritoneal exudate after mice immunized with *T. gondii* homogenate or infected with low virulent strain. Depletion of γδ T cells abrogates hsp65 expression, reduces nitric oxide production, and increases mice susceptibility to infection[269,270,272]. Further, by comparing intrathymic and extrathymic γδ T cells generated from thymus-grafted SCID mice (severe combined immunodeficiency mice transplanted with fetal murine thymus) and nude mice (thymus-dependent T cells are lost) respectively, the protective effects and induction of hsp65 expression on macrophages after *T. gondii* infection are mainly coming from extrathymic γδ T cells[271].

γδ T cells are important in the defense of *T. gondii* in mice. Depletion of γδ T cells leads to the early death of the mice[276]. After infected with an avirulent *T. gondii* strain, the kinetic of γδ T cells is different in different sites. γδ T cells and NK cells in the spleen undergo a dramatic increase after one week of infection[277,276]. Meanwhile, mice CD8 T cells and γδ T cells are more stably enriched in intraepithelial lymphocytes, while γδ T cells undergo a sharp increase that peak at day 4 post-infection in the peritoneal exudate[278]. In addition, γδ T cells exhibit cytotoxicity towards *T. gondii* infected macrophages and produce IFNγ[276-278]. One study using another low virulent strain to intraperitoneally infect mice but found no increase in γδ T cells number from spleen and peritoneal exudate post-infection and depletion of γδ T cells do not influence the survival compared to controls[279]. This might due to the reasons mentioned earlier that different methods to obtain the infection model would influence the outcomes.

γδ T cells expansion after *T. gondii* infection had also been observed in rats of different strains[280,281]. In athymic rats, the extrathymic γδ T cells are recruited to the

brain and associated with a protective performance[281].

γδ T cells are under regulation by different cytokines. Compared to wildtype *T. gondii* infected mice, IL-6 deficient mice after infection have less γδ T cells and CD4 T cells recruited to cerebral inflammation sites[282]. In αβ T cell deficient mice, exogenous IL-15 enhances the survival time of *T. gondii* immunized mice when challenged with lethal dose of parasite and increases γδ T cells in the spleen along with increased cytotoxicity of immunized mice[283].

Intraepithelial γδ T cells also contribute to maintaining the integrity of the intestinal epithelial barrier after infection[284]. In γδ T cell deficient mice, tight junction protein occludin is not increased in the epithelium as controls after *T. gondii* infection and this is associated with increased susceptibility to *T. gondii* invasion[284,285]. Upon infection, γδ T cells quickly migrate to epithelium and directly contact with *T. gondii* [285]. The difference between wildtype and γδ T cell deficient mice on parasite translocation to lamina propria can be seen within one hour after exposure[285].

Human Vγ9Vδ2 T cells from both *T. gondii* seronegative and seropositive adults as well as from *T. gondii* seronegative newborns expand towards *T. gondii* infected cells *in vitro*[286]. Stimulating with cells pretreated with *T. gondii* lysate did not lead to γδ T cells proliferation while stimulating with cells pretreated of killed *T. gondii* had less expansion than with live parasite infected cells; these indicate γδ T cell expansion favors alive parasite. Activation markers CD25 and HLA-DR were increased more strongly on γδ T cells than αβ T cells after stimulation; γδ T cell cytotoxicity towards infected cells and IFNγ production upon stimulation were all increased[286]. One group studied Vγ9Vδ2 T cell responses in congenital toxoplasmosis. The seven patients (age from 2 weeks to 6 years) involved in the study all exhibited classic symptoms as well as serological proofs. Three patients with acute phase congenital toxoplasmosis, two showed dramatic expansion of Vγ9Vδ2 T cells accompanied with increased HLA-DR and CD45RO *in vivo*[287,288]; the other who did not show T cell activation and

expansion *in vivo* was lost due to severe disease progression. Four patients (age 1-6 years) with chronic phase congenital toxoplasmosis did not show upregulation of activation markers and expansion of Vγ9Vδ2 T cells. *In vitro* stimulation with *T. gondii* lysate and infected cells showed comparable proliferation and IFNγ production with seronegative controls. A conclusion of Vγ9Vδ2 T cells are anergic in acute phase congenital toxoplasmosis was drawn due to the PBMC from two acute phase patients who did not respond to *in vitro* stimulation compared to adult controls[287].

1.3 Immune responses to mistletoe extracts

1.3.1 Herbal medicine

1.3.1.1 Herbal medicine – a brief history

When looking at the history of using plants as healing agents, it is basically like looking through the history of mankind, or even before modern humans, as there is evidence in the dental calculus of Neanderthals showing that they apply plants for medical usage[289,290]. Physicians were often referred to as 'medical botanists' before pharmacy evolved as a profession. The switch from botanical-based medicines to chemical-based medicines had happened in the late 1800s when analytical chemistry was advancing[291].

Throughout history, the practice of herbal medicine followed two paths: one is the 'folk-healing' which passes through families and communities; the other is by professional practitioners. Herb usage in the formal medical traditions was based on philosophical understandings about the relationship between humans and the environment as well as integrated with sophisticated theories of anatomy, physiology, and pathology. The ancestor of modern medical theories is the humoralism which introduced by Hippocrates (460-370 BCE, Greece) and spread by Galen (129- around 216 CE, Greece) and Avicenna (980-1037 CE, Persia) and influenced the view about human body among European physicians until 19th century. Basically, in humoral

theory, diseases are believed to be caused by unbalanced humors and herbs are the key method for treatment. The first challenge of this concept started by Paracelsus (1493-1541, Swiss-German physician) and Andreas Vesalius (1514-1564, Flemish anatomist, physician), the former pioneered the use of chemicals and minerals in medicine. Later years, William Harvey (1578-1657, English physician), Claude Bernard (1813-1878, French physiologist), and Rudolf Virchow (1821-1902, German physician, anthropologist, pathologist) all contributed to the shaping of modern medical theories by challenging the humoral theory with new concepts. In 1805, the German pharmacist Friedrich Wilhelm Adam Sertürner first isolated morphine from *Papaver somniferum* L. and started the era of pursuing pure active compounds within plants and eventually lead to the development of modern synthetic drugs[292,293,291].

While the modern Western medicine benefit from standard operation as well as experimental science and becomes dominant in nowadays life, in parallel, traditional herbal medicines are practiced in many countries. In 2019 World Health Organization (WHO) report, 88% (170) of all Member States acknowledged the use of traditional and complementary medicine and 64% have laws or regulations on herbal medicines[294]. Centuries of empiricism, economic consideration along with the craving for developing new drugs by modern pharmaceutical industry make natural products somehow to be an appealing way out[293].

The return to practical herbal usage in Europe is due to a more practical reason. During World War II, the interfering in drug shipments and destroying of infrastructure for modern drug development lead to serious drug shortages throughout Europe; also German company lost patents to commonly used drugs (e.g. aspirin and heroin) due to the Treaty of Versailles after World War I, these all forced the governments to seek substitute measures from traditional herbal medicines[291]. Herbal medicine is prevalent as complementary medicine throughout Europe and the market steadily grew during the past decades; with Germany owning the largest European herbal medicine market[295]. Legal regulations have been developed since late 20th century to ensure

quality, efficacy, and safety of herbal medicinal products[296]. A scientific committee on herbal medicinal products (HMPC) was established at the European Medicines Agency (EMA) in 2004 for development of community monographs harmonized standard. The decision of to which degree the standard is accepted is made by each country[296]. In 2014, around 1500 traditional herbal medicinal products have been granted and about 1000 registration applications were under assessment[296].

1.3.1.2 Definition of herbal medicine

WHO defines herbal medicine as “Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations” (https://www.who.int/health-topics/traditional-complementary-and-integrative-medicine#tab=tab_1). Except provided by WHO, the European regulatory framework also provides definitions for herbal medicinal products, traditional herbal medicinal products, herbal substances, and herbal preparations[296]. Herbal substances and herbal preparations have synonym as herbal drug and herbal drug preparation respectively according to the European pharmacopeia. Extraction, distillation, expression, fractionation, purification, concentration, or fermentation of herbal substances are defined as herbal preparations (or herbal drug preparation)[296].

1.3.1.3 Use of mistletoe

Mistletoe is an obligate hemiparasitic plant which grows on several types of host trees (e.g. apple tree, pine). The term ‘mistletoe’ includes several families in the order Santalales. Originally and commonly it is referred as the *Viscum album* L. species (the term also applied in this thesis), which also includes six sub-species. In Europe, the common mistletoe is *Viscum album subsp. album* (e.g. grow on apple trees) and *subsp. Creticum* (e.g. grow on pines), while in Asia, the common mistletoe is *Viscum album subsp. coloratum Kom*. Medicinally, the species *Viscum album* L. is thought to be the best source. Mistletoe has been used for centuries in traditional medicines both

in Europe and Asia for a variety of conditions including disorders in nervous system such as seizures, and headaches[297], and other abnormalities including arthritis, heart disease, urinary disorders, and high blood pressure[298]. The idea to apply mistletoe in cancer treatment was coming from a philosophy called anthroposophy by the Austrian philosopher Rudolf Steiner in early 20th century. Influenced by Samuel Hahnemann's 'Doctrine of signatures', Steiner thought mistletoe is like cancers that are parasitic on the host and eventually cause the death of the host; he believed that the similarity somehow gives mistletoe extracts the ability to cure cancer[299].

1.3.1.3.1 Mistletoe therapy

Currently, mistletoe is evaluated and granted by HMPC for registered traditional herbal medicinal products in EU by public statement[296]. Extracts of mistletoe are used as an complementary therapy in cancer treatment mainly for the purpose of improving quality of life, reducing adverse reactions from chemotherapies and radiotherapies, and boosting anticancer immunity[299]. In Germany, up to 77% (ranging from 30.6% for lung and 77.3% for breast cancer) of cancer patients receive complementary mistletoe therapy[300]. Treatment normally starts with injecting lower dose of mistletoe extracts subcutaneously or by intravenous infusion and then increases the dose accordingly. Intratumorally injection and instillation have also been applied[301]. The chemical composition of mistletoe extracts depends on the host tree, the time of harvested, and how the extracts are prepared by different commercial producers. The main bioactive compounds are mistletoe lectins (MLs, currently four types are identified: ML-1, ML-2, ML-3, *Viscum album* chitin-binding agglutinin; lectins are sugar-binding proteins, ML-1/2/3 have specificity for galactose/N-acetylgalactosamine[302]), viscotoxins (small proteins that are toxic against a varied number of cell types; they belong to plant thionins, and are produced from the leaves and stems of the European mistletoe), flavonoids, phenolic acids, terpenoids, sterols, phenylpropanoids, and alkaloids[297]. Commercially available extracts are marketed under a variety of brand names, including Iscador AG, Helixor, Iscucin,

Plenosol, ABNOBA GmbH.

1.3.1.3.2 Safety and efficacy

In general mistletoe therapy as an adjuvant therapy is considered as a safe treatment[303-305]. Depending on the dose, up to one-third of patients occur local reactions such as rubor, prurigo, and induration at injection site and around 10% of patients show systemic reactions with mild fever and flu-like symptoms; severe symptoms were rare[303,306]. From the patient's side, self-reported side effects were few and showed good adherence to the therapy; experiences with mistletoe usage were related to changes to physical, emotional, psychosocial well-being and reduction in chemotherapy side effects[307]. Administrating with higher doses also showed no severe adverse effects[308].

Mistletoe therapy is associated with improvement in quality of life, mainly related to the efforts of coping with fatigue, sleep, exhaustion, energy, nausea, vomiting, appetite, depression, anxiety, ability to work, and emotional and functional well-being in general and, less consistently, in regard to pain, diarrhea, general performance, and side effects of conventional treatments[309].

Currently, there are no demonstrated proofs for improvement of survival[310]. Many studies reported a benefit from mistletoe therapy, however the quality such as design, sample size, analyzing methods of most studies was not adequate[310]. In regardless of this, earlier meta-analysis focused on the most prescribed Iscador products showed improved survival though with limitations of the studies as well[311]. The same group updated another systematic analysis recently and found similar results, and mentioned that the mistletoe performance was associated with cancer types, with a better effect for cervical cancer and less relevant effects for lung cancer[312]. In contrast, recent systematic reviews gave a different conclusion as there were no effects of mistletoe therapy[313,314]. However, in a most recent statement, the authors reanalyzed the studies in former review and showed most of the studies gave advantaged

outcomes (Figure 15)[300].

In sum, mistletoe therapy is safe but the efficacy is controversial.

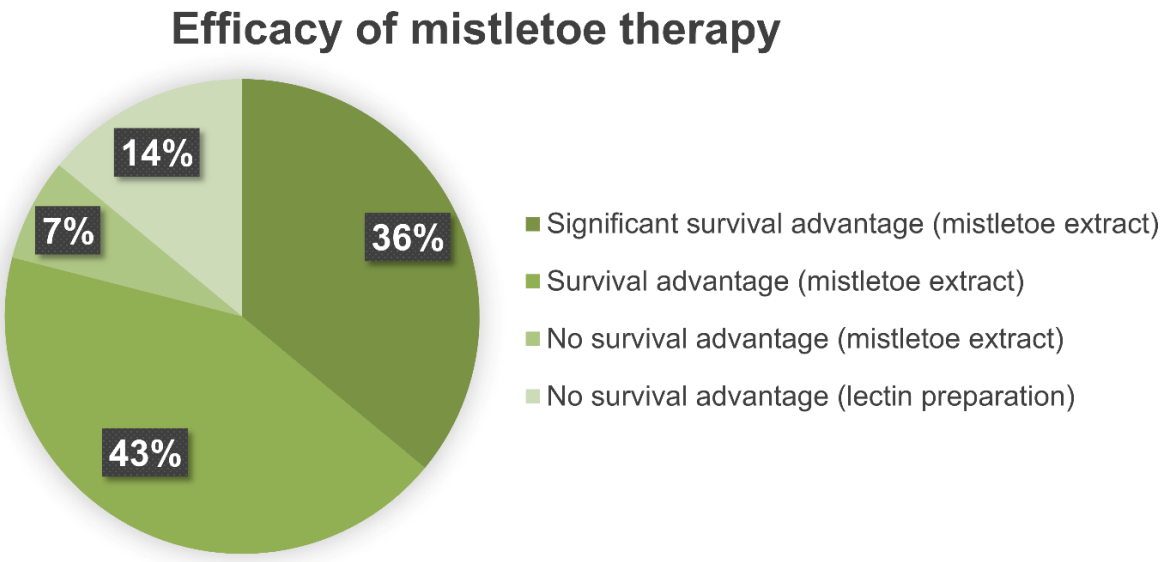


Figure 15 Impact of mistletoe on overall survival in oncological patients.[300]

1.3.2 Immunomodulation by mistletoe

1.3.2.1 Preclinical studies

Mistletoe extracts and lectins have been studied *in vitro* and in mice with focuses on cytotoxicity on cancer cells and anticancer immune responses[310]. Mistletoe preparations (such as Iscador M, Iscador Qu, Iscador P, and Helixor A, Helixor M and Helixor P[315,310]) are dose-dependent toxic to many human cancer cell lines *in vitro* by inducing apoptosis[315,310]. Also, prolonged survival and antitumor effects have been observed in mice studies using preparations from Iscador, Helixor, abnobaVISCUM, Isorel, Eurixor, and Lektinol[316,310]. In the meantime, mistletoe preparations show the potentials to enhance both innate and adaptive anti-cancer immunity[310].

Gene expression analysis showed that with an 8 hours *in vitro* mistletoe extract (from *Viscum album* L. var. *coloratum*) stimulation (along with CD3/CD28 stimulation), gene functions involving cytokine production, cell adhesion, cell motility, cell growth and

maintenance, cell death, and the response to stress and to external stimulus were altered in T cells[317].

Mistletoe lectin (ML-1) induces specific IgA and IgG production in serum and mucosal secretions (such as saliva and nasal washes) following intranasal or oral administration in mice[318]. Also, studies have found mistletoe extracts and MLs induce DCs maturation by upregulating co-stimulatory markers such as CD80, CD40 (varies), CD86 (varies), and promoting Th1 immunity by increasing IL-6, IL-8, IL-1β, IL-12, TNFα production and CCR7 expression, priming CD4 T cells proliferation and cytokine production, enhancing anticancer cytokines (IFNγ, TNFα) production by cytotoxic T cells[319-321]. Mistletoe lipophilic extract modestly regulates tumor cell polarized macrophages by downregulating CD14 and increasing TNFα as well as decreases the migration of monocytes towards MCP-1 (monocyte chemotactic protein-1)[322]. Incubating monocytes with MLs induced IL-1β production[323]. Also, viscotoxins increased NK cell cytotoxicity towards cancer cells through upregulation of perforin, NKG2D[324,325]. Further, the cytotoxic sensitivity of lymphocyte subsets towards ML was different, with $CD62L^{lo}$ CD8 T cells being more killed by ML[326]. CD4 and CD8 T cell mobility were increased following Iscador QuFrF stimulation in a dose-dependent manner[327]. Also, CD28 was upregulated on human CD4 T cells after *in vitro* Iscador P stimulation[321]. Compared to untreated patients, CD4 T cells from patients receiving mistletoe therapy (Abnoba GmbH preparations) by subcutaneous injection were proliferating towards mistletoe extracts (especially vesicles which mainly contain monogalactosyl diglyceride and digalactosyl diglyceride) *in vitro* and the activation was characterized with an oligoclonal pattern[328,329].

Interestingly, study showed ML chain A structurally shared similarities with Shiga toxin from *Shigella dysenteriae*, which may explain the immunogenicity of ML[330].

1.3.2.2 Clinical studies

In a placebo control study conducted on healthy volunteers, counts of leukocyte,

granulocyte, and eosinophil were significantly increased shortly after Iscador Q and purified ML treatment; and GM-CSF, IL-5, and IFNγ production were increased by PBMC[331]. A dramatically reversible increase of eosinophils (cause eosinophilia) and a slightly increased CD4 T cells were observed during Iscucin® Populi treatment in another study that was also tested on healthy volunteers by subcutaneous injection[332]. TNFα and IL-6 levels in serum were increased after a single injection of ML-1 *in vivo* in cancer patients[333]. After four weeks of regular subcutaneous ML-1 injections, T cell (especially CD4 T cell) and NK cell numbers increased and IL-2 receptor expression was upregulated on lymphatic cells from breast cancer patients[334]. Also, mistletoe treatment (Iscador M) gradually increases IFNγ and IL-2 responses by cancer patients' PBMC *in vitro*, although healthy untreated controls have higher stable responses[335]. HLA-DR and IL-2 receptors are upregulated on T cells during Iscador mistletoe treatment from healthy volunteers[336].

1.3.3 γδ T cell immune responses to mistletoe

Studies focused on γδ T cells in mistletoe therapy are rare. One important *in vitro* study showed Vγ9Vδ2 T cells were proliferating towards heated Abnoba mistletoe extracts[337]. They further investigated the chemical properties of the activator and found the activation was sensitive to alkaline phosphatase treatment but not to proteinase K. This indicates that the ligands are similar to those from mycobacteria ligands that are non-proteinaceous phosphate-containing compounds[337]. In addition, an NK cell study used γδ T cells as a control subset to investigate the cytotoxicity towards cancer cells, home-made mistletoe extracts can enhance cancer cell lysis by γδ T cells[324]. A mouse study showed that compared to control, pretreated mice with ML for two weeks before inoculated with colon cancer cells increased γδ T cell percentage in splenocytes[338]. In sum, γδ T cells are responding to mistletoe extracts stimulations.

2. Objectives

Vγ9Vδ2 T cells are known for their TCR-dependent polyclonal recognition of non-peptide pyrophosphate antigens (phosphoantigens, PAg). These PAg are generated from the mevalonate (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway and they are the essential metabolic precursors of isoprenoids biosynthesis which exist in all organisms. Unlike conventional αβ T cells, Vγ9Vδ2 T cells do not need to wait for antigen preparation process so they react quickly and they are poised to become type 1 cytotoxic effector cells in pathogenic events. Vγ9Vδ2 T cells are considered as a very important force in fighting infectious and cancerous diseases. Thus in this thesis, we explored the responses of Vγ9Vδ2 T cells in these two aspects.

We aim to understand:

1. How Vγ9Vδ2 T cells react in congenital PAg-generating pathogen infection, and what kind of role they are playing in early life infection.
2. How Vγ9Vδ2 T cells react to mistletoe extract drugs *in vitro*, and what possible mechanisms are involved.

To address the first aim, we chose *Toxoplasma gondii* (*T. gondii*) infection as the study model. *T. gondii* can vertically transmit to the fetus and cause *in utero* infection (congenital infection). Also, it contains the organelle apicoplast which uses the MEP pathway to generate the most potent Vγ9Vδ2 T cells activator HMBPP. This gives the opportunity to obtain insights into the Vγ9Vδ2 T cell immune responses in early life infection. We investigated the phenotypes (markers regarding expansion, activation, differentiation, and function) and TCR repertoire of congenital *T. gondii* infected infants.

To address the second aim, we compared activation responses of fermented and non-fermented mistletoe extract drugs on healthy adult blood *in vitro*. We investigated

the functional responses and mechanisms along with the mistletoe-reactive TCR repertoire.

3. Materials and Methods

3.1 Antibodies

Marker	*Color*	*Clone*	*Company*	*Project*
CD3	PB	SP34-2	BD	Toxo[1]
CD3	PB	UCHT1	BD	Mistletoe[2]
CD3	BV510	UCHT1	BD	Toxo/Mistletoe
TCR γδ	APC	11F2	Miltenyi Biotec	Toxo/Mistletoe
TCR Vγ9	Pe-Cy5	IMMU 360	Beckman Coulter	Toxo/Mistletoe
TCR Vδ2	FITC	IMMU 389	Beckman Coulter	Toxo/Mistletoe
CD27	PE	M-T271	BD Bioscience	Toxo
CD28	ECD	CD28.2	Beckman Coulter	Toxo
CD45RA	Pe-Cy7	L48	BD Bioscience	Toxo
HLA-DR	V450	G46-6	BD	Toxo
Ki-67	Pe-Cy7	B56	BD	Toxo
T-bet	BV421	4B10	Biolegend	Toxo
Eomes	PE	WD1928	eBioscience	Toxo
Granzyme A	PB	CB9	BioLegend	Toxo
Granzyme B	PE-CF594	GB11	BD	Toxo
Granulysin	PE	eBioDH2 (DH2)	Invitrogen	Toxo
Perforin	Pe-Cy7	dG9 (delta G9)	eBioscience	Toxo
CD4	V500	RPA-T4	BD	Mistletoe
CD4	BV510	RPA-T4	BD	Mistletoe
CD8	Pe-Cy7	SFCI21Thy2D3	Beckman Coulter	Mistletoe
CD56	PE-CF594	NCAM16.2	BD	Mistletoe
CD69	PE	FN50	BD	Mistletoe
IFN-γ	V450	B27	BD	Mistletoe
TNF-α	FITC	MAb11	BD	Mistletoe
IL-17a	PE	eBio64DEC17	eBioscience	Mistletoe
CD107a	PC7	eBioH4A3	eBioscience	Mistletoe

[1] Toxo: Fetal Vγ9Vδ2 T cells immune responses towards congenital *Toxoplasma gondii* infection (Toxo project)

[2] Mistletoe: Vγ9Vδ2 T cells immune responses towards stimulation with mistletoe extract drugs (Mistletoe project)

3.2 Reagents

3.2.1 Complete medium

RPMI 1640 (Gibco, Invitrogen), supplemented with L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 U/mL), and 1% nonessential amino acids (Lonza) and 10% (vol/vol) heat-inactivated FCS (PPA Laboratories).

3.2.2 Other reagents

Name	*Company*	*Stock Concentration*	*Final Concentration*
HMBPP	Echelon Bioscience	1 mM	10 nM
Zoledronate	Novartis	5 mM	1 µM
sec-butylamine	Sigma-Aldrich	99%	0.5 mM
AbnobaViscum Pini	Abnoba GmbH	20 mg/ml	1000 µg/ml
AbnobaViscum Mali	Abnoba GmbH	20 mg/ml	1000 µg/ml
Iscador Pinus	Iscador AG	20 mg/ml	1000 µg/ml
Iscador Malus	Iscador AG	20 mg/ml	1000 µg/ml
IL-2	Norvatis	100000 U/ml	100 U/ml
mevastatin	Sigma-Aldrich	5 mM	2 µM
apyrase	Sigma-Aldrich	100 U/ml	0.2 U/ml
Anti-BTN3 clone 103.2	ImCheck Therapeutics	810 µg/ml	10 µg/ml
IgG2A isotype control	BD	1 mg/ml	10 µg/mL

3.3 Sample collection

3.3.1 Ethics statement

<u>For samples of the Toxo project:</u>

This study was approved by IRCCS San Matteo Polyclinic Foundation ethical committee number 20160017812. All parents were provided with written and oral information about the study and gave their consensus. Research was conducted in accordance with the Declaration of Helsinki.

<u>For samples of the Mistletoe project:</u>

This study was approved by Ethics Commission CHU Tivoli (ethical code number 917, 29 October 2013). All donors were in consent of the use of the blood. The study was conducted in accordance with the Declaration of Helsinki.

3.3.2 Samples of the Toxo project

Peripheral blood was collected in the Microbiology and Virology outpatients of IRCCS San Matteo Hospital Foundation, Pavia, Italy. The diagnosis of congenital toxoplasmosis was performed with Liaison®XL Toxo IgG II / IgM CLIA, Novalisa IgA (DiaSorin, Saluggia, Italy), VIDAS Toxo IgG II, ISAGA IgM ((bioMérieux, Marcy l'Etoile, France), IgG-IgM western blot (LDBio, Lyon, France) and homemade Interferon gamma release assay[339]. IGRA test was performed same as the test developed by Chapey and colleagues[339] with some modification: the antigen employed (the same antigen utilized for Liaison commercial tests) with a final concentration of 3µg/ml, kindly provided by DiaSorin®: this concentration yielded the best results according to previous studies. The ELISA test to evaluate IFN-γ production is a commercial kit (Qiagen, GMBH Germany).

All the mothers from both infected and non-infected groups were diagnosed with *T. gondii* infection during pregnancy and were treated in the same way (either with pyrimethamine+sulfodiazine or with spyramicin)[340]. Depending on the volume of collected blood, samples were either directly lysed (~0.5ml) with FACS-lysing solution (BD FACS™ Lysing Solution) or processed (~1ml) to isolate peripheral blood mononuclear cells (PBMC) with Lymphoprep gradient centrifugation (Lymphoprep™, Stemcell Technology) and stored in liquid nitrogen. Frozen FACS lysed blood samples (FACS lysed samples) and PBMC samples were then sent to the Institute for Medical Immunology (IMI) of the Université Libre de Bruxelles (ULB) in Belgium.

3.3.3 Samples of the Mistletoe project

PBMC were isolated by Lymphoprep gradient centrifugation (Lymphoprep™, Stemcell

Technology) and cryopreserved in liquid nitrogen from healthy adult donors (> 18 years) were isolated from blood donations at the CHU Tivoli (La Louvière, Belgium)

3.4 Flow cytometry

3.4.1 FACS lysed samples

FACS lysed samples were thawed from liquid nitrogen at 37°C and washed with PBS containing 0.1% bovine serum albumin (BSA) (Sigma). For surface staining, cells were incubated with antibody mix at 4°C for 15-20min, then washed and resuspended with 0.1%BSA/PBS. For intracellular staining, after surface staining, Phosflow™ Perm Buffer II (BD) was used to permeabilize cell membrane.

3.4.2 PBMC samples

PBMC were first washed with PBS, then labeled with 1000 times diluted Zombie NIR™ dye (Biolegend) at room temperature for 20 min and washed with 0.1% BSA/PBS. For surface staining, cells were incubated with antibody mix at 4°C for 15-20 min, then washed and resuspended with 1% PFA/PBS. For intracellular staining, after surface staining, CytofixCytoperm™ (BD) was used to permeabilize cell membranes.

Dead cells were excluded (negative for Zombie NIR) (Figure 16). Gating CD3+Vγ9+ lymphocytes identify the vast majority of Vγ9Vδ2 T cells in adult blood[341].

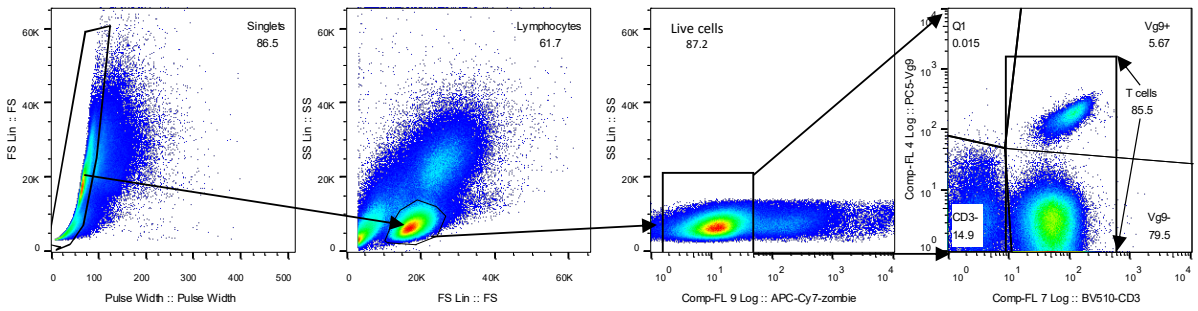


Figure 16 Gating strategy for live cells and for the main subsets studied in mistletoe extract drug stimulation.

3.4.3 Analysis

Staining results were acquired either on CyAn ADP cytometer (Dako Cytomation) or LSRFortessa (BD); analysis was done using FlowJo software and R.

3.5 Cell culture and treatment

3.5.1 Cell culture

PBMC were thawed at 37°C in complete medium and cultured in 96-well round bottom plates at 2×10^5 cells/well (1×10^6 cells/mL). Before treatment, PBMC were rested for 2 hours or overnight after thawing.

3.5.2 CD69 test

After resting, PBMC were stimulated with following compounds: HMBPP, zoledronate, sec-butylamine, AbnobaViscum Pini, AbnobaViscum Mali, Iscador Pinus and Iscador Malus. Cells were cultured at 37°C and 5% CO_2 for 1 day and harvested for surface staining.

3.5.3 Proliferation test (including CFSE test)

PBMC were either cultured in the presence of heat-treated mistletoe extracts for 7 days or washed 3 times after 1 day of incubation with non-heated extracts to remove stimulants (pulse stimulation), and then complete medium with IL-2 was added (re-added at day 3 or day 4) and cultured at 37°C and 5% CO_2 for 7 days and prepared for surface staining (see 3.4.1).

Carboxyfluorescein succinimidyl ester (CFSE) labeling was done with CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher Scientific): after resting, cells were labeled at a cell concentration of 15×10^6 cells/mL and 1.5 µM CFSE for 5 min at room temperature and washed 3 times after which the stimulants were added and the cells were cultured at 37°C and 5% CO_2 for 5 days and prepared for surface staining.

3.5.4 Cytokine test

PBMC were stimulated with the former indicated compounds for 4 hours or 1 day at 37°C and 5% CO_2 without IL-2. Monensin (2 µM) was added 4 hours before harvest. Cells were intracellularly stained.

3.5.5 CD107a test

PBMC were stimulated with the former indicated compounds for 4 hours or 1 day at 37°C and 5% CO_2 without IL-2. In the 4 hours stimulation assays, CD107a antibody was added right after the addition of the stimulant, the cells were incubated for an hour after which monensin was added; in 1 day stimulation assays, CD107a and monensin were added 4 hours before harvesting the cells. Cells were either surface stained or intracellularly stained.

3.5.6 Mevastatin test

PBMC were pretreated with 2 µM mevastatin for 1 hour, and then proceeded to stimulation and CD107a staining as above mentioned. Cells were either surface stained or intracellularly stained.

3.5.7 Apyrase treatment

Each stimulant was incubated with 0.2 U/mL apyrase at 37°C for 2 hours, control stimulants were incubated at 37°C for 2 hours without apyrase. Apyrase treated stimulants were preserved at -20°C. PBMC were treated with apyrase treated stimulants or controls, and then proceeded to CD107a staining as above mentioned. Cells were surface stained.

3.5.8 BTN3 blocking test

Zoledronate-expanded (10–14 days, cryopreserved) PBMC were used to restimulate with the indicated stimulants. BTN3 [72] and isotype control antibodies were added 1

hour before stimulation. PBMC were then proceeded to stimulants and CD107a staining as above mentioned. Cells were intracellularly stained.

3.5.9 Heat treatment for mistletoe extracts

Mistletoe products were heated in dry baths at 80°C for 5 min and then preserved at 4°C.

3.5.10 Cell expansions

After resting, PBMC were exposed to HMBPP, zoledronate, and AbnobaViscum P for 1 day, washed 3 times, after which complete medium containing IL-2 was added. IL-2 was added every 3-4 days, cells were cultured for 14 days to allow an expansion of Vγ9Vδ2 T cells.

3.6 Cell sorting

For Toxo samples:

PBMC were thawed at 37°C in complete medium, then labelled with Zombie NIR™ dye at room temperature for 10 min, and stained with CD3/TCR γδ/TCR Vγ9/TCR Vδ2 antibodies at 4°C for 15 min. Zombie NIR-/CD3+/TCR γδ+ T cells were sorted on FACS Aria III (BD) with mean purity of 98.0%. Cells were snap frozen in liquid nitrogen and preserved at -80°C.

For Mistletoe samples:

Harvested cells were labeled with Zombie NIR™ dye at room temperature for 10 min, and stained with CD3/TCR γδ/TCR Vγ9/TCR Vδ2 antibodies at 4°C for 15 min. Zombie NIR-/CD3+/TCR γδ+/TCR Vγ9+/TCR Vδ2+ T cells were sorted on FACS Aria III (BD) (purity range 94.4-100% (%Vγ9Vδ2 of T cells)). Cells were snap frozen in liquid nitrogen and preserved at -80°C.

3.7 RNA isolation and CDR3 analysis

RNA was isolated from sorted cells (~10000 cells each sample) with the RNeasy Micro Kit (Qiagen). cDNA was generated performing a template switch anchored RT-PCR. RNA was reverse transcribed via a template-switch cDNA reaction using TRGC (5'-CAAGAAGACAAAGGTATGTTCCAG-3') and TRDC (5'-GTAGAATTCCTTCACCAGACAAG-3') specific primers in the same reaction tube, a template-switch adaptor (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3') and the Superscript II RT enzyme (Invitrogen). The TRGC primer binds both TRGC1 and TRGC2. The cDNA was then purified using AMPure XP Beads (Agencourt). Amplification of the TRG and TRD region was achieved using a specific TRGC primer (binding also both TRGC1 and TRGC2 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATAGTGGGCTTGGGGGAAACATCTGCAT-3', adapter underlined) and a specific TRDC primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGGATGGTTTGGTATGAGGCTGACTTCT-3', adapter underlined) and a primer complementary to the template-switch adapter (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGCAGTGGTATCAACGCAG-3', adapter underlined) with the KAPA Real-Time Library Amplification Kit (Kapa Biosystems). Adapters were required for subsequent sequencing reactions. After purification with AMPure XP beads, an index PCR with Illumina sequencing adapters was performed using the Nextera XT Index Kit. This second PCR product was again purified with AMPure XP beads. High-throughput sequencing of the generated amplicon products containing the TRG and TRD sequences was performed on an Illumina MiSeq platform using the V2 300 kit, with 150 base pairs (bp) at the 3'end (read 2) and 150 bp at the 5'end (read 1) [at the GIGA center, University of Liège, Belgium].

Raw sequencing reads from fastq files (read 1 and read 2) were past the quality check using fastqc (version 0.11.8,

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then the sequences were aligned to reference V, D and J genes from GenBank database specifically for 'TRG' or 'TRD' to build CDR3 sequences using the MiXCR software (version 3.0.3)[342]. Default parameters were used except to assemble TRDD gene segment where 3 instead of 5 consecutive nucleotides were applied as assemble parameter. CDR3 sequences were then exported and analyzed using VDJtools software (version 1.2.1) using default settings[343]. Sequences out of frame and containing stop codons were excluded from the analysis. Data for CDR3 length, treemaps and (D)J usage are generated by the VDJtools routine 'annotate'; normalized Shannon Wiener Index by the routine 'CalcDiversityStats'; Top shared clonotypes by the routine 'TrackClonotypes' and multidimensional scaling analyzing by the routine 'ClusterSamples')[343]. Files generated from VDJtools were uploaded into Rstudio (version 1.1.463, R version 3.5.2) and analysis involved following packages: Treemap (https://CRAN.R-project.org/package=treemap) to generate Treemap plots, ggplot2[344] for data visualization, ggpubr (https://CRAN.R-project.org/package=ggpubr) for statistical analysis .

3.8 Dimensionality reduction and clustering

Flow cytometry results generated from Flowjo were uploaded into Rstudio. Package ggfortify (https://CRAN.R-project.org/package=ggfortify) and ggbiplot (http://github.com/vqv/ggbiplot) were used to generate principal-components analysis (PCA). The expression feature of each marker (eigenvectors) is indicated by arrows in the PCA figures using loadings.label. Package Rtsne (https://github.com/jkrijthe/Rtsne) was used to generate t-distributed stochastic neighbor embedding (t-SNE) clustering analysis[345]. Parameters were adapted according to sample size. t-SNE analysis were run multiple times using different parameters.

3.9 Statistics

Statistical analysis was performed with R package ggpubr

(https://CRAN.R-project.org/package=ggpubr). Student's t-test and paired t-test were used for normally distributed data (determined by the Shapiro-Wilk test, $p>0.05$) and with equal variances (determined by the Levene's test, $p>0.05$). Otherwise, Mann Whitney U test or Wilcoxon signed-rank test was used.

4. Results

4.1 Vγ9Vδ2 T cells provide immunity to *Toxoplasma gondii* infection in the fetus

Introduction

Although Vγ9Vδ2 T cells express a potent cytotoxic effector phenotype and are activated and expanded in a TCR-dependent manner by microbe- and host-derived PAg, the fetal Vγ9Vδ2 T cells that with a distinct developmental origin are hyporesponsive towards phosphoantigen stimulation *in vitro*, they are highly regulated by PD-1 and they do not show a cytotoxic effector phenotype, all features that are likely related to (tolerance) requirements of the fetal immune system[3,346-348,168,349]. Thus it is unclear whether Vγ9Vδ2 T cells in the human fetus can provide anti-microbial immunity.

Here, we investigated whether human fetal Vγ9Vδ2 T cells, despite having a highly regulated phenotype, have the capacity to react towards microbial infections using the model of congenital *Toxoplasma gondii* (*T. gondii*) infection. We found that Vγ9Vδ2 T cells with fetal public TCR clonotypes expanded strongly and differentiated towards potent killer effector cells in infants with congenital *T. gondii* infection.

Results

Study population

Pregnant mothers with primary *T. gondii* infection were enrolled in this study. In order to address the fetal/newborn immune response towards *T. gondii* infection, blood from their infants were collected from birth till 2 years of age. Twelve out of 70 infants (17%) were diagnosed with congenital *T. gondii* infection ('Toxo+'). The Toxo+ infants were age-matched with their non-infected ('Toxo-') counterparts (< 1 month: $p=0.397$; > 1 month: $p=0.140$). Clinical characteristics such as age at the moment of diagnosis and treatment schedules of the Toxo+ infants can be found in Table 2.

Table 2. Clinical information regarding infants congenitally infected with T. gondii

Sample	Sample type	Sex	Gestation time at diagnosis (Mother)	Start of treatment (Mother)	Treatment (Mother)	Age at diagnosis (infant)	Start of treatment (infant)	Treatment (infant)	Symptom (infant)	Age at sampling
I-Toxo-001	FACS-lysed	M	20gw	28gw	pyr+sulf	At birth	3 weeks	pyr+sulf+FA	No	5 days
I-Toxo-011	FACS-lysed	F	28gw	29gw	pyr+sulf	At birth	3 weeks	pyr+sulf+FA	No	1 day
I-Toxo-012	FACS-lysed	F	34gw	34gw	spyr	At birth	3 weeks	pyr+sulf+FA	No	1 day
I-Toxo-024	FACS-lysed	M	34gw	35gw	spyr	2 months	2 months	pyr+sulf+FA	No	99 days
I-Toxo-072	FACS-lysed	M	At delivery	no	no	At birth	At birth	pyr+sulf+FA	No	93 days
I-Toxo-075	FACS-lysed	F	26gw	28gw	pyr+sulf	*In utero*	At birth	pyr+sulf+FA	No	29 days
I-Toxo-080	FACS-lysed PBMC	M	24gw	26gw	spyr	4 months	4 months	pyr+sulf+FA	Retinitis	511 days
I-Toxo-085	FACS-lysed PBMC	F	35gw	36gw	spyr	At birth	1 month	pyr+sulf+FA	No	73 days
I-Toxo-090	PBMC	F	12gw	no	no	At birth	3 months	pyr+sulf+FA	No	434 days
I-Toxo-106	PBMC	M	-	-	pyr+sulf	7 days	15 days	pyr+sulf+FA	No	82 days
I-Toxo-108	PBMC	F	36gw	37gw	spyr	At birth	15 days	pyr+sulf+FA	No	755 days
I-Toxo-122	PBMC	M	27gw	28gw	Pyr+sulf (only 1 month)	15 days	21 days	pyr+sulf+FA	No	78 days

gw: gestation week; "-": no information; pyr: pyrimethamine; sulf: sulfodiazine; spyr: spyramicin; FA: folic acid.

Vγ9Vδ2 T cells expand strongly and specifically upon congenital *T. gondii* infection

First, we determined the percentage of γδ T cells and found that they were significantly increased in Toxo+ infants (Figure 17A, left panel). We noticed a high variation in percentage of γδ T cells in both the Toxo+ and Toxo- group that appeared to be age-related (Figure 18-19). Therefore, we analyzed the percentage of γδ T cells in newborns (<1 month) and infants older than 1 month separately. Strikingly, the percentage of γδ T cells was only different between the newborn Toxo+ and Toxo- group (Figure 17B, left panel). No differences could be observed anymore in infants older than 1 month, due to both an increase of γδ T cells in infants of the Toxo- group and a decrease within the Toxo+ group (Figure 17B-C, left panels; Figure 18-19). We could not detect higher expression of the proliferation marker Ki-67 in γδ T cells from Toxo+ newborns; and in the >1 month-infants, the percentage of Ki-67+ γδ T cells was even lower in the Toxo+ group (Figure 17, right panels), indicating that the increased percentage of γδ T cells was mainly due to prior γδ T cell proliferation *in utero*.

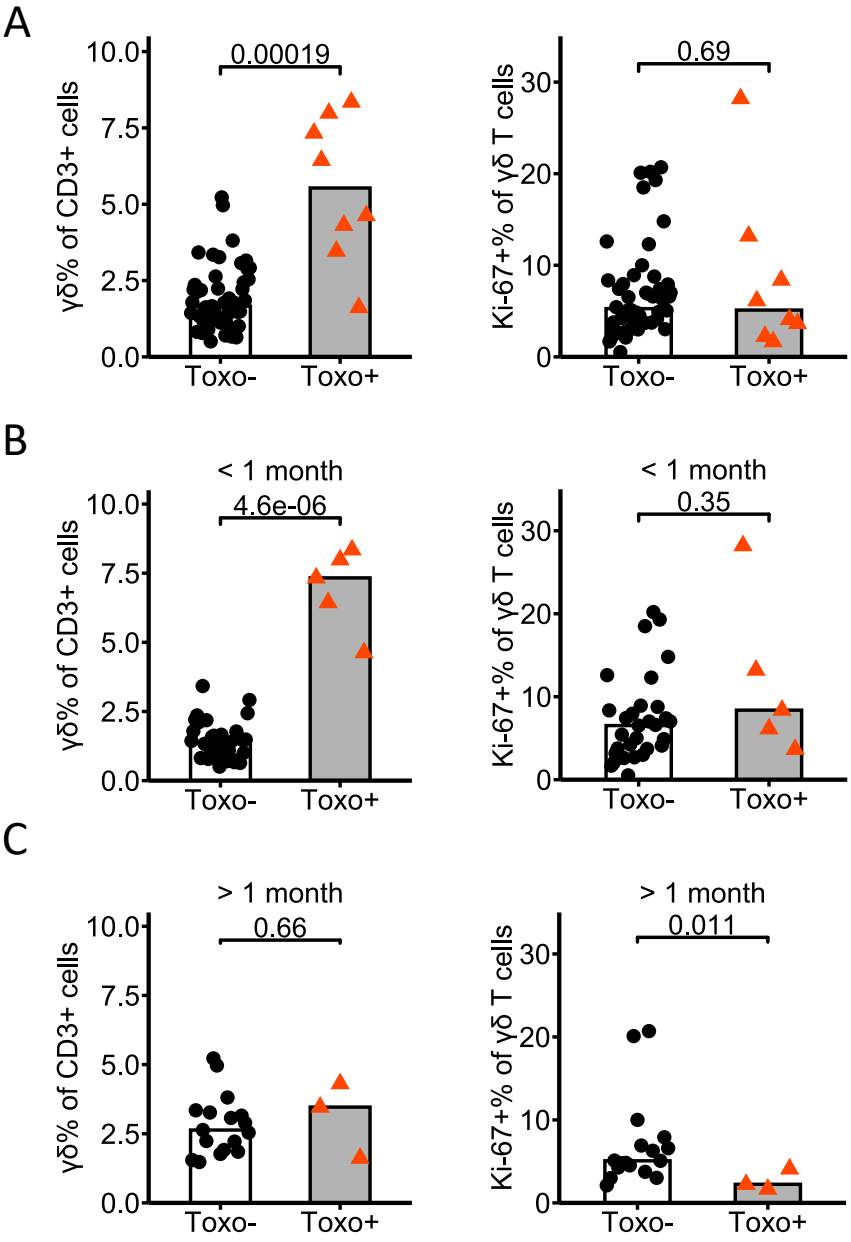


Figure 17 Congenital *T. gondii* infection induces expansion of γδ T cells *in utero*.
(A) Left panel: percentage of γδ T cells (of total CD3+ T cells), (Toxo-: n=49, Toxo+: n=8); right panel, expression of the proliferation marker Ki-67 in γδ T cells (Toxo-: n=48, Toxo+: n=8). **(B)** Left panel: percentage of γδ T cells (of total CD3+ T cells) in <1 month infants (Toxo-: n=32, Toxo+: n=5); right panel, expression of the proliferation marker Ki-67 in γδ T cells in <1 month infants (Toxo-: n=31, Toxo+: n=5). **(C)** Left panel: percentage of γδ T cells (of total CD3+ T cells) in >1 month infants (Toxo-: n=17 , Toxo+: n=3); right panel, expression of the proliferation marker Ki-67 in γδ T cells in >1 month infants (Toxo-: n=17 , Toxo+: n=3). Data from FACS-lysed samples; bar indicates median.

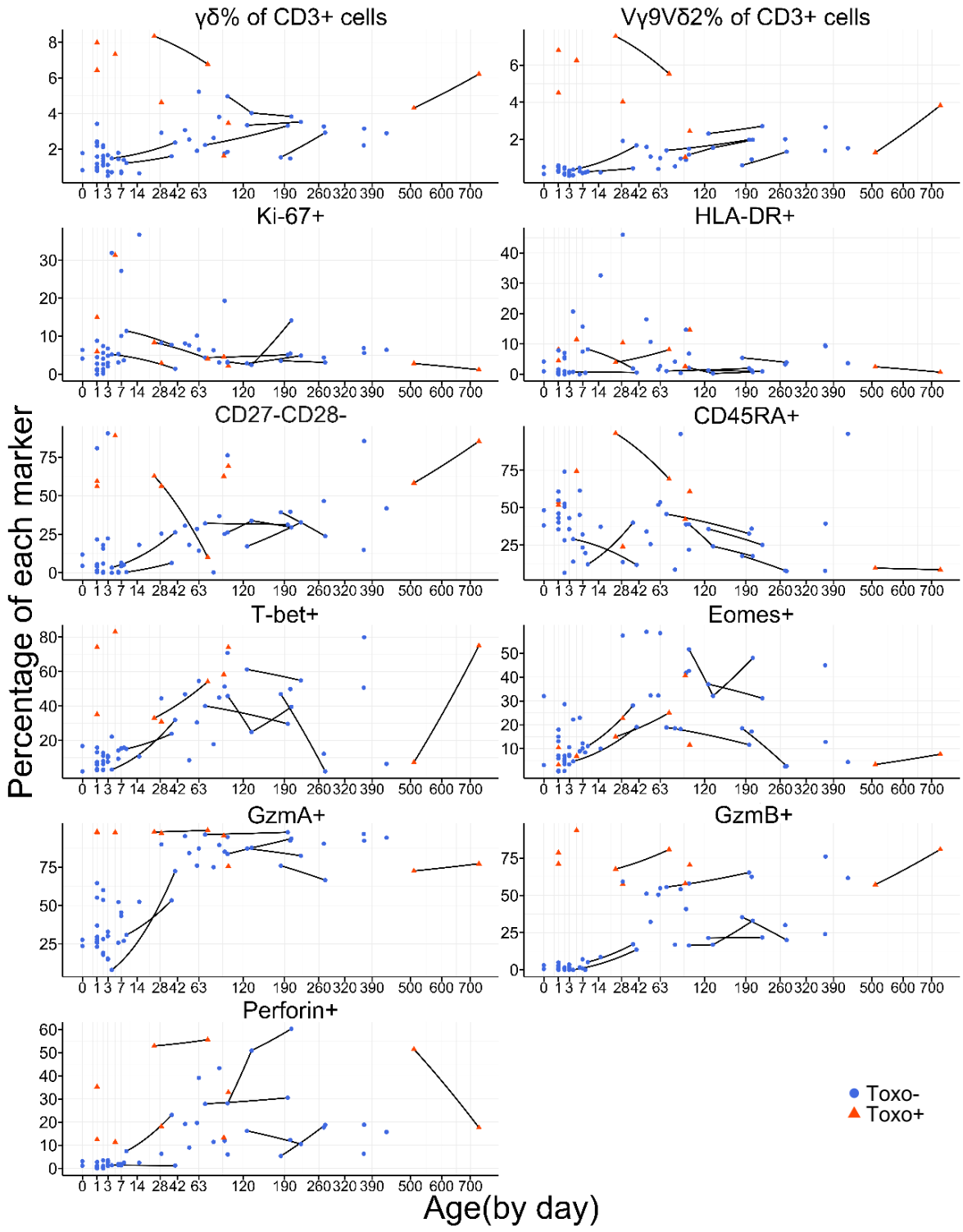


Figure 18 Percentage of γδ T cells, Vγ9Vδ2 T cells (of CD3+) and expression of a series of markers on Vγ9Vδ2 T cells according to age.

Flow cytometry results of γδ and Vγ9+Vδ2+ percentage of T cells (n=66) and percentage of HLA-DR+, CD27-CD28-,CD45RA+ (n=60), Ki-67+,T-bet+, eomes+ (n=65), granzyme A+, granzyme B+, perforin+ (n=63) on Vγ9+Vδ2+ T cells. Age is expressed in days after birth. Lines connect samples of the same subject. Toxo- samples are in blue dots, Toxo+ samples in orange triangles. The eldest Toxo+ sample has ocular toxoplasmosis.

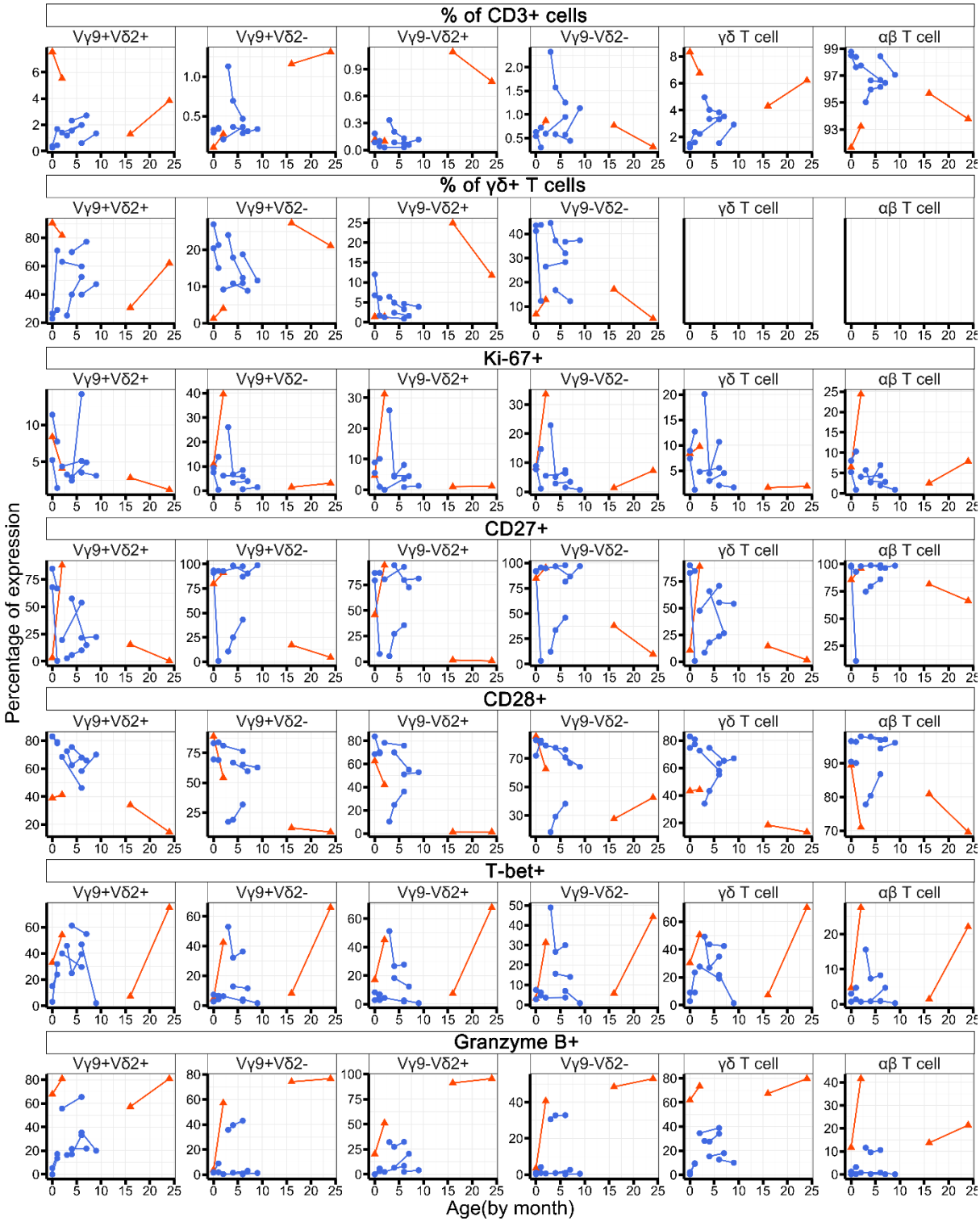


Figure 19 Phenotype results on all T cell subsets of subjects with multiple samples at different ages.

Percentage of γδ subsets, total γδ T cells and αβ T cells in T cells (first row), γδ subsets percentage of γδ T cells (second row), and percentage of Ki-67+, CD27+, CD28+, T-bet+ and granzyme B+ expression on each subsets (3-7 rows). Toxo- samples are in blue round dot, Toxo+ samples are in orange triangle dot. Toxo-: n=6, Toxo+: n=2. Toxo+ subject of age 16 months and 24 months has ocular toxoplasmosis.

Next, we investigated more specifically the Vγ9+Vδ2+ subset and compared it to other γδ subsets. Using antibodies specific against the Vγ9 and Vδ2 chain (combined with CD3 and pan-γδ antibodies), we could delineate four different populations: Vγ9+Vδ2+, Vγ9+Vδ2-, Vγ9-Vδ2+, and Vγ9-Vδ2- γδ T cells (Figure 20). The increase in newborn (<1 month) γδ T cells upon congenital *T. gondii* infection was highly restricted to the Vγ9+Vδ2+ T cell subset; the other subsets did not show different percentages compared to Toxo- newborns, including the more abundant Vγ9-Vδ2- γδ T cell subset (Figure 20). In infants older than 1 month, no difference in γδ T cell subset frequencies could be observed between Toxo+ and Toxo- subjects (Figure 21A). Gating on newborn Vγ9Vδ2 T cells, again no increase in the Ki-67 proliferation could be observed between Toxo+ and Toxo- subjects (Figure 21B), supporting the notion that the proliferation of Vγ9Vδ2 T cells has already occurred *in utero*.

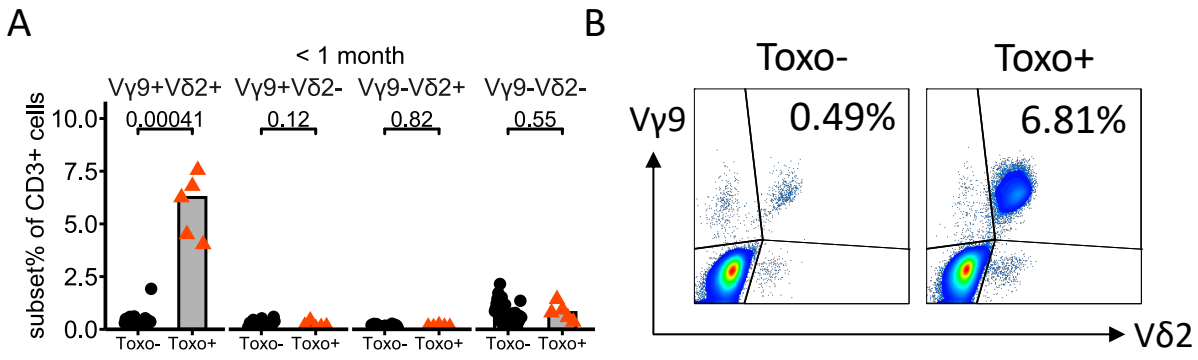


Figure 20 The expansion of newborn (<1 month) γδ T cells upon congenital *T. gondii* infection is highly restricted to Vγ9+Vδ2+ T cells.

(A) γδ subset percentages (of T cells) in <1 month infants (Toxo-: n=32, Toxo+: n=5). Resutls generated from FACS-lysed samples; bar shows median. **(B)** Representative flow cytometry plots of a Toxo- (2 days old) and Toxo+ (1 day old) newborn. Gate is put on CD3+ T lymphocytes, percentages of Vγ9+Vδ2+ T cells are indicated.

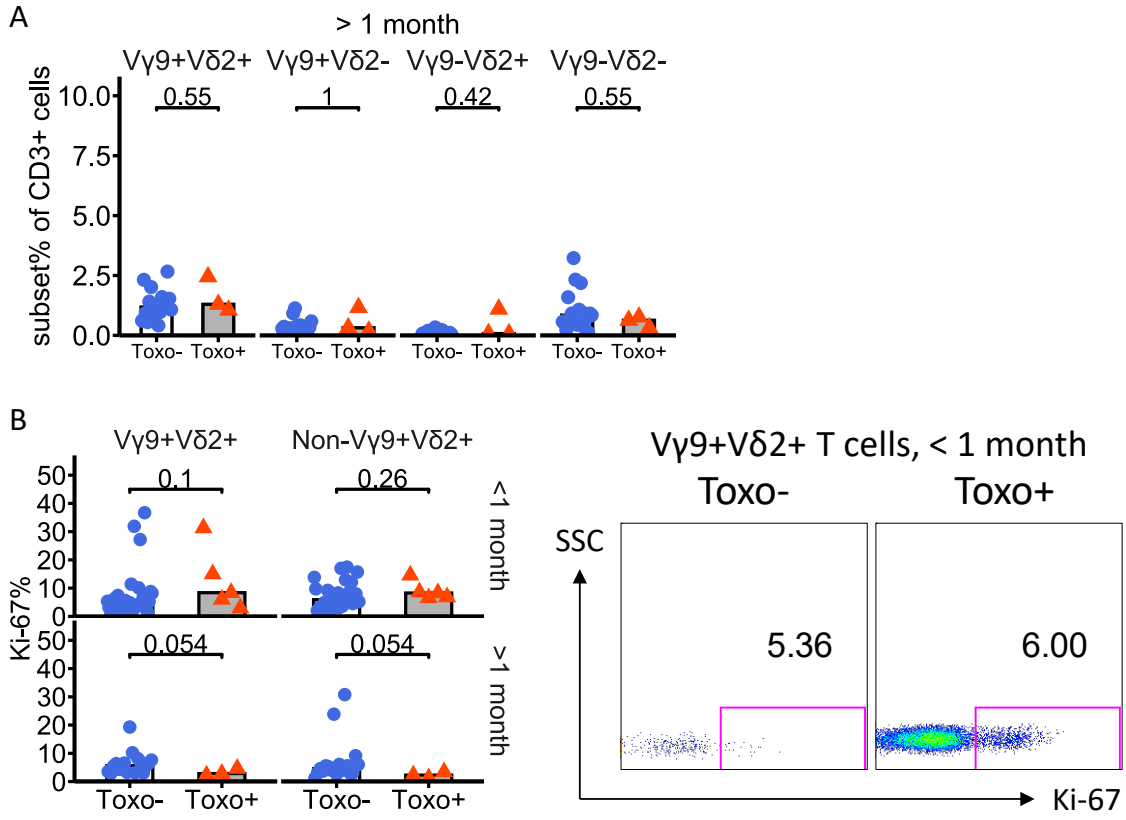


Figure 21 γδ T cell subset percentages and Ki-67 expression.

(A) γδ T cell subset percentages (of CD3+ cells) on >1 month FACS-lysed samples (Toxo-: n=17, Toxo+: n=3). **(B)** Left panel: expression of the proliferation marker Ki-67 in Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells of <1 month samples (upper panel, Toxo-: n=31, Toxo+: n=5) and >1 month samples (lower panel, Toxo-: n=17, Toxo+: n=3); bar indicates median. Right panel: flow cytometry plots of Ki-67 expression, gated on Vγ9+Vδ2+ T cells from representative <1 month samples.

Newborn Vγ9Vδ2 T cells are highly differentiated upon congenital *T. gondii* infection

Next, we examined whether the expanded Vγ9Vδ2 T cells upon congenital *T. gondii* infection were differentiated, as assessed by the downregulation of CD28 and CD27[350]. The Vγ9Vδ2 T cells of Toxo+ newborns (<1 month) were highly differentiated (CD27-CD28-) compared to Toxo- newborns (Figure 22A, left panel; Figure 22C). While a Toxo+ vs Toxo- difference could still be observed in infants older than 1 month, this difference became less pronounced due to an increase of differentiated Vγ9Vδ2 T cells in >1 month Toxo- infants (Figure 22B, left panel). A relatively small difference in differentiation status could also be observed in nonVγ9Vδ2 T cells in <1 month but not in >1 month infants (Figure 22A-B, left panels). In contrast to differentiation, the activation status (HLA-DR expression) of Vγ9Vδ2 T cells and nonVγ9Vδ2 T cells of Toxo+ and Toxo- infants, both in the <1 month and >1 month group (Figure 22A-B, right panels), were similar, like the expression of the proliferation marker Ki-67 (Figure 18-19; Figure 21B). Thus, it appears that the Vγ9Vδ2 T cells were activated and differentiated *in utero* along with the strong expansion, after which the proliferation and activation declined while the differentiation status remained stable and high till early after birth.

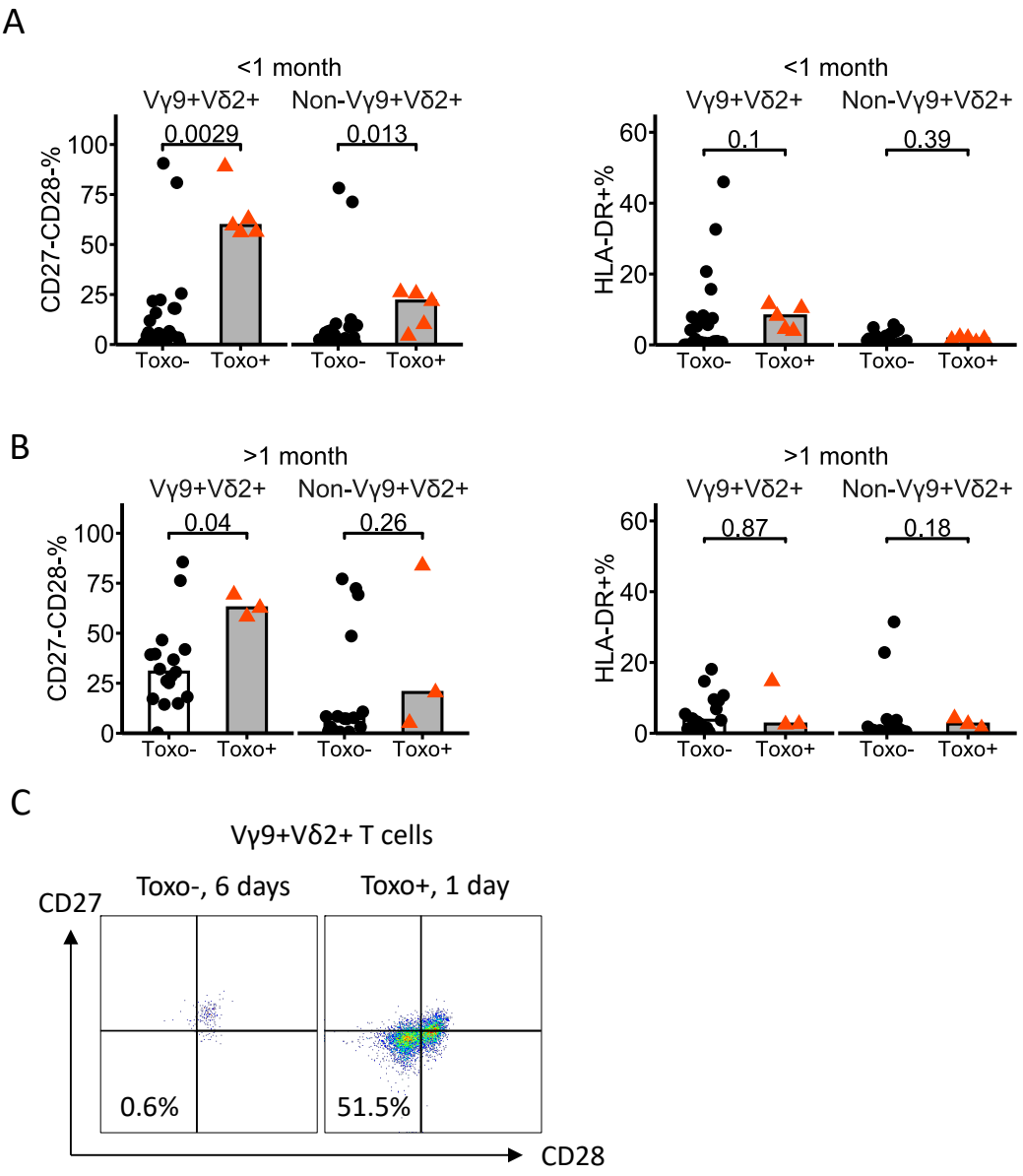


Figure 22 Vγ9Vδ2 T cells are differentiated upon congenital *T. gondii* infection.

(A) Left panel: percentage of CD27-CD28- cells of Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells on <1 month newborns (Toxo-: n=26, Toxo+: n=5); right panel: expression of activation marker HLA-DR on Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells of <1 month newborn samples (Toxo-: n=26, Toxo+: n=5). Bar shows median. **(B)** Left panel: percentage of CD27-CD28- cells of Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells on >1 month infants (Toxo-: n=17, Toxo+: n=3); right panel: expression of activation marker HLA-DR on Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells of <1 month infant samples (Toxo-: n=17, Toxo+: n=3). Bar shows median. **(C)** Representative flow cytometry plots of CD28 vs CD27 staining of Toxo- (left) and Toxo+ (right) newborn Vγ9Vδ2 T cells. Values indicate the percentages of CD27-CD28- cells (of Vγ9Vδ2 T cells).

In addition, we also tested CD45RA expression on those subjects, as CD27 and CD45RA co-expression is also commonly used to separate the differentiation status of T cells[351]. we include the results of CD27-CD45RA+ subset as supplemental information in Figure 23. In this scheme, CD27-CD45RA+ cells represent the terminally differentiated T cells. All T cell subsets from *T. gondii* infected subjects increased CD27-CD45RA+ cells at <1 month of age, but Vγ9Vδ2 T cells were the most differentiated (Figure 23). We did not observe differences between *T. gondii* infected and non-infected subjects for CD27-CD45RA- (effector memory) expression (data not shown).

Furthermore, CD27 and CD28 expression were both downregulated on Vγ9Vδ2 T cells of Toxo+ samples and remained stable in all age group samples; while the dynamics of CD27 and CD28 expression on γδ T cells were different in Toxo- samples. CD27 expression was downregulated on Toxo- samples (Figure 24) during aging. We include separate figures for CD27 and CD28 expression in Figure 24-25, and display the comparison between infection groups and age groups.

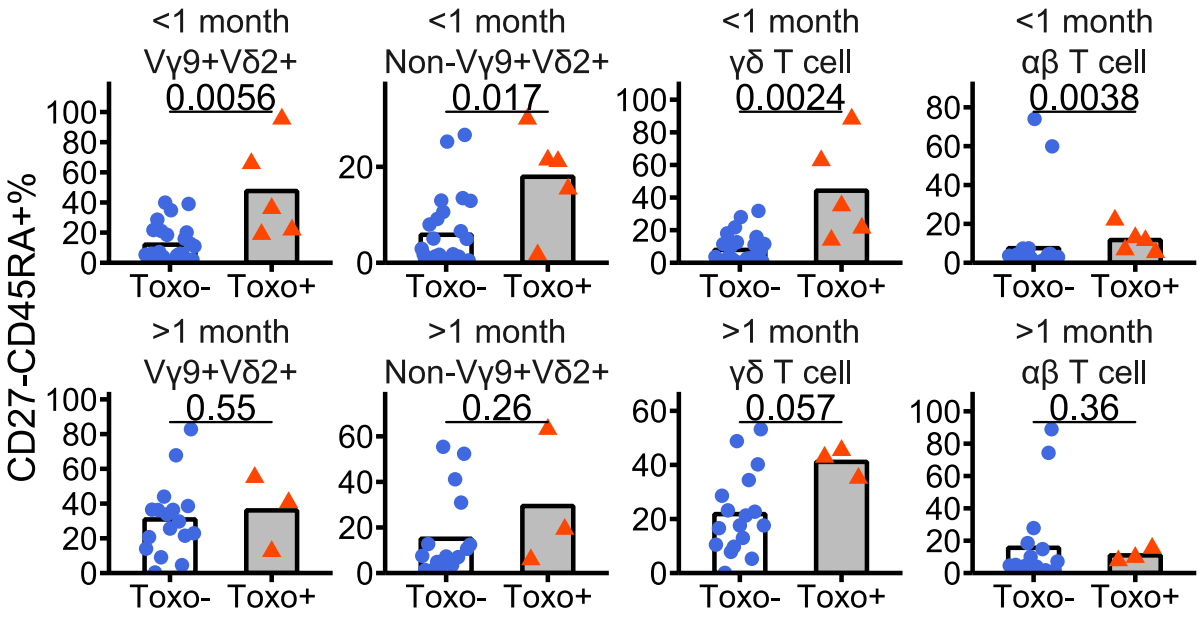


Figure 23 CD27-CD45RA+ expression on T cells.

Percentage of CD27-CD45RA+ expression on Vγ9Vδ2 T cells, nonVγ9Vδ2 T cells, total γδ T cells and αβ T cells in <1 month subjects (Toxo-: n=26, Toxo+: n=5; top row) and >1 month subjects (Toxo-: n=17, Toxo+: n=3; bottom row). Bar indicate mean value, statistic: Mann Whitney U test.

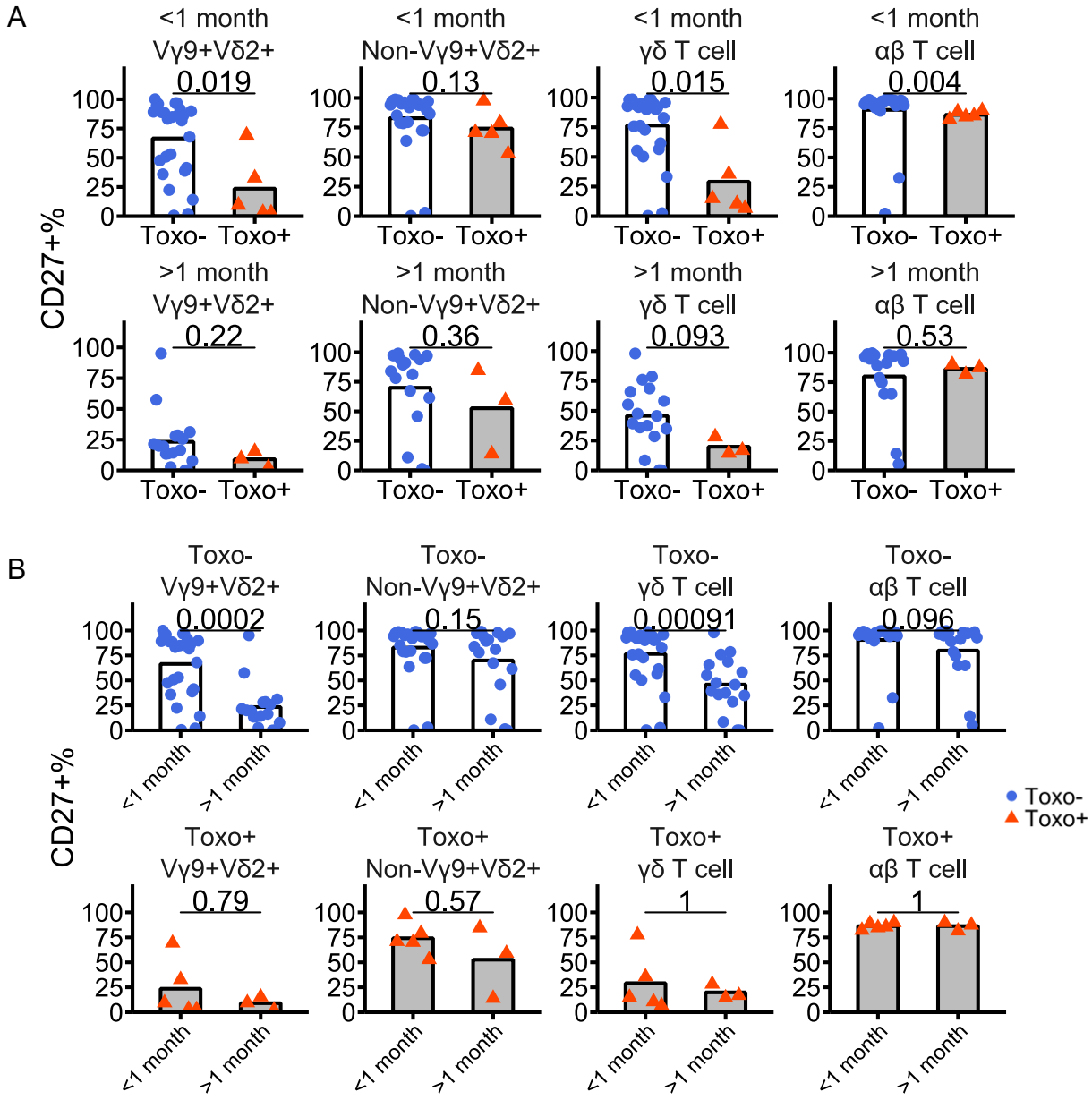


Figure 24 CD27+ expression on T cells compared by *T. gondii* infection and age.

(A) Percentage of CD27+ expression on Vγ9Vδ2 T cells, nonVγ9Vδ2 T cells, total γδ T cells and αβ T cells in <1 month subjects (top row) and >1 month subjects (bottom row). **(B)** CD27+ expression compared by age. Comparisons on Vγ9Vδ2 T cells, nonVγ9Vδ2 T cells, total γδ T cells and αβ T cells in *T. gondii* non-infected subjects are at the top row, comparisons between infected subjects are at the bottom.

For <1 month subjects, Toxo-: n=26, Toxo+: n=5; and >1 month subjects, Toxo-: n=17, Toxo+: n=3. Bar indicates mean value, statistic: Mann Whitney U test

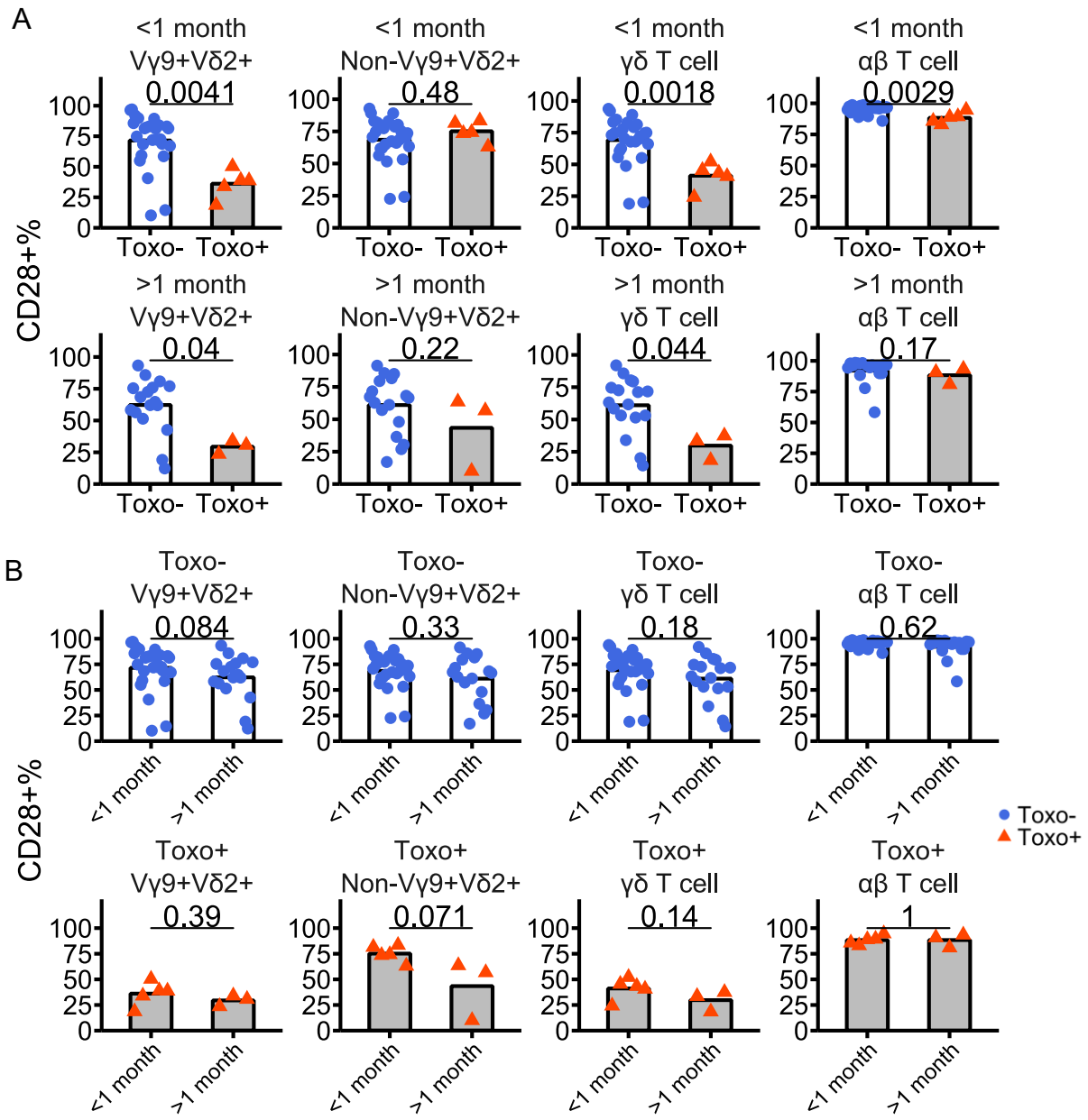


Figure 25 CD28+ expression on T cells compared by *T. gondii* infection and age.

(A) Percentage of CD28+ expression on Vγ9Vδ2 T cells, nonVγ9Vδ2 T cells, total γδ T cells and αβ T cells in <1 month subjects (top row) and >1 month subjects (bottom row). **(B)** CD28+ expression compared by age. Comparisons on Vγ9Vδ2 T cells, nonVγ9Vδ2 T cells, total γδ T cells and αβ T cells in *T. gondii* non-infected subjects are at the top row, comparisons between infected subjects are at the bottom.

For <1 month subjects, Toxo-: n=26, Toxo+: n=5; and >1 month subjects, Toxo-: n=17, Toxo+: n=3. Bar indicates mean value, statistic: Mann Whitney U test.

Vγ9Vδ2 T cells develop potent cytotoxic effector functions upon congenital *T. gondii* infection

We investigated whether the differentiation of the Vγ9Vδ2 T cells *in utero* was associated with effector functions. A main function of adult Vγ9Vδ2 T cells is to kill infected cells, including cells infected with *T. gondii* [286]. While fetal Vγ9Vδ2 T cells express granzyme A (GzmA), they do not express granzyme B (GzmB) and perforin[3], the main cytotoxic effector molecules that can efficiently kill infected cells[352]. Indeed, also newborn Vγ9Vδ2 T cells did not express GzmB and perforin (Figure 26A, left panels; Figure 26B, left plot; Figure 18-19). However, upon congenital *T. gondii* infection, the expressions of these two cytotoxic mediators were strikingly increased: a vast majority of (newborn) Vγ9Vδ2 T cells expressed GzmB, while perforin was co-expressed in the GzmB^{high} Vγ9Vδ2 T cells (Figure 26A, left panels, Figure 26B, right plot). The co-expression of GzmB and perforin is in line with the need of their combined action in order to mediate their cytotoxic activity[352]. At older ages (infants >1 month), the Vγ9Vδ2 T cells of Toxo- infants started also to express GzmB and perforin and thus no difference could be observed anymore with Toxo+ infants (Figure 26A, right panels; Figure 18-19). Since the transcription factor T-bet is important for the expression of GzmB and perforin[352], we investigated the expression of this transcription factor in Vγ9Vδ2 T cells. In line with its role in the expression of these cytotoxic mediators, the expression of T-bet followed the same expression pattern as GzmrB and perforin (Figure 26C, upper panels; Figure 18-19). In contrast, this was not observed for the transcription factor eomes (Figure 26C, bottom panels; Figure 18). Granulysin is a cytotoxic granule pore-forming peptide that can directly permeabilize bacteria and parasites and delivers death-inducing GzmB into these pathogens[353]. Furthermore, adult Vγ9Vδ2 T cells that are expanded in blood stage of malaria (*Plasmodium falciparum*)-infected patients are able to reduce parasite reinvasion in a granulysin-dependent manner[354,355]. However, in contrast to GzmB and perforin, granulysin remained low in Toxo+ infants, even in >1 month infants

(Figure 26D), indicating that this cytotoxic mediator does not play an important role in the defense of fetal Vγ9Vδ2 T cells against congenital *T. gondii* infection. Finally, we confirmed the programmed expression of $GzmA^3$ in Toxo- newborns, which was further increased upon congenital *T. gondii* infection (Figure 26E; Figure 18).

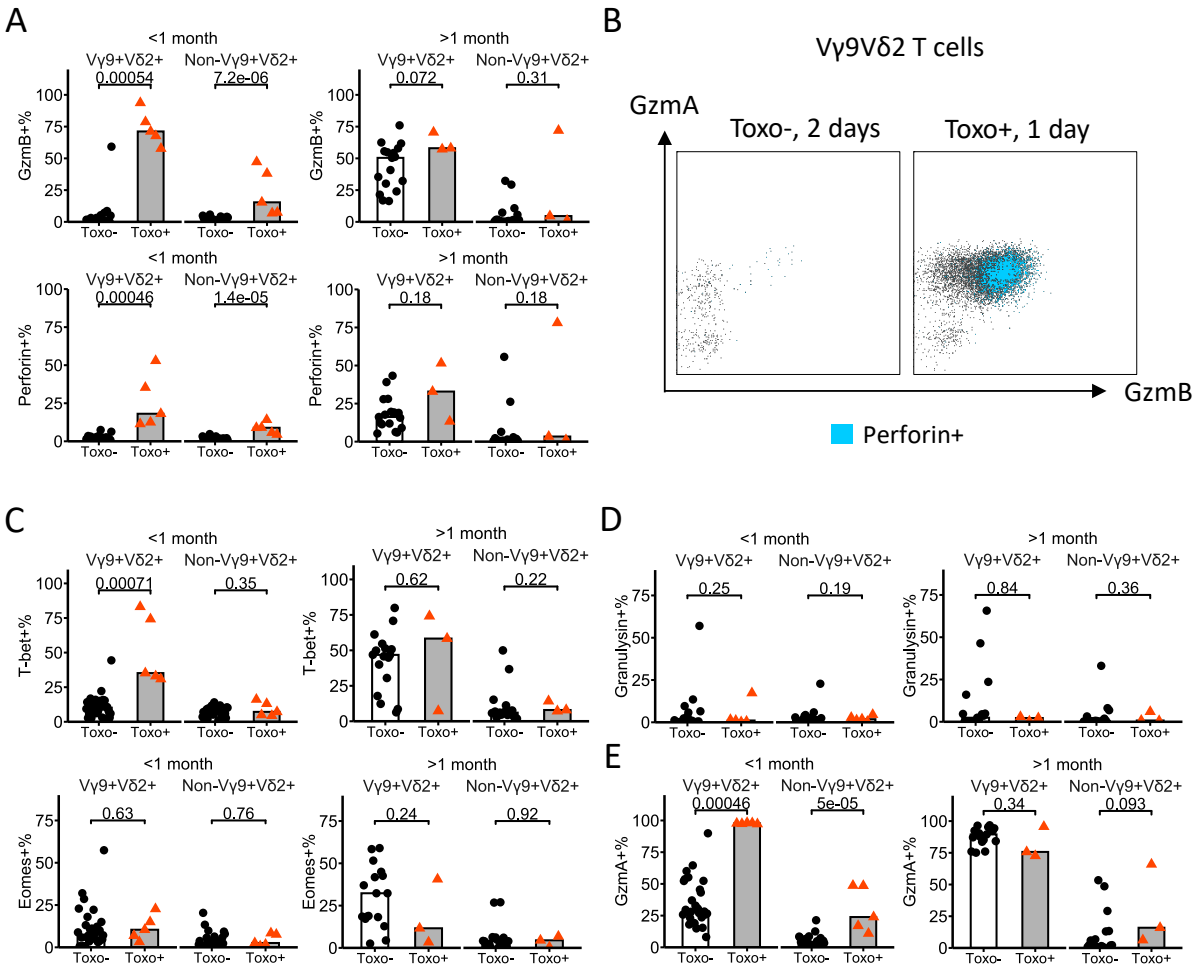


Figure 26 Vγ9Vδ2 T cells develop potent cytotoxic effector functions upon congenital *T. gondii* infection.

(A) Expression of GzmB (upper row) and perforin (lower row) in Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells. Left panels: <1 month; right panels: >1 month. **(B)** Flow cytometry plots gated on Vγ9Vδ2 T cells of a representative sample of Toxo- (2 days old) and Toxo+ (1 day old) newborns illustrating the expression of GzmA, GzmB and perforin. **(C)** Transcription factors T-bet (upper row) and eomes (lower row) expression on Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells. Left panels: <1 month; right panels: >1 month. **(D-E)** Expression of granulysin **(D)** and GzmA **(E)** on Vγ9+Vδ2+ and nonVγ9+Vδ2+ γδ T cells. Left panels: <1 month; right panels: >1 month. For **(A)(D)(E)**, <1 month: Toxo-: n=29, Toxo+: n=5; >1 month: Toxo-: n=17, Toxo+: n=3; for **(C)**, <1 month: Toxo-: n=31, Toxo+: n=5; >1 month: Toxo-: n=17, Toxo+: n=3. Bar indicates median.

Due to the limitation in obtaining PBMC samples, we only tested *in vitro* Vγ9Vδ2 T cells functional response to HMBPP stimulation in four subjects at 2 months of age (Figure 27). Only one of the infected samples had sufficient live cells. We observed similar CD107a, IFNγ, and TNFα responses on this Toxo+ sample and the other two Toxo- samples.

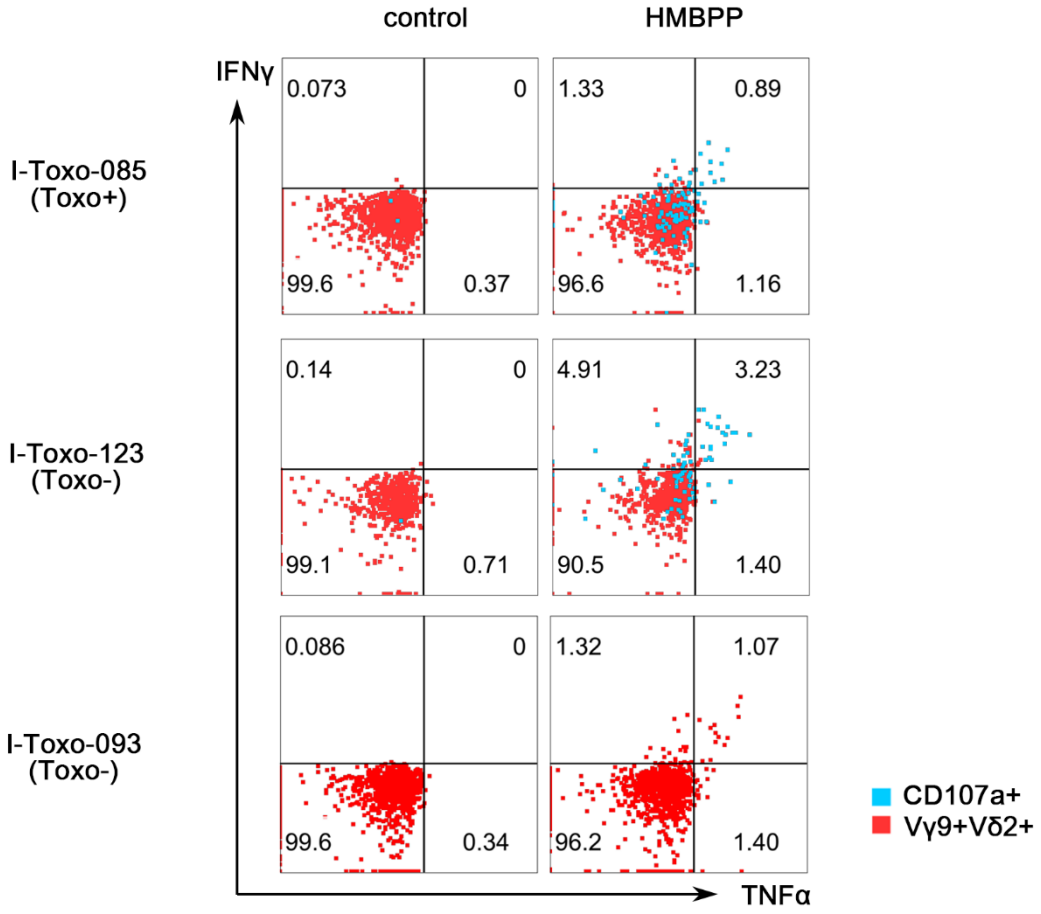


Figure 27 *In vitro* stimulation of 2 months old subjects.

PBMC samples were stimulated with HMBPP (10nM) in 96-well U-bottom plate overnight. Flow cytometry plots of CD107a (n=2), IFNγ and TNFα expressions on Vγ9Vδ2 T cells are showed. Percentage of IFNγ and TNFα co-expression are indicated in each gate. CD107a+% in I-Toxo-085 (Toxo+) and I-Toxo-123 (Toxo-) are 8.62% and 10.5% respectively.

In order to have a global overview of all the flow cytometry data of Vγ9Vδ2 T cells in Toxo+ and Toxo- infants at <1 month and >1 month of age and how this compares to the data obtained in nonVγ9Vδ2 T cells and conventional αβ T cells, we performed t-SNE and PCA analysis (Figure 28-29). This analysis revealed that early after birth (<1 month) Vγ9Vδ2 T cells from Toxo+ infants are clearly forming a distinct cytotoxic effector-related cluster, while this is not the case for nonVγ9Vδ2 T cells and αβ T cells. Later in life, the Vγ9Vδ2 T cells from Toxo- and Toxo+ infants grouped together into one cluster (Figure 28-29). Thus this global analysis highlights the early and potent response of Vγ9Vδ2 T cells towards congenital *T. gondii* infection.

Notably, one of the Toxo+ subjects showed retinitis symptom (Table 2, Figure 18-19), and we did not observe any difference on its phenotypes and functional status from other Toxo+ samples.

In summary, Vγ9Vδ2 T cells develop potent cytotoxic (GzmB+perforin+) effector functions upon congenital *T. gondii* infection.

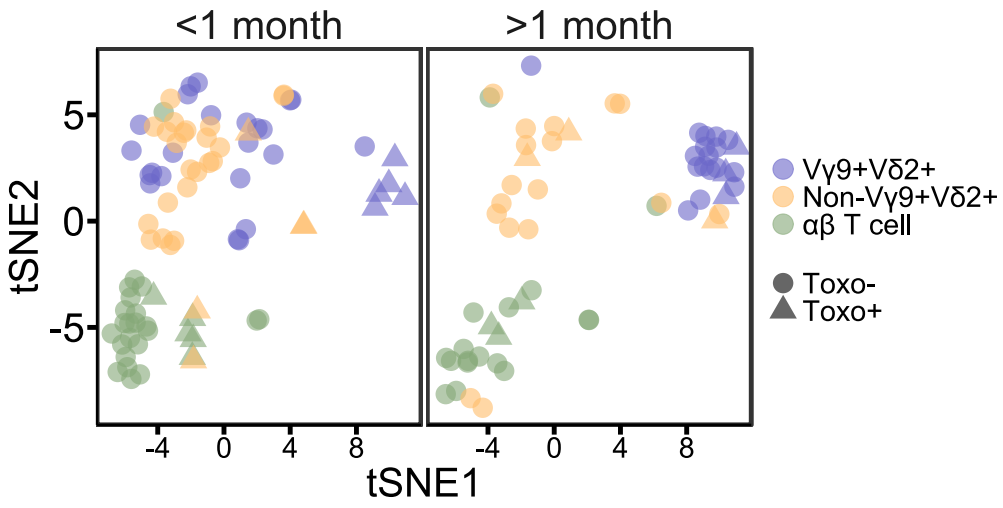


Figure 28 Vγ9Vδ2 T cells in congenital *T. gondii*-infected newborns form a distinct cluster in t-SNE analysis of flow cytometry data.

t-SNE analysis of flow cytometry results (11 markers [HLA-DR, CD27, CD28, CD45RA, Ki-67, T-bet, eomes, GzmA, GzmB, granulysin, perforin], n=48 subjects). Color indicate T cell subsets, Toxo- samples in round dot, Toxo+ samples in triangle dot.

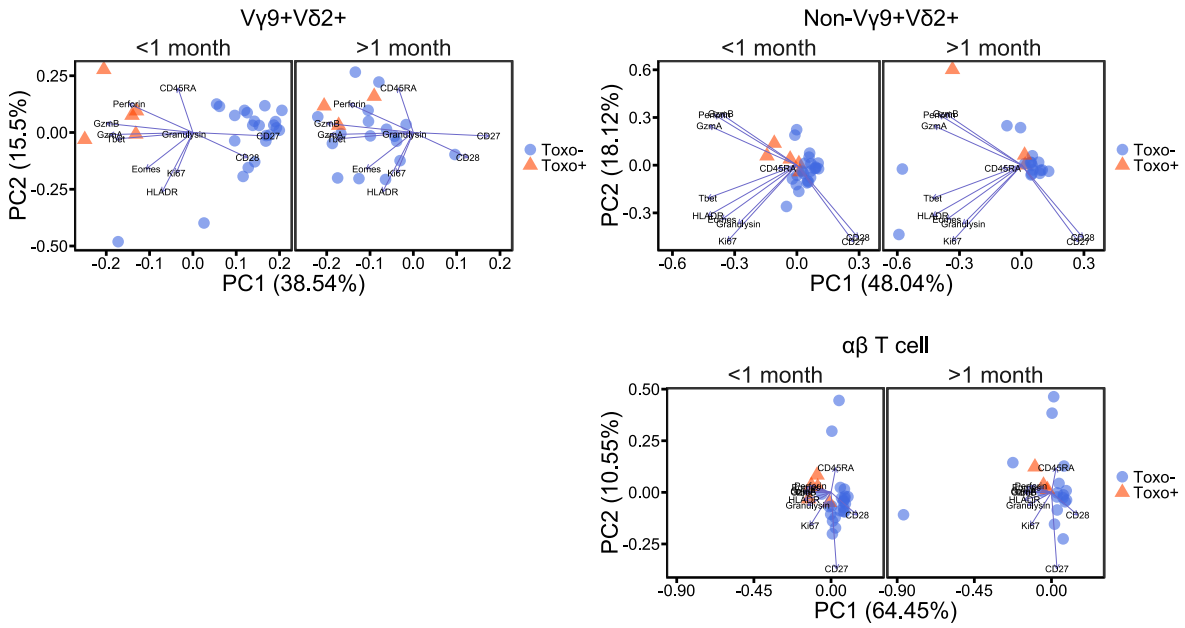


Figure 29 Toxo+ newborn Vγ9Vδ2 T cells are clustered by the high expression of cytotoxic molecules and low expression of CD27 and CD28.

PCA analysis of all flow cytometry results (11 makers [HLA-DR, CD27, CD28, CD45RA, Ki-67, Tbet, eomes, GzmA, GzmB, granulysin, perforin], n=48 subjects), with Vγ9Vδ2 T cells at the top left, nonVγ9+Vδ2+ γδT cells at the top right and αβ T cells at the bottom right; each time with <1 month samples on the left, >1 month samples on the right. Toxo- in blue dot, Toxo+ in red triangles.

The Vγ9Vδ2 TCR repertoire of Toxo+ infants is different from Toxo- infants

The detailed flow cytometry analysis (Figure 17-26, 28-29) showed that Vγ9Vδ2 T cells are strongly expanded and differentiated in <1 month infants with congenital *T. gondii* infection. But this impact disappeared in older (>1 month) infants, probably due to a more general post-natal phosphoantigen exposure that affects as well the Toxo- infants. We wondered whether this difference in timing of phosphoantigen exposure (fetal in Toxo+ infants versus post-natal in Toxo- infants) and thus timing of Vγ9Vδ2 T cell expansions can lead to a different infant TCR repertoire. Therefore we analyzed the TCR repertoire of sorted γδ T cells at 2 months and 1 year after birth of Toxo+ and Toxo- infants (note that, because of limited material, we could not assess the repertoire in <1 month newborns). Analyzing the TRGV (Vγ) and TRDV (Vδ) usage did not show a difference in the usage of the TRGV9 (the gene segment encoding the Vγ9 chain) or TRDV2 (the gene segment encoding the Vδ2 chain) between Toxo+ and Toxo- subjects at these time points (Figure 30A), which is in line with flow cytometry data (Figure 30B). It is known that TRGV9 is almost exclusively associated with the joining gene segment TRGJP[23], known for its importance for phosphoantigen reactivity of the Vγ9Vδ2 TCR[26]. Therefore we focused our analysis on TRGV9-TRGJP- and TRDV2-containing CDR3 sequences in order to assess the Vγ9Vδ2 TCR-associated repertoire[23].

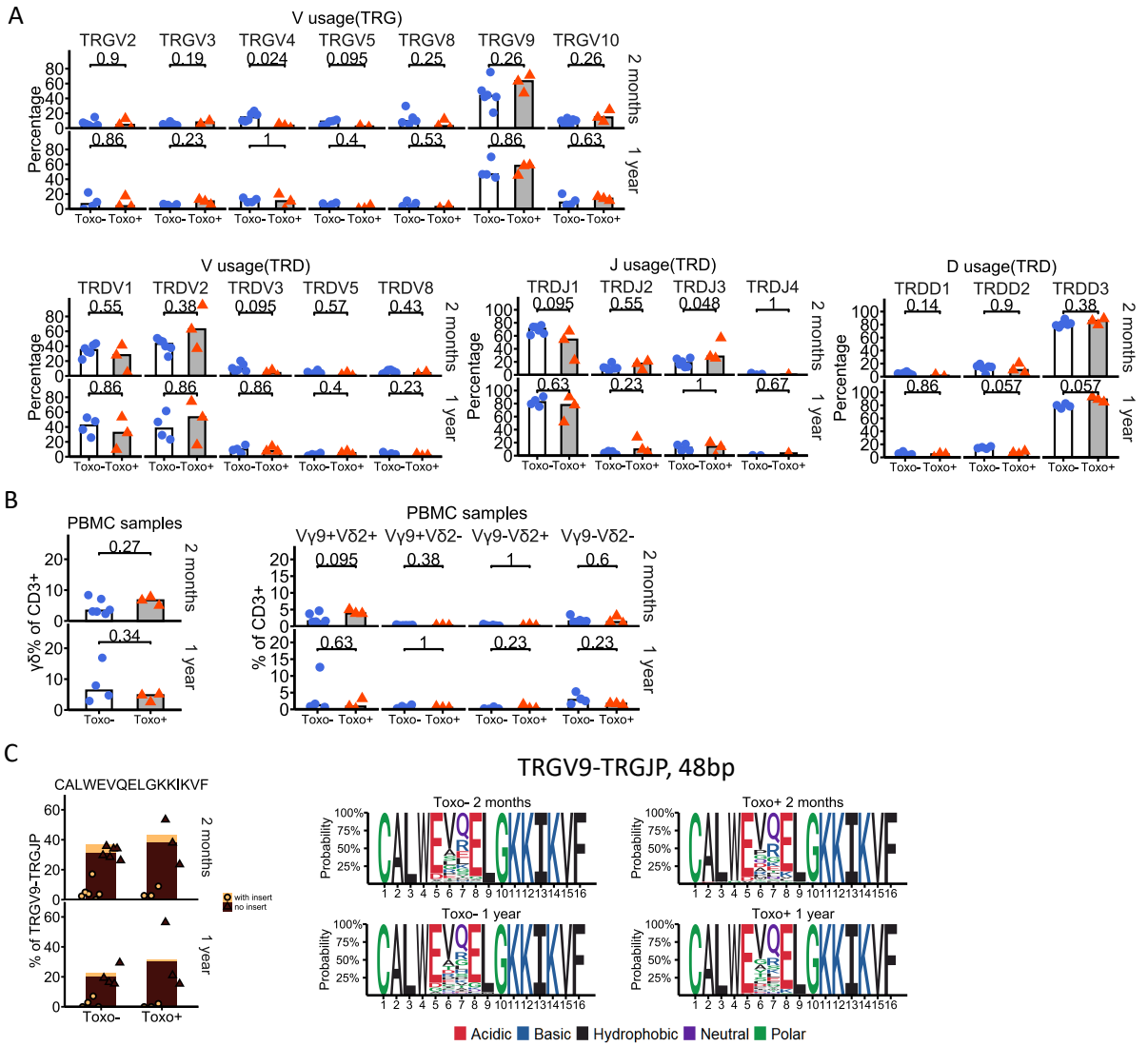


Figure 30 γδ T cell percentages of the PBMC samples used for the sorting of γδ T cells and CDR3 repertoire derived from the sorted γδ T cells.

(A) Segment usage of γ chain (top row, Variable gene) and δ chain (bottom row, Variable gene (left), Joining gene (middle), Diversity gene (right)). **(B)** Total γδ (left) and γδ subsets (right) percentage of CD3+ cells (n=16). **(C)** Left panel: prevalence of the TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N addition in dark brown bar/triangle dot (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') or encoded with N additions in light orange bar/round dot, bar shows accumulated mean. Right panel: logos of 48bp (in nucleotides) TRGV9-TRGJP sequences from each indicated group; the dominant motif is the public clonotype CALWEVQELGKKIKVF. Bar in (A)-(B) indicates median.

The random insertion of nucleotides (denoted by N) by the enzyme terminal deoxynucleotidyl transferase (TdT) into the junctions of the joining V(D)J gene segments can increase significantly the junctional diversity of the CDR3 region[356]. The number of these N additions was significantly lower in TRGV9-TRGJP- and, especially, in TRDV2-containing CDR3 sequences of Toxo+ compared to Toxo- infants at 2 months after birth (Figure 31A, upper panels). These differences waned at 1 year of age where both Toxo+ and Toxo- infants contained CDR3 sequences with a higher number of N additions compared to 2 month infants (Figure 31A, lower panels). Despite the clear lower number of N additions in Toxo+ 2 month infants, this did not result in significantly lower mean CDR3 lengths (Figure 31B), which can be (at least partially) explained by longer TRGJP and TRDJ1 segment lengths (caused by less trimming during V(D)J recombination) used for the formation of the CDR3 sequences (Figure 31C). Analysis of the CDR3δ length distribution, however, revealed a clear peak (at 42bp nucleotide length) in Toxo+ infants (Figure 31D-E, right panels), while no obvious differences could be observed for CDR3γ (Figure 31D, left panel). Note that, as described previously[23], the CDR3γ length distribution was clearly more restricted compared to the CDR3δ distribution (Figure 31D). The prominent peak at 48bp of the CDR3γ corresponded to the fetal public clonotype CALWEVQELGKKIKVF[3,23], which was present at similar frequencies in Toxo+ and Toxo- infants (Figure 31 E, left panel; Figure 30C). While the D50 value (percentage of unique clonotypes required to account for 50% of the total repertoire) did not change upon congenital *T. gondii* infection for the CDR3γ repertoire, the CDR3δ D50 value clearly decreased at 2 months (Figure 31F-G), indicating a repertoire focusing and thus specific expansion, which could be due to the increased presence of CDR3δ lengths at 42bp (Figure 31D-E, right panels). The degree of repertoire sharing between different subjects did not differ significantly between the Toxo- and Toxo+ groups, but a high level of variation was observed in the TRDV2-associated CDR3 sequences at 2 months of age (Figure 31H, right panel).

In summary, while Vγ9Vδ2 T cells from 2 months Toxo- and Toxo+ infants show a similar phenotype (as assessed by flow cytometry), their TCR repertoire show clear differences.

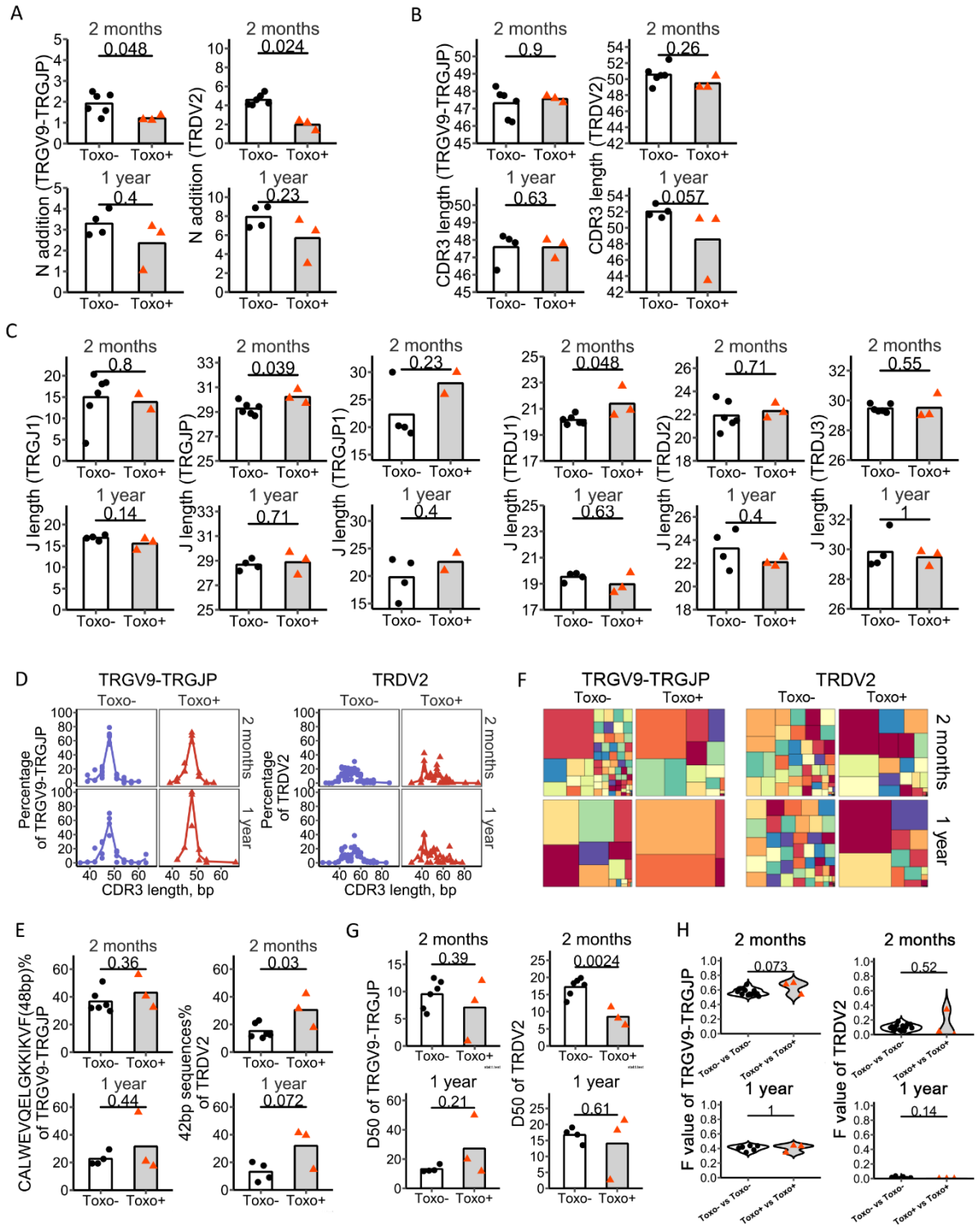


Figure 31 The Vγ9Vδ2 TCR repertoire of Toxo+ infants is different from Toxo- infants.

(A-B) CDR3 TRGV9-TRGJP (left) and TRDV2 (right) N additions (A) and CDR3 length (B); top row: 2 month-old infants (Toxo-: n=6; Toxo+: n=3), bottom row: 1 year-old infants (Toxo-: n=4, Toxo+: n=3). Bar indicates mean, dots show the weighted mean by nucleotide sequences of each sample. **(C)** Joining segment length of TRGV9-TRGJP (left 3) and TRDV2 (right 3); top row: 2 month-old infants, bottom row: 1 year-old infant samples. Bar indicates mean; weighted mean was used for each sample. **(D)** CDR3 length (in nucleotides) distribution of TRGV9-TRGJP (left) and TRDV2 (right). Lines connect median for each CDR3 length. Top row: 2 month-old infant samples; bottom row: 1 year-old infant

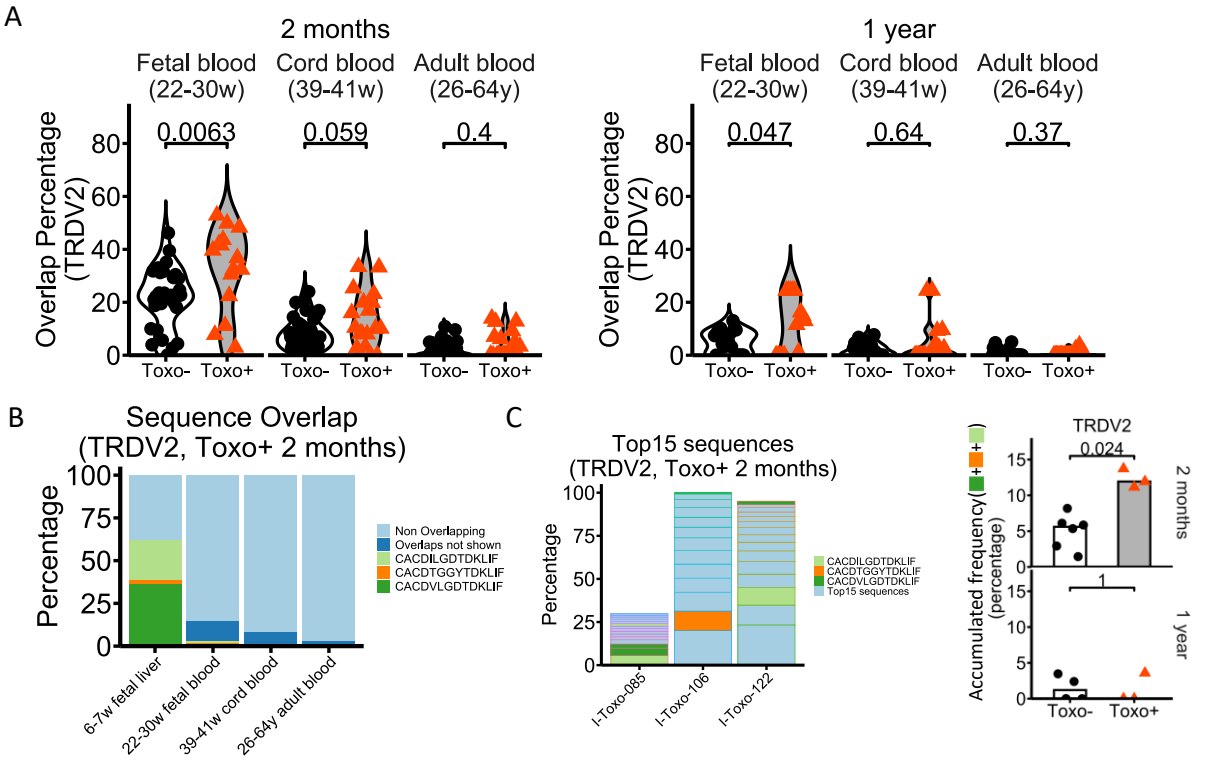
(continued from previous page) samples. **(E)** Percentage of the public CDR3γ sequence CALWEVQELGKKIKVF (48bp) among TRGV9-TRGJP-containing CDR3γ sequences (left) and percentage of CDR3δ sequences with a length of 42bp among TRDV2-containing CDR3δ sequences (right). Bar indicates median. **(F)** Clonotypes of the TRGV9-TRGJP and TRDV2 repertoire from representative Toxo- and Toxo+ samples are illustrated by tree maps. Top row: 2 month-old infant samples; bottom row: 1 year-old infant samples. Each square represents one clonotype and the square size represents the frequency of each clonotype (rectangle colors are chosen randomly and do not match between plots) **(G)** D50 index (percentage of unique clonotypes required to account for the top 50% of the total repertoire) of TRGV9-TRGJP (left) and TRDV2 (right) from 2 month-old (top row) and 1 year-old (bottom row) infant samples. Bar indicates median. **(H)** Geometric mean of relative overlap frequencies (F metrics by VDJtools) within pairs of Toxo- and Toxo+ samples from 2 month-old (upper row) and 1 year-old (lower row) infants for TRGV9-TRGJP (left) and TRDV2 (right) repertoire. Each dot represent the F value for each comparison between 2 samples, violin plots show the distribution by density.

The Vγ9Vδ2 TCR repertoire of Toxo+ infants contains a fetal footprint

The lower number of N additions and enrichment of CDR3δ at 42bp of 2 month-old Toxo+ infants indicated a fetal origin of their Vγ9Vδ2 TCR repertoire since these features are known to be enriched in fetal blood Vγ9Vδ2 T cells[3,23]. In order to address this more directly, we investigated the overlap of the TRGV9-TRGJP and TRDV2 CDR3 infant repertoires with the repertoires derived from fetal blood (22w-30w gestation), cord blood (39w-41w gestation), and adult blood (26-64y)[23]. Compared to Toxo- infants, the TRDV2 CDR3 repertoire of Toxo+ infants at 2 months was much more shared with the fetal blood repertoire (Figure 32A, left panel). A tendency for such increased sharing was also observed when compared with term delivery cord blood, but was completely absent when compared to adult blood (Figure 32A, left panel). These Toxo+ vs Toxo- differences waned at 1 year of age (Figure 32A, right panel). The TRGV9-TRGJP repertoire did not show differences in sharing between Toxo+ and Toxo- infants, both at 2 months and 1 year (Figure 33). These data indicated that the Toxo+ TRDV2 repertoire at 2 months had an origin early in fetal life. Therefore, we verified in the earliest TRDV2 CDR3 sequences generated in human, namely from 6-7 weeks gestation fetal livers[12], the presence of the 2 month Toxo+ TRDV2 sequences. Strikingly, the majority of the 6-7w fetal liver TRDV2 repertoire contained 2 month Toxo+ sequences (Figure 32B, bar at the left). A high prevalence of 2 month Toxo+ sequences were also observed in the fetal blood (22-30w gestation) repertoire, while these were virtually absent from the adult blood repertoire (Figure 32B). Importantly, the 6-7w fetal liver-associated sequences were more prevalent in 2 month Toxo+ compared to 2 month Toxo- infants; this difference waned at 1 year of age (Figure 32C). A possible explanation for the high prevalence of the CACDVLGDTDKLIF and CACDILGDTDKLIF TRDV2 CDR3 sequences in early fetal life is that the P nucleotide(s) needed to form these sequences can be derived from both the TRDV2 and the TRDD3 gene segment (Figure 32D). This can occur in an efficient way in the absence of N additions because of low expression of the TdT

enzyme[357]. In addition, there is a short-homology repeat present between the TRDD3 and TRDJ1 gene segments (Figure 32D). The prevalence of the third fetal liver sequence, CACDTGGYTDKLIF, can be explained by short-homology recombination (Figure 32D). Note that the short-homology repeat between TRDV2 and TRDD3 (the nucleotides ac) to form CACDTGGYTDKLIF has been described previously[357]. These three fetal liver sequences all have a CDR3 nucleotypic length of 42bp, thus contributing to the 42bp peak in the CDR3 length distribution at 2 months (Figure 31D, right panel). This more detailed analysis at the CDR3 sequence level provided also an explanation for the high variation observed in the sharing of TRDV2 CDR3 sequences among 2 month Toxo+ infants (Figure 31H): one of the Toxo+ infants (I-Toxo-106) showed a high enrichment of one of the 6-7w fetal liver sequences (CACDTGGYTDKLIF), that was not among the top 15 sequences of the other two Toxo+ infants (I-Toxo-085 and I-Toxo-122); the I-Toxo-085 and I-Toxo-122 infants were rather enriched for CACDVLGDTDKLIF and CACDILGDTDKLIF (Figure 32C, left panel).

In summary, it appears that Vγ9Vδ2 clonotypes with an early fetal origin highly expand *in utero* upon congenital *T. gondii* infection, resulting in a TCR repertoire footprint that is still present at 2 months after birth.



D

Germline sequence:

TRDV2	TRDD3	TRDJ1
TGT GCC TGT GAC ACC	ACT GGG GGA TAC G	AC ACC GAT AAA CTC ATC TTT GGA AAA GGA ACC CGT GTG ACT GTG GAA CCA A
C A C D T	T G G Y	T D K L I F G K G T R V T V E P

CACDVLGDTDKLIF

C A C D V L G D T D K L I F
TGT GCC TGT GAC GTA CTG GGG GAT ACC GAT AAA CTC ATC TTT
TGT GCC TGT GAC GTA CTG GGG GAC ACC GAT AAA CTC ATC TTT

TGT GCC TGT GAC ACT GGG GGA TAC G
TG TG
TGT GCC TGT GAC GT
GT ACT GGG GGA TAC G
AC ACC GAT AAA CTC ATC TTT
GT ACT GGG GGA TAC G
AC ACC GAT AAA CTC ATC TTT

CACDILGDTDKLIF

C A C D I L G D T D K L I F
TGT GCC TGT GAC ATA CTG GGG GAT ACC GAT AAA CTC ATC TTT
TGT GCC TGT GAC ATA CTG GGG GAC ACC GAT AAA CTC ATC TTT

TGT GCC TGT GAC A ACT GGG GGA TAC G
T T
TGT GCC TGT GAC A T
T ACT GGG GGA TAC G
AC ACC GAT AAA CTC ATC TTT
T ACT GGG GGA TAC G
AC ACC GAT AAA CTC ATC TTT

CACDTGGYTDKLIF

C A C D T G G Y T D K L I F
TGT GCC TGT GAC ACT GGG GGA TAC ACC GAT AAA CTC ATC TTT

TGT GCC TGT GAC ACC
ACT GGG GGA TAC G
AC ACC GAT AAA CTC ATC TTT

(legend on the next page)

Figure 32 The Vγ9Vδ2 TCR repertoire of Toxo+ newborns contains a fetal footprint.

(A) Overlapping comparisons of the TRDV2 repertoire between fetal/cord/adult blood samples and Toxo samples (Toxo+ and Toxo-). Each symbol represent a comparison showing the percentage in Toxo- and Toxo+ infant samples of overlapping clonotypes from the indicated age source (fetal, cord or adult). Left panel: comparison with 2 month-old infant samples; right panel: comparison with 1 year-old infant samples. Violin plots show the density distribution. Data are obtained with the CalcPairwiseDistances routine of VDJtools. **(B)** Accumulated mean percentage of 2 month Toxo+ TRDV2 CDR3 sequences present in 6-7 weeks gestation fetal livers, 22-30 weeks gestation fetal blood, 39-41 weeks gestation cord blood and 26-64 year adult blood. Data are obtained with the CalcPairwiseDistances routine of VDJtools. Three abundant sequences present in 6-7 week gestation fetal liver are highlighted (CACDVLGDTDKLIF in dark green, CACDILGDTDKLIF in light green, CACDTGGYTDKLIF in orange). Note that in 6-7 fetal liver sequences, only CDR3 sequences were analyzed that combined TRDV2 with TRDJ1[12]. **(C)** Left panel: accumulated percentage of top 15 TRDV2 CDR3 sequences from three Toxo+ 2 month-old infant samples. The same three sequences are highlighted as in (B). Right panel: accumulated percentage of the three fetal liver sequences (CACDVLGDTDKLIF + CACDILGDTDKLIF + CACDTGGYTDKLIF) in Toxo+ and Toxo- 2 month (top panel) and 1 year (bottom panel) infants. Bar indicates median. **(D)** Formation of the CACDVLGDTDKLIF, CACDILGDTDKLIF and CACDTGGYTDKLIF CDR3 clonotypes via usage of P nucleotides and/or short-homology repeats. All nucleotypes found in 2 month Toxo+ samples are listed for each clonotype. P-nucleotides are in green letters, short-homology repeats are indicated with a green square and the repeated nucleotides in the germline region in orange letters; nucleotype sequences are underlined.

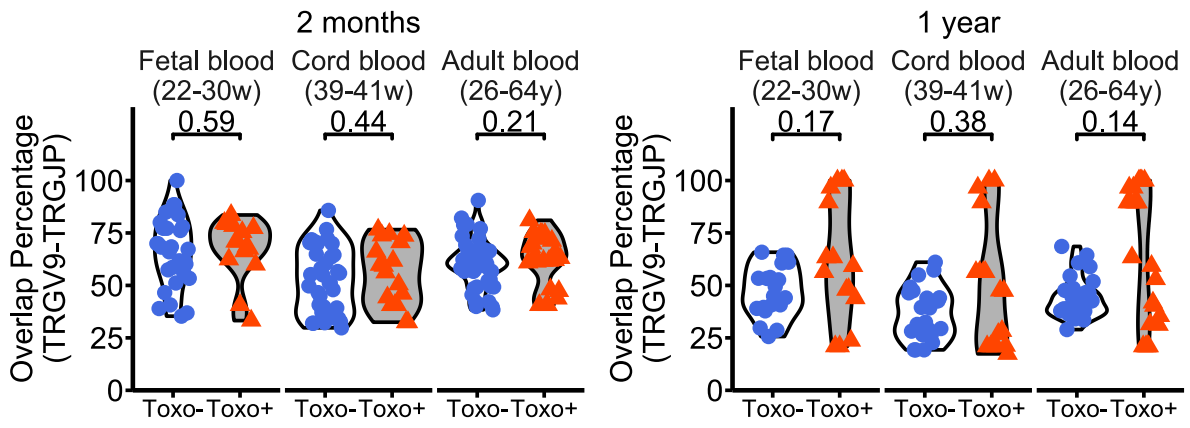


Figure 33 Overlapping analysis of the TRGV9-TRGJP repertoire.

Overlapping comparisons of the TRGV9-TRGJP repertoire between fetal/cord/adult blood samples and Toxo samples (Toxo+ and Toxo-). Each symbol represent a comparison showing the percentage in Toxo- and Toxo+ infant samples of overlapping clonotypes from the indicated age source (fetal, cord or adult). Left panel: comparison with 2 month-old infant samples; right panel: comparison with 1 year-old infant samples. Violin plots show the density distribution. Data are obtained with the CalcPairwiseDistances routine of VDJtools.

Discussion

Despite their high activation threshold *in vitro*, we show here that fetal Vγ9Vδ2 T cells can provide immunity to a parasite infection *in utero*. A main finding of our study was the enrichment among congenital Toxo+ infants of particular germline-encoded (no N nucleotide additions) and public (shared between individuals) CDR3δ sequences (CACDVLGDTDKLIF, CACDILGDTDKLIF, CACDTGGYTDKLIF), which have been described to be highly prevalent in 6-7 week gestation human fetal livers[12]. In parallel, the CDR3γ repertoire was highly enriched for the phosphoantigen-reactive germline-encoded clonotype CALWEVQELGKKIKVF (TRGV9-TRGJP), described previously to be highly prevalent in fetal liver, thymus, and blood[3,23,12]. In line with our *in vivo* observations in Toxo+ infants, Vγ9Vδ2 T cell clones expressing these germline-encoded public TCR sequences are responsive *in vitro* towards phosphoantigen-containing mycobacterial extracts[12]. We propose that protection against congenital *T. gondii* infection may have provided a selective pressure during evolution for the maintenance of the germline-encoded genetic elements needed for the generation of phosphoantigen-reactive TCRs early during fetal development[23,78,77], in line with the high level of heritability of Vγ9Vδ2 T cells compared to other innate-like T cells[358]. In contrast to newborns (<1 month), older congenitally Toxo+ infants showed similar Vγ9Vδ2 T cell percentages as their age-matched controls. This probably relates to the more general phosphoantigen exposure (e.g. microbiome) at birth and/or the treatment that the Toxo+ infants received. However, there was still a fetal footprint in the Toxo+ infants Vγ9Vδ2 TCR repertoire due to the expansion of Vγ9Vδ2 T cells *in utero*.

In contrast to our data, a previous study, mainly based on *in vitro* re-stimulation data, indicated that congenital *T. gondii* infection induces an anergic state in infant Vγ9Vδ2 T cells[287]. However, other Vγ9Vδ2 T cell functions were not assessed and age-matched controls were lacking[3,287,348,359]. Our data indicate that a major wave of proliferation of fetal public Vγ9Vδ2 T cells has occurred *in utero* upon *T. gondii*

encounter, accompanied by the acquirement of potent cytotoxic effector functions (GzmB+perforin+). These effector functions can be used to kill *T. gondii*-infected cells, as illustrated by the *in vitro* study of Subauste et al with Vγ9Vδ2 T cell lines and clones[286]. Such a response that combines innate (germline-encoded TCR acting as a pathogen recognition receptor) and adaptive (high proliferation upon pathogen encounter) features has been referred to as 'adaptate' biology[360]. After birth, the ex-vivo proliferation rate of Vγ9Vδ2 T cells (as assessed by Ki-67 staining) was not different between Toxo+ and Toxo- newborns (<1 month) and even lower in Toxo+ compared to Toxo- in >1 month infants, in line with a lower proliferation rate upon *in vitro* re-stimulation[287]. Hara et al suggested that Vγ9Vδ2 T cells are susceptible towards anergy induction because of their extra-thymic development[287]. However, we have shown recently that the human thymus clearly contains Vγ9+Vδ2+ T cells[23]. Based on our data in this study we conclude that congenital *T. gondii* infection does not induce an anergic state of fetal Vγ9Vδ2 T cells but rather transforms them into lymphocytes with a potent CTL (cytotoxic lymphocytes) phenotype that contributes to protection against infection by killing *T. gondii*-infected cells.

We have previously shown that fetal nonVγ9Vδ2 γδ T cells, such as the public Vγ8Vδ1 T cells, play a major role in the response towards congenital human cytomegalovirus (HCMV) infection[350]. Together with the data of our current study, it appears that the human fetus is equipped with γδ T cell subsets that show a division of labor in their response to congenital infections: (i) the Vγ9Vδ2 T cells respond to *T. gondii* and possibly other phosphoantigen-generating pathogens and (ii) the nonVγ9Vδ2 T cells that target HCMV-infected cells. Data from human *in vitro* studies[286,350] and *in vivo* studies in the mouse[276,361,362] indicate that γδ T cells play a protective role against infections with *T. gondii* and HCMV, but it cannot be excluded that the potent effector γδ T cells contribute to the development of pathologies observed upon congenital infections[363,364].

A main correlate of protection of the malaria vaccine PfSPZ (attenuated *Plasmodium*

falciparum sporozoite) is Vγ9Vδ2 T cells[365,366]. Our data, showing the importance of Vγ9Vδ2 T cells in the response towards congenital *T. gondii* infection, indicate that vaccine or other strategies could be developed targeting these cells to protect infants against (congenital *T. gondii*) infections. Tools to manipulate Vγ9Vδ2 T cells *in vivo* are becoming increasingly available and include modified phosphoantigens with improved pharmacological characteristics and monoclonal antibodies targeting BTN3A1[367,72]. Both *Plasmodium falciparum* and *T. gondii* contain an organelle, the apicoplast, which has specific metabolic functions including the MEP pathway of isoprenoid synthesis. In this pathway, the metabolite HMBPP is generated, the most potent natural phosphoantigen[368,369]. This indicates that *T. gondii*-derived HMBPP is a major driving force for the expansion of fetal Vγ9Vδ2 T cells *in utero*. However, in contrast to our observations in congenital *T. gondii* infection, Cairo et al observed rather a depletion of phosphoantigen-reactive Vγ9Vδ2 T cells in placental malaria[370]. A main difference between congenital *T. gondii* infection and placental malaria is that the malaria parasite very rarely crosses the placenta into the fetal circulation to establish an infection[371]. Furthermore, the type of placental malaria infection can have opposing effects on the immune system in early life, thus possibly contributing to the differential effect on the fetal Vγ9Vδ2 T cells[370,371].

In immunocompromised (adult) patients (AIDS and transplant patients), *T. gondii* infection is a major cause of morbidity and mortality[191]. HIV specifically targets Vγ9Vδ2 T cells[116], but it is not clear to which extent the depletion of these potential *T. gondii*-responsive cells contributes to *T. gondii* induced morbidities. HCMV infection is a major driving force of nonVγ9Vδ2 γδ T cell expansion in organ transplant and hematopoietic stem cell transplant patients[372-374]. These expansions have been associated with reduced cancer development[375,376]. In contrast, the role of *T. gondii* infection in driving Vγ9Vδ2 T cell expansion/differentiation in these transplant settings and their potential anticancer role is not known. Thus the role of Vγ9Vδ2 T cells in *T. gondii* infection in transplant and AIDS patients deserves further investigation.

4.2 Mistletoe-extract drugs stimulate anti-cancer Vγ9Vδ2 T cells

Introduction

Recently, T cell-based cancer immunotherapy has become a main therapy arm in the clinic besides surgery, radio- and chemotherapy. While mostly conventional αβ T cells are considered, it has become increasingly clear that γδ T cells have a large potential, which is illustrated by the interest of commercial partners[377]. The antitumor function of γδ T cells is generally associated with their cytotoxic potential and their production of both interferon γ (IFNγ) and tumor necrosis factor α (TNFα)[377,378]. Vγ9Vδ2 T cells respond (expansion, release of cytotoxic granules, cytokine production) in a TCR-dependent manner towards PAg that critically depends on the transmembrane protein butyrophilin3A1 (BTN3A1)[368,379]. The Vγ9Vδ2 T cell subset has been a target for cancer clinical trials, mainly through their *in vivo* activation via the administration of aminobisphosphonates (N-BPs) such as zoledronate. N-BPs inhibit the farnesyl pyrophosphate synthase enzyme (FPPS/FPS) leading to the intracellular accumulation of endogenous IPP[377,380]. Interestingly, Vγ9Vδ2 T cells also respond to alkylamines such as sec-butylamine (SBA) that are found in edible plants and tea[56,381]. These alkylamines, like N-BPs, activate Vγ9Vδ2 T cells indirectly through inhibition of the FPPS enzyme[59]. The stimulation of the antitumor function of γδ T cells by such plant-derived compounds are thought to play an important role in the prevention of cancer development[382-384].

Mistletoe treatment has been suggested to increase the survival of cancer patients, but this is controversial and thus an increased understanding of its mechanism of action is needed to guide further *in vivo* studies and clinical trials[303,385,386]. Here we show that non-fermented mistletoe extract drugs (AbnobaViscum) stimulate and expand specifically Vγ9Vδ2 T cells, induce the release of cytotoxic granules and promote the production of the cytokines IFNγ and TNFα. Furthermore, we show that this mistletoe-mediated activation of anti-cancer Vγ9Vδ2 T cells is rapid and direct

(i.e., not dependent on the accumulation of endogenous phosphoantigens) and is completely BTN3A-dependent.

Results

AbnobaViscum but not Iscador mistletoe extracts induce specific expansion of Vγ9Vδ2 T cells

We obtained four commercially available *Viscum album* L. (VA) extracts from two companies that derive them from the same host trees but use different preparatory methods: non-fermented extracts are AbnobaViscum Pini (AP) and AbnobaViscum Mali (AM) and fermented products are Iscador Malus (IM) and Iscador Pinus (IP). In order to perform short-term (4 hours, 1 day) assays to assess T cell activation (CD69) and function (cytokine production and release of cytotoxic granules) and long-term (7 days) expansion cultures, we first titrated each extract to assess the potential cytotoxic effect and dose-response in PBMC cultures. After 1 day of culture, none of the extracts showed a cytotoxic effect (Figure 34A, left panels). At 7 days, however, all the VA extracts except IP showed a clear dose-dependent cytotoxicity (Figure 34A, right panels). Heat treatment of mistletoe extracts[337] prevented the cell death induction in the long-term PBMC cultures (Figure 34B). As heat-treatment is not performed on the mistletoe-extracts that are injected in cancer patients, we preferred to test an alternative method to prevent the cytotoxic effects in order to verify whether the heat-treatment was essential for possible effects on Vγ9Vδ2 T cells[337]. Exposure of the cells for one day to the VA extracts (at the highest concentration, 1000 µg/mL) followed by washing (‘pulse’), instead of continued exposure, prevented or reduced significantly the cytotoxic effects in longer term cultures (Figure 34B). Thus, we included results from both heat treatment and pulse stimulation in the 7 days expansion cultures since both methods resulted in similar expansion levels (%Vγ9+ of CD3+ T cells, data not shown). Based on short-term activation experiments with the lymphocyte activation marker CD69, we selected 1000 µg/mL concentration of mistletoe extracts for further experiments (Figure 35). Both AbnobaViscum (AP and AM) and Iscador (IA and IM) VA extracts activated Vγ9+ γδ T cells, with no or minimal effects on Vγ9− γδ T cells and αβ T cells (Figure 36A). A different trend was observed

for NK cells: here the activation was more pronounced with Iscador compared to AbnobaViscum extracts (Figure 36A). Surprisingly, while both AbnobaViscum and Iscador extracts activated Vγ9Vδ2 T cells (Figure 36A), and despite being derived from the same host trees, only AbnobaViscum extracts induced proliferation of Vγ9Vδ2 T cells (Figure 36B-C). AP was the strongest stimulant (Figure 36C; AP vs AM: $p = 0.0078$, Wilcoxon signed-rank test) and the expansion was highly restricted to Vγ9Vδ2 T cells (Figure 36 B for CD3+ T cells, data not shown for CD3− NK cells). We therefore focused further on the stimulation of Vγ9Vδ2 T cells upon treatment with AP. Of note, AP-induced expansion levels were similar to expansion levels observed with known Vγ9Vδ2 T cell stimulants (HMBPP, zoledronate) and individual AP-, HMBPP-, and zoledronate-induced expansions showed a strong correlation (Figure 36D).

In sum, although both AbnobaViscum (non-fermented) and Iscador (fermented) VA extracts can activate Vγ9Vδ2 T cells, only exposure to AbnobaViscum VA extracts results in their proliferation. This strong expansion was highly specific for Vγ9Vδ2 T cells.

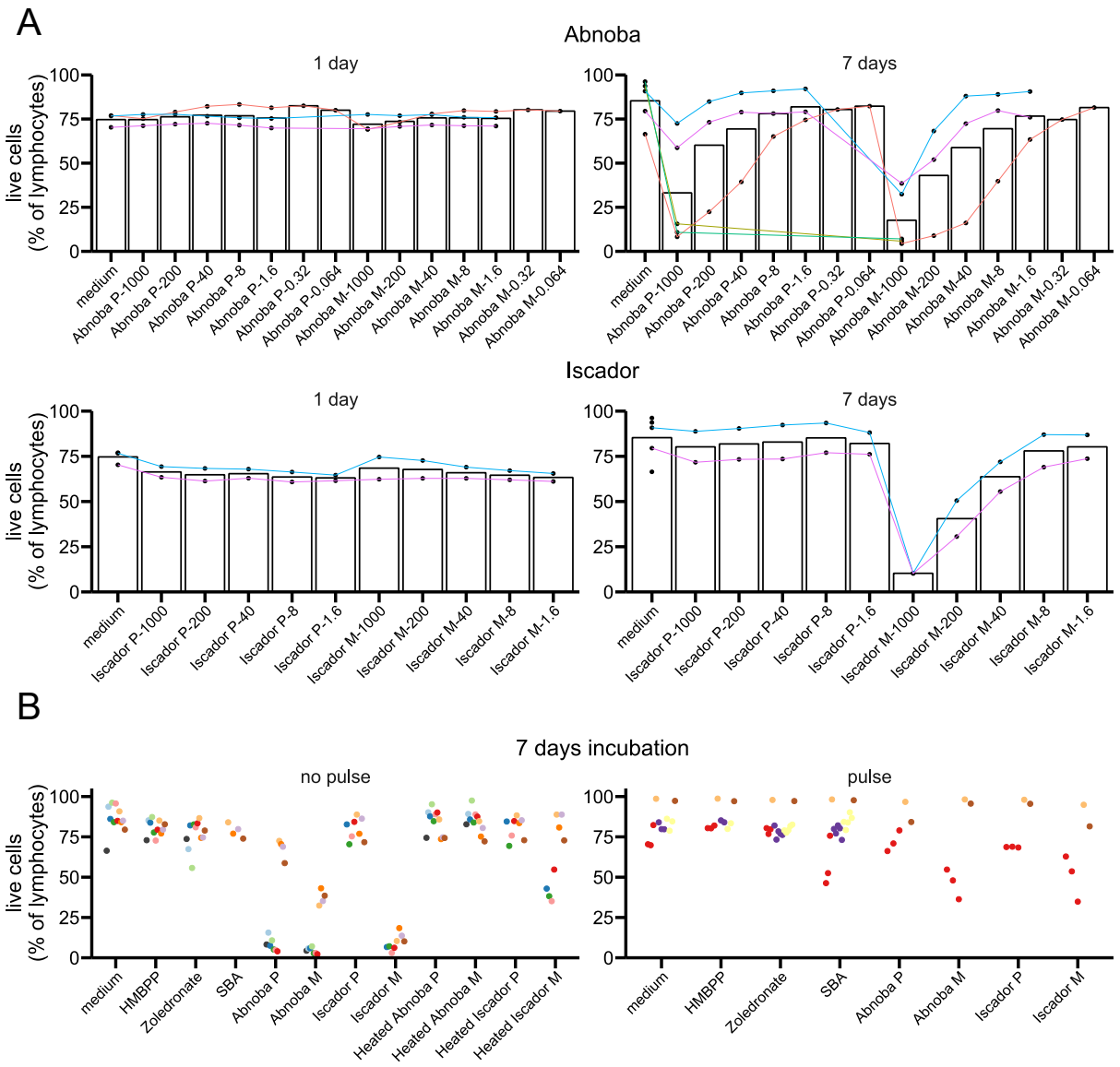


Figure 34 *In vitro* cytotoxicity on PBMCs of each mistletoe-extract drug.

(A) Percentage of lymphocytes that are negative for staining with the Zombie-NIR viability dye. Lines connect the same subjects (n = 2-5), bars indicate mean values. Concentrations (in µg/ml) are indicated after the extract names on the X-axis. Incubation times (1 day or 7 days) are indicated. **(B)** Zombie-NIR negative percentage of lymphocytes within each stimulation after 7 days incubation. Data are from all the 5 or 7 days tests used in the results. Each color represent one subject (n = 13); each dot represents an independent experiment.

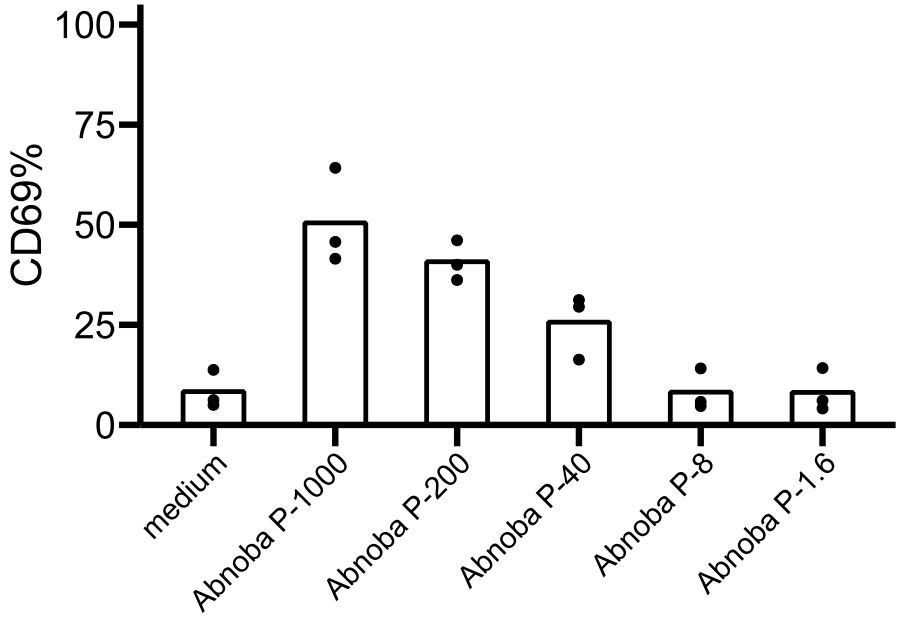


Figure 35 CD69 expression on Vγ9+ T cells after 1-day stimulation with different concentrations of AbnobaViscum Pini (Abnoba P).

Bars indicate mean value; concentrations are indicated after the stimulant name on the X-axis. Each dot represent the data of one subject.

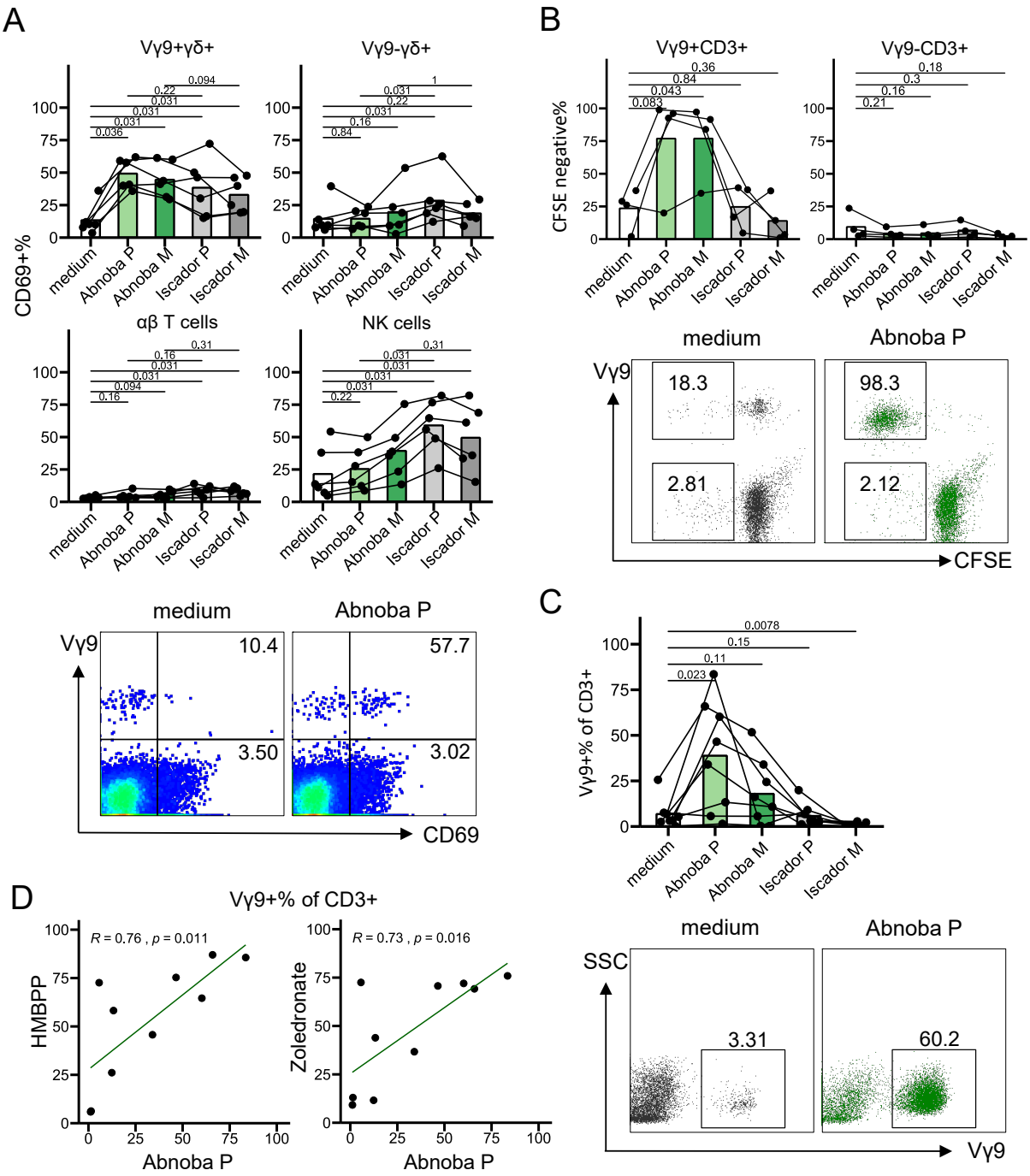


Figure 36 AbnobaViscum but not Iscador mistletoe extracts induce specific expansion of Vγ9Vδ2 T cells.

(A) Percentage of CD69 expression on different cell types after stimulation with different mistletoe extracts for 1 day. Upper left: Vγ9+ γδ T cells (CD3+γδ+Vγ9+); upper right: Vγ9− γδ T cells (CD3+γδ+Vγ9−); lower left: αβ T cells (CD3+γδ−); lower right: natural killer (NK) cells (CD3−CD56+). Lines connect the same subjects (n = 6), bars indicate mean values. Values on the graphs indicate p values (obtained with the Wilcoxon signed-rank test). Bottom panels show representative flow cytometry plots (gated on CD3+ T cells), numbers indicate percentages of CD69+ cells, expressed as a percentage of Vγ9+CD3+ cells (top) and as a percentage of Vγ9−CD3+ cells (bottom). **(B)** Percentage of CFSE-negative Vγ9+ T cells (CD3+Vγ9+, upper left) and Vγ9− T cells (CD3+Vγ9−, upper right) after stimulation for 5 days with different mistletoe extracts. Lines connect the same subjects (n = 4), bars

(continued from previous page) indicate mean values. Values on the graphs indicate p values (obtained with the paired T-test). Bottom panels show representative flow cytometry plots (gated on CD3+ T cells), numbers indicate percentages of CFSE negative cells, expressed as a percentage of Vγ9+CD3+ (top) and as a percentage of Vγ9−CD3+ (bottom). **(C)** Percentage of Vγ9+ cells (of total CD3+ T cells) after stimulated with different mistletoe extracts for 7 days. Lines connect the same subjects (n = 8), bars indicate mean values. Values on the graphs indicate p values (obtained with the Wilcoxon signed-rank test). Bottom panels show representative flow cytometry plots (gated on CD3+ T cells), numbers indicate percentages of positive cells in the indicated gates. **(D)** Correlation between AbnobaViscum P- and HMBPP-, AbnobaViscum P and zoledronate-induced expansion (7 days). Each dot represents one subject (n = 10).

AbnobaViscum rapidly stimulates the release of cytotoxic granules and the production of IFNγ and TNFα in Vγ9Vδ2 T cells

As VA-extract drugs are used as complementary cancer therapy, we assessed the induction of the two main anti-cancer functions of Vγ9Vδ2 T cells: degranulation of their cytotoxic granules (by analyzing the surface expression of the granule-associated CD107a) and the induction of the cytokines IFNγ and TNFα. AP induced a rapid (4 hours) and striking upregulation of CD107a, IFNγ, and TNFα in Vγ9+ T cells, but not on Vγ9− T cells (Figure 37A–D). The release of cytotoxic granules and production of cytokines were largely co-expressed (Figure 37D). While some studies have ascribed an anti-tumor role for IL-17-producing γδ T cells like when they act in concert with immunogenic cell death-inducing chemotherapeutic drugs[387], in a range of other settings a pro-tumor role has been proposed[377,378,388]. Here, we could not find significant upregulation of this cytokine in Vγ9Vδ2 T cells upon exposure to AP (Figure 38). Of note, the stimulation kinetics of AP (4 hours rather than 1 day) was similar to the stimulation kinetics of HMBPP (direct activation) but not with the kinetics of the indirect stimulatory compounds zoledronate and SBA (Figure 37E). In sum, AP rapidly stimulates the degranulation of cytotoxic granules and the production of the anti-cancer cytokines IFNγ and TNFα in Vγ9Vδ2 T cells but not within other T cells.

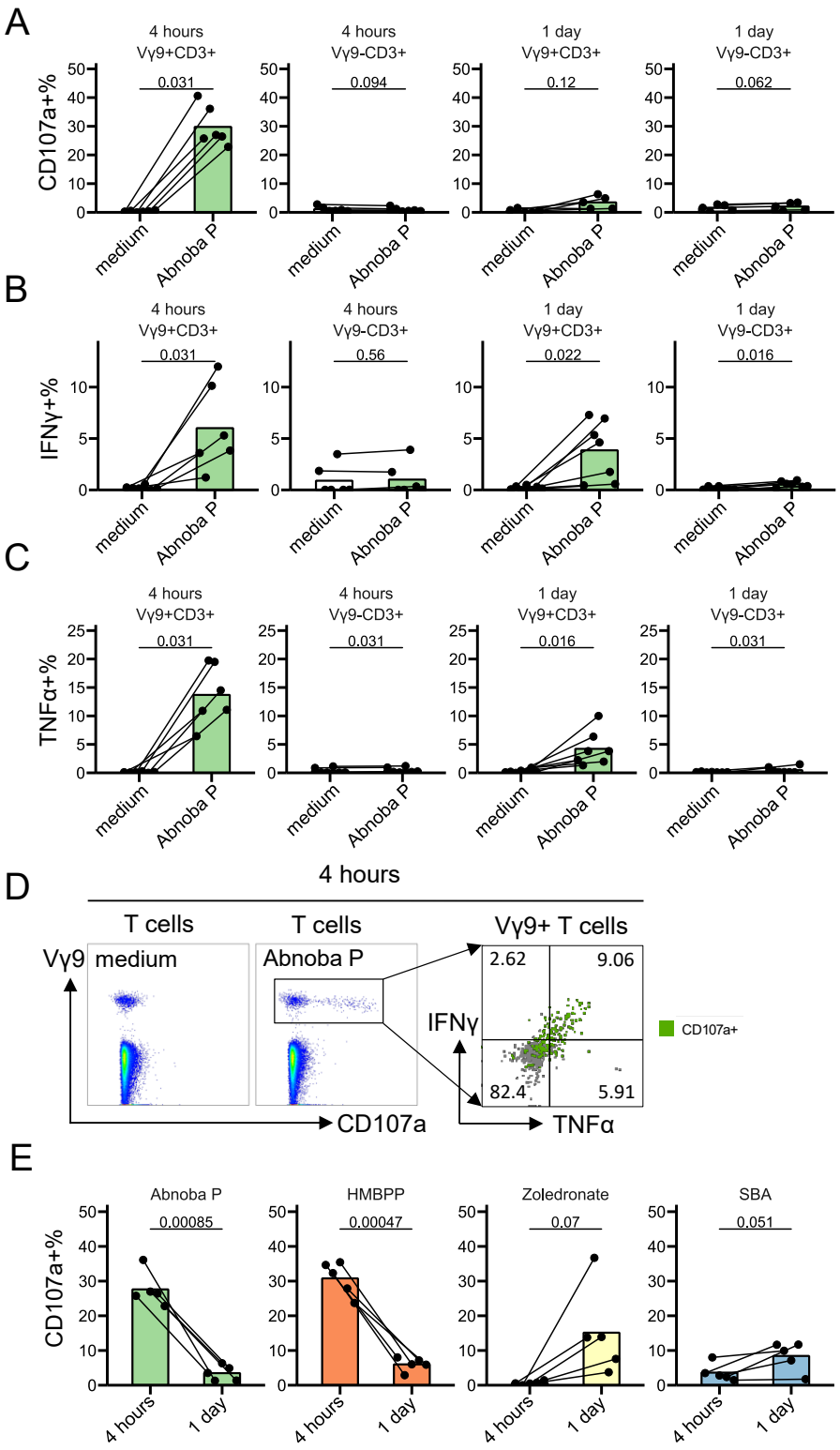


Figure 37 AbnobaViscum rapidly stimulate the release of cytotoxic granules and the production of IFNγ and TNFα in Vγ9Vδ2 T cells.

(A–C) CD107a **(A)**, IFN-γ **(B)** and TNF-α **(C)** expression on Vγ9+ T cells and Vγ9− T cells after AbnobaViscum Pini (Abnoba P) stimulation. Lines connect the same subjects (n = 6), bars indicate mean value. Values on the graphs indicate p values (obtained with the Wilcoxon signed-rank test). **(D)** Representative flow cytometry plots (4 hours stimulation): the first two plots are gated on T cells (medium control on the left, Abnoba P on the right), the third plot is gated on Vγ9+ T cells (Abnoba P),

(continued from previous page) illustrating CD107a, IFNγ and TNFα co-expression **(E)** Kinetics of CD107a expression on Vγ9+ T cells by Abnoba P, HMBPP, zoledronate and sec-butylamine (SBA). Lines connect the same subjects (n = 5), bars indicate mean values. Values on the graphs indicate p values (obtained with the paired T-test).

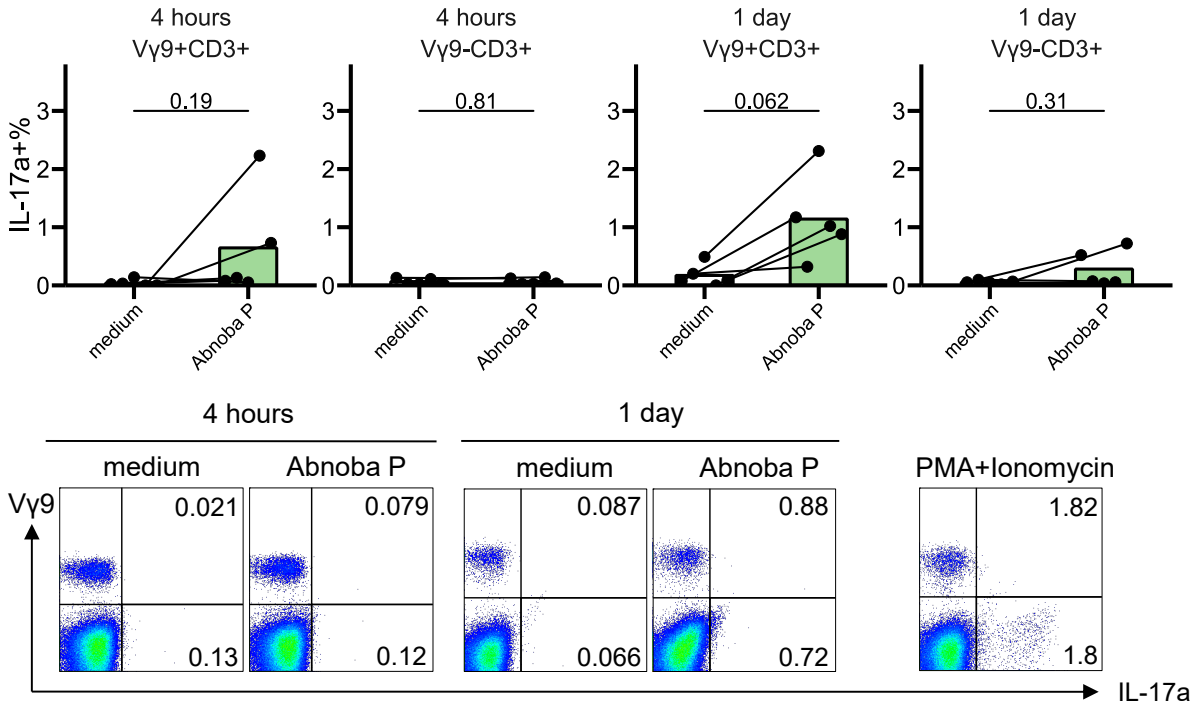


Figure 38 Absence of IL-17a induction by AbnobaViscum Pini (Abnoba P) stimulation in Vγ9+ T cells and Vγ9- T cells.

Lines connect the same subject (n = 5), bars indicate mean value. Values on the graphs indicate p values (obtained with the Wilcoxon signed-rank test). Bottom panels show representative flow cytometry plots (gate on CD3+ T cells). Upper and lower numbers indicate percentages of IL-17-positive cells in Vγ9+ T cells and Vγ9- T cells respectively. Incubation time (4 hours or 1 day) is indicated.

AbnobaViscum stimulation of Vγ9Vδ2 T cells is direct and BTN3A-dependent

Alkylamines such as SBA of edible plants and tea have been described as main Vγ9Vδ2 T cell-stimulating compounds[56,381] and to act, like N-BPs, indirectly by endogenous phosphoantigen (IPP) accumulation[59]. Whether the same kind of 'indirect' Vγ9Vδ2 activating compounds or rather more direct mechanism are involved in the AP-induced activation is not clear. In order to verify the involvement of endogenous accumulation of IPP, we used mevastatin to inhibit HMG-Coenzyme A reductase activity upstream of IPP synthesis and thus to inhibit IPP production[389]. To our surprise, inhibiting IPP synthesis did not decrease the AP-induced stimulation (Figure 39A). As expected, direct HMBPP-induced activation was not influenced by mevastatin treatment as well, while SBA- and zoledronate-induced Vγ9Vδ2 T cell activation were inhibited (Figure 39A). These results rather indicate that AP contains (a) direct activating pyrophosphate compound(s). To address this further, pretreatment of AP with apyrase, that sequentially releases inorganic phosphate groups from phosphorylated molecules, completely abolished the Vγ9Vδ2 T cell response, while the same pretreatment of zoledronate and SBA did not influence their Vγ9Vδ2 T cell-activation potential (Figure 39B). To investigate further the mechanism of activation of Vγ9Vδ2 T cells by AP, we verified the involvement of BTN3A, that plays a crucial role in the phosphoantigen-mediated activation via the Vγ9Vδ2 TCR: the blocking BTN3A antibody 103.2 completely abolished AP-induced degranulation and cytokine production (Figure 39C). Thus overall, the AP-induced activation of Vγ9Vδ2 T cells does not depend on the accumulation of endogenous IPP production and is mediated via BTN3A.

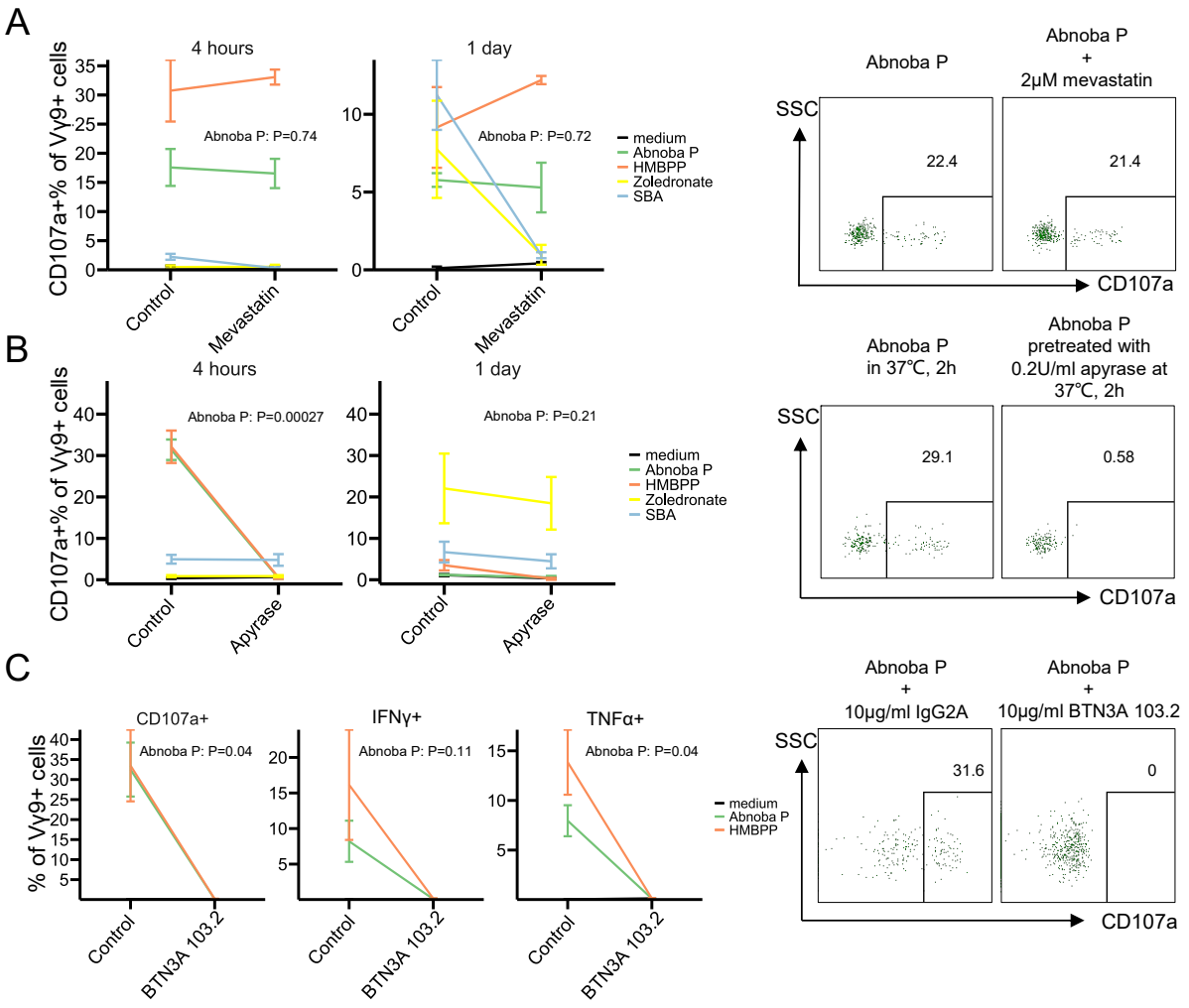


Figure 39 AbnobaViscum stimulation of Vγ9Vδ2 T cells is direct and BTN3A-dependent.

(A) CD107a expression on Vγ9+ T cells upon mevastatin treatment within each stimulation. Lines connect the mean values between control and mevastatin treatment within the same stimulation, error bars show mean±sem (n = 3). Representative flow cytometry plots after 4 hours stimulation are on the right of the graphs (gate on CD3+Vγ9+ T cells). **(B)** CD107a expression on Vγ9+ T cells upon apyrase treatment within each stimulation. Lines connect the mean values between control and apyrase treatment within the same stimulation, error bars show mean±sem (n = 5 for 4 hours, n = 3 for 1 day). Representative flow cytometry plots after 4 hours stimulation are on the right of the graphs (gate on CD3+Vγ9+ T cells). **(C)** CD107a (left), IFNγ (middle), TNFα (right) expression in Vγ9+ T cells upon blocking BTN3A within each stimulation for 4 hours. Lines connect the mean values between isotype control and BTN3A 103.2 mAb within the same stimulation, error bars show mean±sem (n = 3). Representative CD107a stainings (4 hours stimulation) after are on the right of the graphs (gate on CD3+Vγ9+ T cells). Values on the graphs indicate p values (obtained with paired T-test).

In addition, we were wondered whether the buffer from AbnobaViscum would have an influence on apyrase digestion. Thus, as supplemental information, we tried to dilute HMBPP with the mistletoe extract drug buffer, and then treated with apyrase to compare with other conditions (Figure 40). Interestingly, buffer-diluted HMBPP maintained the stimulation capacity after apyrase digestion (Figure 40).

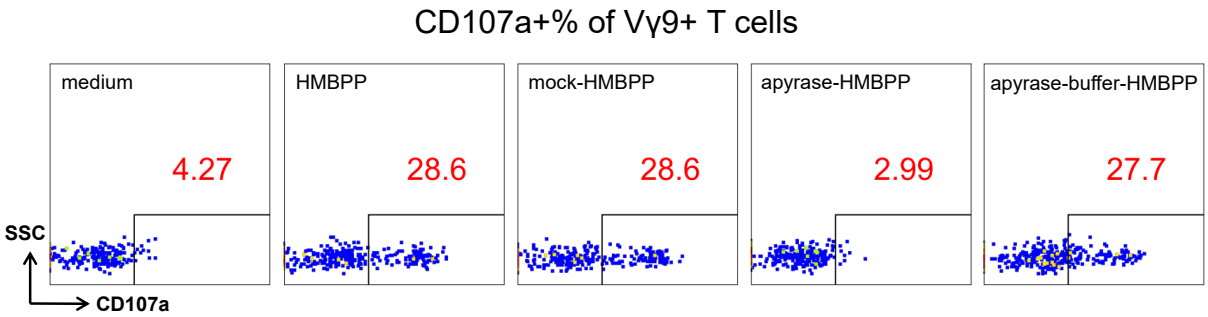


Figure 40 CD107a expression on Vγ9+ T cells after apyrase treatment of HMBPP.
HMBPP diluted in medium (apyrase-HMBPP) or the buffer from AbnobaViscum mistletoe extracts (apyrase-buffer-HMBPP) were pretreated with 0.2 U/ml apyrase at 37°C for 2 hours. Mock-HMBPP was pretreated at 37°C for 2 hours without apyrase. Percentage of CD107a expression on Vγ9+ T cells were shown in flow cytometry plot. N=1.

The AbnobaViscum-responsive Vγ9Vδ2 TCR repertoire is similar to the phosphoantigen responsive repertoire

Next, we wondered whether the BTN3A/Vγ9Vδ2 TCR-dependent AP stimulation induces a similar polyclonal Vγ9Vδ2 T cell response as HMBPP and zoledronate[24] or whether AP targets rather a subset of Vγ9Vδ2 T cells. In order to address this issue, we investigated the CDR3γ and CDR3δ repertoire by high-throughput sequencing of Vγ9Vδ2 T cells expanded with AP, and compared it to HMBPP- and zoledronate-expanded Vγ9Vδ2 T cells. The CDR3 length distributions were highly similar between AP-, HMBPP- and zoledronate-expanded Vγ9Vδ 2 T cells (Figure 41A). Compared to HMBPP and zoledronate- expanded Vγ9Vδ2 T cells, AP-expanded CDR3γ and CDR3δ sequences showed the same diversity levels (Figure 41B), the same TRGJ and TRDJ usage (Figure 41C), and were highly shared (Figure 41D). This high sharing between the different treatments was mainly due to public clonotypes in the TRGV9 repertoire, i.e., shared between the different individuals (Figure 41D, left panels), while the shared AP/HMBPP/zoledronate TRDV2 repertoire was private for each individual (Figure 41D, right panels). This was further shown by a global analysis via multidimensional scaling (MDS): the AP-, HMBPP- and zoledronate-expanded TRD repertoire were grouped in subject-based clusters (Figure 41E, right panel), highlighting again the similarities between the Vγ9Vδ2 TCR responses induced by AP, HMBPP, and zoledronate.

In sum, the AP-responsive Vγ9Vδ2 TCR repertoire is similar to the direct (HMBPP) and indirect (zoledronate-induced IPP accumulation) phosphoantigen-responsive repertoire.

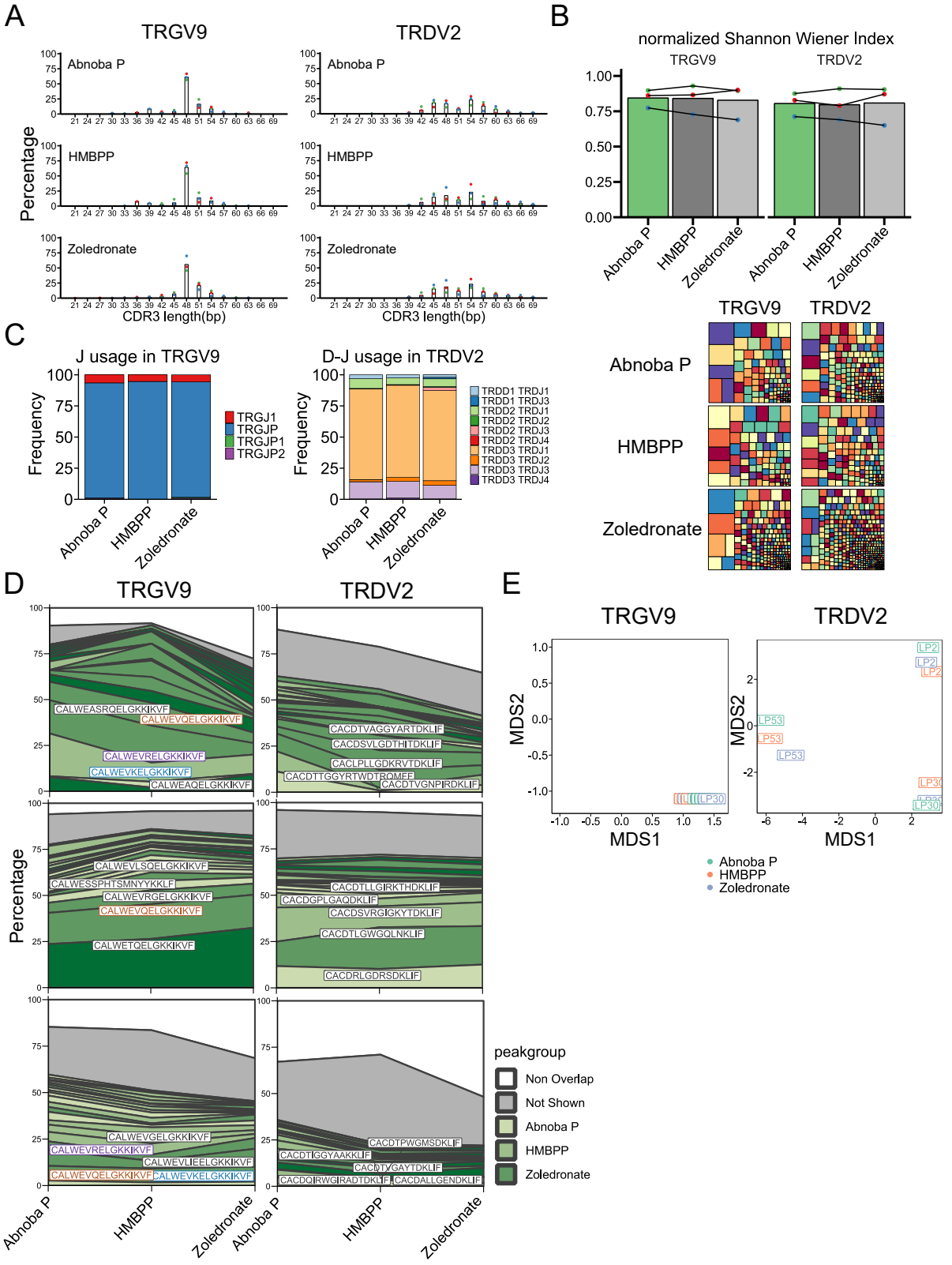


Figure 41 The AbnobaViscum-responsive Vγ9Vδ2 TCR repertoire is similar to the phosphoantigen-responsive repertoire.

(A) Distribution of CDR3 length for TRGV9- and TRDV2-containing CDR3 sequences after expansion with the indicated Vγ9Vδ2 T cell stimulators. Each color of the dots represents the same subject, bar indicates mean percentage for each CDR3 length (expressed in nucleotides). **(B)** Diversity of TRGV9- and TRDV2- containing CDR3. Normalized Shannon Wiener index: each color of the dots represents

(continued from previous page) the same subject, lines connect each subject, bars indicate mean value. Representative treemaps for the indicated stimulators are below the graphs: each small square represents a CDR3 sequence of which the size is related to the frequency of the sequence within the repertoire within each sample (rectangle colors are chosen randomly and do not match between plots). **(C)** Mean J gene segment usage in TRGV9-containing CDR3 (left) and mean D-J gene segment usage in TRDV2-containing CDR3 sequences (right) (n = 3). **(D)** Sequence overlap between AP-, HMBPP- and zoledronate-induced expansions for TRGV9-containing CDR3 (left) and TRDV2-containing CDR3 (right). The top 20 sequences are filled with different green shades, the remaining overlapping sequences are indicated in grey and the non-overlapping sequences are in white. Top 5 shared sequences are provided on the plots for each subject: colored sequences occur in more than one subject while black sequences indicate unique sequences. **(E)** Multidimensional scaling (MDS) analysis of TRGV9-containing CDR3 sequences (left) and TRDV2-containing CDR3 sequences (right). Colors indicate each expansion; the subject number is indicated within each small square.

Discussion

Non-fermented mistletoe-extract drugs (AbnobaViscum) induced the specific expansion of Vγ9Vδ2 T cells, the rapid release of their cytotoxic granules and production of IFNγ and TNFα. All these features are known to be associated with anti-cancer activity[377,378]. AbnobaViscum has been shown to upregulate the expression of maturation markers on dendritic cells (DC), but failed to increase important cytokines such as IL-12p70 needed to stimulate and differentiate αβ T cells[390]. However, the promotion of IFNγ production by Vγ9Vδ2 T cells by AbnobaViscum may indirectly promote the full maturation of DC including IL-12p70 production[155,391,156,392] that is initiated by the direct DC-stimulation by mistletoe-derived lectins[390]. These fully mature DC could then in turn promote the development of (tumor) antigen-specific αβ T cell responses[392]. IL-17 production by γδ T cells has been associated with the promotion of tumor development[378,388], but we did not find evidence for significant production of this cytokine by Vγ9Vδ2 T cells upon AbnobaViscum exposure.

The Vγ9Vδ2 T cell response appeared to be specific to the type of preparation of the mistletoe extracts. Despite being derived from the same host trees (pine or apple tree), the bacterial-fermented extracts from Iscador did not result in the expansion of Vγ9Vδ2 T cells. This was rather unexpected as the fermentation process could be a source of bacterial phosphoantigens[368,393] and thus indicates that these bacteria are not a source of Vγ9Vδ2 T cell-activating phosphoantigens in mistletoe-extract drugs. Possibly, the fermentation process leads to a degradation of mistletoe-derived pyrophosphates and rather induce compounds that are stimulatory for NK cells. Indeed, it has been suggested that Iscador preparations are stimulatory while AbnobaViscum preparations are inhibitory for NK cells[385,394,395]. This is in line with our observation that Iscador induced higher CD69 expression on NK cells than AbnobaViscum. It is known that fermented and non-fermented mistletoe extracts can be very different in terms of their composition[396] and our recent metabolomics analysis

on a series of mistletoe-extract drugs indicate that the composition of the extracts is much more dependent on the producer (company) than on the host tree (manuscript in preparation). Thus, the main immune cell target for fermented mistletoe extracts such as Iscador could be NK cells. The CD69 induction observed on Vγ9Vδ2 T cells upon exposure towards Iscador extracts could therefore be secondary to NK cell activation and thus rather a bystander effect. While this bystander effect could be sufficient for the increased cell surface expression of the sensitive activation marker CD69, this effect may not be sufficient for the more robust signaling needed to induce expansion of the Vγ9Vδ2 T cells[160] and thus provide a possible explanation why we did not observe any expansion of Vγ9Vδ2 T cells upon exposure to Iscador extracts.

Alkylamines that are present in plants such as sec-butylamine have been shown to activate Vγ9Vδ2 T cells indirectly by inhibiting the enzyme farnesyl pyrophosphate synthase resulting in the upregulation of endogenous phosphoantigens[56,59]. However, using approaches that verified the role of endogenous and exogenous phosphoantigens in the activation of Vγ9Vδ2 T cells by AbnobaViscum, we show here that the activation is mediated directly and thus not depend on the intracellular accumulation of phosphoantigens. Our findings are in line with the observed sensitivity towards alkaline phosphatase of heat-treated mistletoe extract-induced γδ T cell expansion[337]. The absence of expansion without heat treatment[337] is likely due to the cytotoxic effects of the mistletoe extracts in longer term cell cultures needed to study proliferation. Indeed, we showed that the mistletoe-extract drugs as such (thus without heat treatment) are sufficient to stimulate Vγ9Vδ2 T cells. In general, Vγ9Vδ2 T cell-activating phosphoantigens can be derived from the mevalonate pathway or the non-mevalonate pathway, the latter also known as the methylerythritol phosphate (MEP) pathway. Most organisms only use one of the two pathways for their isoprenoid synthesis. The MEP pathway is the one present in most (pathogenic) eubacteria and parasites of the phylum *Apicomplexa*, but it is absent from archaebacteria, fungi, and animals, which synthesize their isoprenoids exclusively through the operation of the

mevalonate pathway. By contrast, plants use both the MEP pathway and the mevalonate pathway for isoprenoid biosynthesis, although they are localized in different compartments: the MEP pathway is active in the plastids while the mevalonate pathway in the cytosol[43]. These plastids are likely derived from once free-living bacteria by endosymbiosis[397,398]. *Viscum album* L, used for the generation of mistletoe-extract drugs, contains the gene expression profile of the enzymes needed for the MEP pathway[399]. HMBPP, an MEP pathway-derived Vγ9Vδ2 T cell-activator, is up to 10,000 times more potent than the mevalonate pathway-derived Vγ9Vδ2 T cell-activator IPP and is thus described as a main compound allowing Vγ9Vδ2 T cells to sense cells infected with bacteria or parasites such as *Plasmodium* [368,153,400]. We propose that AbnobaViscum contains HMBPP or metabolites with a similar structure derived from the plastid-derived MEP pathway and thus that its administration mimics the presence of bacterial- or parasite-derived HMBPP resulting in the stimulation of Vγ9Vδ2 T cells that are cross-reactive with cancer cells[401]. Since the AbnobaViscum-induced Vγ9Vδ2 T cell stimulation was completely dependent on BTN3A, we propose that mistletoe-derived phosphoantigens such as HMBPP act in a direct and rapid manner via this ubiquitously expressed butyrophilin[379,402]. These phosphoantigens could act in concert with other mistletoe-derived compounds such as lectins (glycoproteins) and viscotoxins (polypeptides) targeting other immune cells such as DC and NK cells[390,324].

It has been recently described that both HMBPP and zoledronate stimulate polyclonal TCR responses as assessed by high-throughput sequencing of the Vγ9Vδ2 TCR repertoire[24]. We wondered whether AbonabaViscum stimulated similar TCR responses as HMBPP and/or zoledronate, or whether it would act only on a restricted Vγ9Vδ2 TCR repertoire as described for the tuberculosis vaccine BCG[403]. The AbnobaViscum-expanded Vγ9Vδ2 TCR repertoire showed a high level of similarity with the HMBPP- and zoledronate-expanded repertoire at the level of CDR3 length, CDR3 diversity, and (D)J gene segment usage. Furthermore, the same top expanded

TRGV9-associated CDR3 sequences could be found among AbnobaViscum-, HMBPP- and zoledronate-expanded TCR repertoires, that were highly shared among subjects (i.e., public). In contrast, the TRDV2-response was highly private: the expanded TCR repertoire was specific for each subject, confirming previous studies[24,23,404], but again the same top TRDV2-associated CDR3 sequences could be found in the AbnobaViscum-, HMBPP- and zoledronate-expanded TCR repertoires. Thus, AbnobaViscum appears to act on the same polyclonal Vγ9Vδ2 TCR repertoire in adult peripheral blood as does HMBPP and IPP (upregulated by zoledronate), and not on a small subset of Vγ9Vδ2 T cells. Despite significant recent progress regarding the molecular basis of phosphoantigen recognition by Vγ9Vδ2 T cells, it is still not known to which molecular structures the CDR3 regions of both the TRGV9 and TRDV2 chains bind[405,406]. It remains thus unclear why the TRGV9 and TRDV2 repertoire of adult Vγ9Vδ2 T cells, also after expansion with AbnobaViscum, are public and private respectively.

While bone targeting of N-BPs can be useful in the treatment of bone-related diseases, including cancer metastasis to the bones, this is rather a disadvantage for the treatment of most cancers that do not show bone metastasis. Furthermore, treatment with N-BP such as zoledronate can lead to medication-related osteonecrosis of the jaw, a serious adverse reaction[407]. Thus, alternatives are being developed such as the ex-vivo expansion of Vγ9Vδ2 T cells in order to re-infuse in cancer patients or the development of Vγ9Vδ2 T cell-activators with potential improved pharmacokinetic properties[377,408,409]. However, these compounds are till now not administrated *in vivo* and the injection of expanded Vγ9Vδ2 T cells have only been performed in the context of small phase 1/2 clinical trials[377]. In contrast, the administration of mistletoe-extract drugs is safe and about 500,000 cancer patients each year in Germany receive this treatment[303]. Our *in vitro* data regarding the stimulation of anti-cancer Vγ9Vδ2 T cells by AbnobaViscum encourages the inclusion of these cells in future immunophenotyping studies upon AbnobaViscum treatment *in vivo*. Such

immunophenotyping data and their possible correlation with clinical outcome will allow the stratification of cancer patients and are expected to provide insight into the controversial anti-cancer activities of mistletoe-extract drugs in cancer patients.

5. Discussion

5.1 Vγ9Vδ2 T cells – born to work?

One major question we wanted to explore is whether Vγ9Vδ2 T cells are protective in congenital infections. To address this question, we investigated the responses of these T cells during congenital infection with the parasite *T. gondii*. Currently, the only study regarding γδ T cells immune responses in congenital *T. gondii* infection was a cohort with 7 congenital toxoplasmosis subjects within an age ranged from 2 weeks to 6 years, of which 3 subjects were within one month of age[287]. They checked HLA-DR and CD45RO of these samples, and tested *in vitro* stimulation responses compared to different control groups and declared that Vγ9Vδ2 T cells are anergic in the acute phase congenital *T. gondii* infection[287]. However, T cell anergy is a rather obscure term in history which is used to describe any tolerance phenomenon. In one systematic review, anergy was defined as “a tolerance mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in the hyporesponsive state”[410]. It is intensively studied in CD4+ αβ T cells and can be observed *in vitro* by clonal anergy and *in vivo* by adaptive tolerance[410]. There isn’t a clear-cut boundary to consider a T cell in anergy state. In general, proliferation, differentiation, and cytokine production are key factors to characterize the anergy state. Furthermore, T cell anergy, exhaustion, and regulation share many similarities, but they involve different signaling pathways and gene expression profiles. The major differences include anergy can be induced very quickly while exhaustion takes time; anergy is cell-autonomous activity while exhaustion and regulation involve other cells[410-412].

In this work, we included 5 congenital toxoplasmosis subjects within one month of age and investigated their Vγ9Vδ2 T cell phenotypes regarding function status. We observed that Vγ9Vδ2 T cells were highly expanded in these ‘acute phase’ congenitally infected subjects, and these Vγ9Vδ2 T cells were highly differentiated

towards a cytotoxic effector phenotype. Although we did not perform true counts of the samples, according to the standard operation during test (for example, samples were collected from the same volume of blood, and for each batch, samples were tested in a similar volume of buffer), this can give an estimated idea of the cell number for each sample. The event counts in Toxo+ group are dramatically higher than Toxo- group, thus the increased frequency in congenital toxoplasmosis group was highly unlikely due to the decrease of other T cell subset. This is in line with the former study which demonstrated the increase of γδ T cells in number[363]. Due to the difficulties to acquire a sufficient number of PBMC samples within this age group, *in vitro* functional tests were not feasible, thus it is difficult to draw a definitive conclusion about their functional reactivities. However, the obtained phenotyping results do not fully support an anergic status. First, Vγ9Vδ2 T cells in congenital toxoplasmosis subjects exhibited an increased terminally differentiated effector phenotype (CD27-CD45RA+) compared to age-matched controls. CD27- Vγ9Vδ2 T cells are considered to be associated with lower proliferation and higher functional properties[413] and they are homing to infection sites[351]. To be noticed, in chronic virus infections, adult Vγ9Vδ2 T cells were found to exhibit effector phenotype (CD27-CD45RA+) and associated with increased cytotoxicity[127,123]. Second, we observed dramatically increased granzymes and perforin expression within these Vγ9Vδ2 T cells, along with the upregulated expression of the transcription factor T-bet. These phenotypes strongly support an effector potential within these cells[414]. Further, one indirect support might come from the ‘bystander effects’ exhibited by other immune subsets. We observed other γδ T cell subsets and also αβ T cells in ‘acute phase’ congenital toxoplasmosis group showed a certain level of increased activation and differentiation, but the responses never as much as found in Vγ9Vδ2 T cells. This mild and broad activation might be mediated by the proinflammatory cytokines produced by the highly responsive Vγ9Vδ2 T cells, while adaptive tolerance normally has reduced cytokine responses[410].

Regarding proliferation capacity, neonate Vγ9Vδ2 T cells were expanded toward *T.*

gondii infection. This expansion highly likely happened *in utero* because two of the Toxo+ samples with intense expansion of Vγ9Vδ2 T cells were sampled at day 1 after birth. Interestingly, we did not observe Ki-67 upregulation within this age group, which supports the observed expansion was happened before, but might also support a proliferatively 'anergic' state. Especially, in >1 month toxoplasmosis subjects, Ki-67 expression had a tendency to decrease compared to age-matched controls and to <1 month subjects. But, as mentioned earlier, late differentiated cells have lower proliferation but they are not nonresponsive[413], so the tendency to have a decreased proliferation capacity might be more related to a change in effector functions rather than to an anergic state. Also, we tested *in vitro* stimulating with HMBPP on four around 2 months old (73-82 days) subjects (2 Toxo- vs 2 Toxo+). One of the Toxo+ sample had lots of dead cells, thus only one Toxo+ result acquired. We did observe CD107a, IFNγ, TNFα upregulation within this sample. Although we cannot draw a clear conclusion based on this result, it did not provide evidence to support the anergic of Vγ9Vδ2 T cells from Toxo+ infant.

Another observation was that the dynamics of CD27 and CD28 expression are different in Toxo- samples, while the expressions of CD27 and CD28 are both stably low in Toxo+ samples. Toxo- samples downregulated CD27 expression during aging, while CD28 expression did not change during the observed age range. These expression dynamics are consistent with the observation on healthy adults γδ T cells[415]. For Toxo+ subjects, they had stably lowered CD28 expression which is a reflection of their functional status. CD28 is downregulated on activated Vγ9Vδ2 T cells. CD28-B7(CD80/CD86) signaling is important for Vγ9Vδ2 T cell proliferation upon activation *in vitro*[416,417] and CD28 is crucial for IL-2 mediated γδ T cell proliferation and cytokine responses in mice infected with malaria parasite[416]. Further, CD28- expression is positively correlate with CD5-, CD8+, CD16+, CD56+ expression on γδ T cells[415]. CD28+ Vγ9Vδ2 T cells are associated with higher proliferation and cytokine production, while CD28- Vγ9Vδ2 T cells are more cytotoxic[112]. Thus, the

stably lowered CD28 expression on Vγ9Vδ2 T cells from congenital toxoplasmosis infants fits with the lower Ki-67 expression tendency observed in >1 month Toxo+ subjects. Overall, congenital *T. gondii* infection might lead to dampened proliferation capacity within the expanded Vγ9Vδ2 T cells, but our data indicate that these expanded Vγ9Vδ2 T cells are responsive and highly possibly being protective against infection.

5.2 Vγ9Vδ2 T cells and *T. gondii* – evolutionary rivals?

One of the most interesting observations we found in congenital toxoplasmosis subjects is that certain sequences are abundant in the CDR3 region from their TCRδ chain[12,13]. The top shared TRDV2 CDR3 sequences (containing the motifs VLGD/TGGY/ILGD) observed in the peripheral blood of two months old congenital toxoplasmosis infants were found abundant in fetal intestinal and liver γδ T cells at around 6-8 weeks of gestation, when the fetal thymus is not formed yet. Interestingly, in a mice study, extrathymic γδ T cells were found important in the defense towards *T. gondii* infection[271]. These mice γδ T cells predominantly use Vγ7 gene segment[271], which is specific for mouse gut intraepithelial γδ T cells. Thus, these provoke the hypothesis about the evolutionary origin spot of the abundantly shared Vδ2 T cells we observed and the importance of those earliest extrathymic Vδ2 T cells in human fetus life.

Former studies showed that fetal γδ T cells in general prefer TRDJ2 and TRDJ3 during TRD chain rearrangement[418,13,23]. However, pre-thymic fetal intestinal γδ T cells (especially Vδ1 T cells) seem to have a preferred taste with the ‘mature’ TRDJ1 gene segment during rearrangement, as this segment is normally seen in adult blood or post-natal thymus TRD chain rearrangement[13,23,20,24]. Notably, the three abundantly shared Vδ2 sequences all use TRDJ1 gene segment, which makes it even more ‘strange’ as fetal Vδ2 rearrangement normally more favors TRDJ3 than TRDJ1. One possibility is that these earliest rearranged Vδ2 T cells with the ‘mature’ form TRDJ1

segment normally do have higher chances (for example higher affinity) to be PAg responsive sequences after pairing with Vγ9 chain and thus been selected during pathological events. These abundant Vγ9Vδ2 T cells might in greater chance originate from fetal intestine. One support for this origin is that in former study, at 8 weeks gestation, VLGD sequence was more abundant in intestine than liver from the same donor (Annex)[13]. Also, interestingly, TRDJ1 is predominant in Vδ2 T cells in the pre-thymic fetal intestine and switched to TRDJ3 in later trimester[13]. However, the fetal circulation between liver and intestine starts after 4 weeks of gestation, so either site could be the original generation point[13]. Besides, in another study which focus on the pre-thymic liver, all the three top shared Vδ2 sequences were found abundant in pre-thymic liver[12]. Although it seems TRDJ3 was more used compare to TRDJ1 in fetal liver Vδ2 T cells, the authors only sequenced TRDJ1-containing sequences[12]. Interestingly, we indeed did not observe *T. gondii* top shared TRDJ3 Vδ2 sequences among 2 months infected subjects in the fetal liver and fetal intestine TRDJ3 Vδ2 sequences. This might be because those abundant TRDJ3-containing sequences found in Toxo+ samples are generated later than the top abundant TRDJ1-containing sequences. This in a way supports the hypothesis that the TRDJ1 Vγ9Vδ2 T cells are kind of guarantee responders that are equipped in the very first wave, and selected during common PAg-generating pathogenic events in early prenatal times.

Further, in human fetus, well-oxygenated and nutrient fetal blood comes back from placenta through the umbilical vein and heads to the fetal liver, and from there branches into two paths, one goes directly to the liver, the other bypass the liver and heads to the heart[419]. Thus the vertical invasion of *T. gondii* might through the blood flow in the umbilical vein and spread in the fetus. This emphasizes the importance of the blood type γδ T cells in response to the parasite. The pre-thymic Vγ9Vδ2 T cells with a mature-like character extend the efficient pattern ‘surveillance’ to pre-thymic time. So even when early *in utero* infection happens, these Vγ9Vδ2 T cells might ready to be quickly selected to expand and protect.

The innate-like fast response and Th1 biased defense mode already put Vγ9Vδ2 T cells in a very unique position in prenatal defense. Most human congenital infections, typically refer to the TORCH infections (stand for **T**oxoplasmosis, **O**ther (syphilis, varicella-zoster, parvovirus B19), **R**ubella, **C**ytomegalovirus (CMV), and **H**erpes infections)[420], are caused by viruses. *T. gondii* is one of the exceptions. This lead to the thinking that γδ T cells might be specially designed to provide prenatal protections, with Vγ9Vδ2 T cells in charge of defending against congenital parasitic and bacterial infections; meanwhile the nonVγ9Vδ2 T cells contribute to the congenital virus surveillance[350].

Furthermore, in mice, TLR11 and TLR12 within DCs and macrophages are the most important *T. gondii* profilin recognition receptors, which initiate the mice IL-12/IFNγ anti-*T. gondii* defense[216,213]. However, humans do not have these TLRs, instead, the primate unique Vγ9Vδ2 T cells and the BTN molecules (BTN3A1 and BTN2A1)[69,67] recognize PAgs that generated by apicomplexan parasites[421]. In sensing the level of PAgs, Vγ9Vδ2 T cells do not need to directly contact the parasites, and they can produce IFNγ to quickly initiate the systematic attack.

5.3 Vγ9Vδ2 T cells in mistletoe therapy – new target?

With the fast, broadly responsive, and intrinsic cytotoxic effector features, Vγ9Vδ2 T cell wins its place in cancer immunotherapy[422]. In this work, we investigated *in vitro* Vγ9Vδ2 T cells immune responses towards mistletoe extracts, one of the most used complementary herbal drug preparations in cancer therapy. We tested two kinds of mistletoe extracts (one from apple tree, the other from pine) and each prepared by two companies using different extraction methods. Although fermented mistletoe preparations (especially Iscador P) induced more general activation responses measured by CD69 expression from all types of immune cells (Vγ9Vδ2 T cells, αβ T cells, NK cells), we did not see proliferation from any of these subsets. This is consistent with former *in vitro* observation on proliferation in healthy donors[423]. In

contrast, non-fermented mistletoe extracts (especially Abnoba P) only induce Vγ9Vδ2 T cells activation and successfully lead to proliferation. It seems the specificity of activation is correlated with proliferation potency. One possible explanation for these observations is that CD69 expression is not necessarily linked to proliferation[424,425]. Former studies showed that CD69 deficient mice have similar T cell proliferation responses compared to wild-type mice[424], indicating that other routes of signaling are sufficient and important for T cell proliferation. Thus it is possible that fermented mistletoe extracts lack the key compound for proliferation signal, but can sufficiently induce CD69 expression. In contrast, non-fermented mistletoe extracts possess the key antigen(s) (e.g. PAg) that lead to the successful proliferation of the particular subset (i.e. Vγ9Vδ2 T cells). In addition, another possibility for not seeing proliferation after activation on other types of immune cells might be due to the activation-induced cell death (AICD). Mistletoe lectins can induce FasL on T cells[426], and they do not influence activation markers expression[427]. The apoptosis signs can be seen within 24 hours of incubation[427], so it is possible even after we removed the extracts at day 1, the apoptosis on certain subsets of immune cells was still ongoing. Fermented mistletoe extracts might indeed possess antigens for certain proliferation response on nonVγ9Vδ2 T cells, but AICD might overcome the stimulation effects. Also, the possible ongoing apoptosis may induce PD-L1 expression on APCs which sending inhibitory signals to lymphocytes to prevent proliferation[90].

The mistletoe-expanded Vγ9Vδ2 TCR repertoires were similar to the PAg and N-BP expanded Vγ9Vδ2 TCR repertoires. This is consistent with a recent study which compared HMBPP and zoledronate expanded Vγ9Vδ2 T cells from neonates and adults[24]. Also, in line with former studies[19,20,24,23,404], we observed the CDR3 of Vγ9 chain is more restricted than Vδ2 chain on mistletoe-expanded cells. Common Vγ9 CDR3 sequences were found in different subjects among the top shared mistletoe, HMBPP, and zoledronate induced expansions, while the Vδ2 CDR3 sequences were more private to each subject. This intrinsic diversity difference between CDR3 region

of Vγ9 and Vδ2 chain might be related to BTN and PAg binding capacity. It seems the main task for Vδ2 chain is to provide diversity (for example different levels of affinity), while Vγ9 chain provides some kind of insurance for PAg reactivity. One possible support for this hypothesis is that even in the most diverse CDR3 region, KKIK motif in Vγ9 CDR3 region is evolutionally restricted for PAg reactivity[29,26], while the important PAg reactivity-related position 97 in Vδ2 CDR3 allows different amino acids with similar physiochemical properties. The polyclonal mistletoe-reactive Vγ9Vδ2 T cells indicate the rapid response capacity of these cells in stimulating anti-tumor immunity.

5.4 Vγ9Vδ2 T cells 'in practice'

Congenital toxoplasmosis normally does not show any symptoms at birth, but the patients are under higher risks to have symptoms later in life[199,428]. The key cytokine IFNγ in controlling *T. gondii* infection is also associated with pathological lesions[429]. Even the very young fetal immune system is facing the balance between controlling the infection and controlling the immune attack. The former congenital toxoplasmosis study focused on patients with symptoms, with patient 1 showed no activation and expansion of Vγ9Vδ2 T cells and lead to fatal outcome[287]. This indicates the protective effects of active anti-parasite immune response. Among the 12 congenital toxoplasmosis subjects we have, only one is with ocular manifestation, the other were all asymptomatic when sampling. We observed reduced CD27, CD28 expression and increased granzyme B, T-bet in γδ T cells and to a more minor extent in αβ T cells at 16 months and 24 months with this patient. Also, the different dynamic between γδ T cells and αβ T cells, for example, Ki-67 expression increased in αβ T cells while it was stable in total γδ T cells and slightly decreased in Vγ9Vδ2 T cells. We could not give any conclusions based on one case. However, these observations, if could be repeated in more ocular toxoplasmosis samples, support the not anergic state of the fetal immune system and the delicate immune regulations. The earliest, meanwhile, also the strongly responsive Vγ9Vδ2 T cells showed signs for reduced proliferation capacity. Comparing to continue producing highly activated Vγ9Vδ2 T cells, reduced

proliferation might be good to the infants as effector cells might produce large amount of proinflammatory cytokines and exhibit cytotoxic behaviors which once last long enough would eventually not benefit to the host. But their fast reactions along with drug interventions might provide the infants enough time to have *T. gondii* specific CD4 and CD8 αβ T cells ready and which would become the protectors in controlling the parasite in the long run. Notably, different virulence of the parasite might also contribute to differential T cell responses. Too strong activation might lead to lymphocytes tolerance. This might be related to the more serious disease burden in South American countries[198].

In addition, early diagnosis of congenital toxoplasmosis is facing many challenges. For example, varied immune responses in newborns and the transplacental *T. gondii* specific IgG can both influence the accuracy of the routine serologic diagnosis method[339]. Multiple strategies for testing and a follow-up until one year of age are needed[339]. Also, many congenital infections share similar symptoms at birth[183]. Thus, with the specific and dramatic expansion reaction towards congenital *T. gondii* infection at birth, and the easy-to-approach way for testing, Vγ9Vδ2 T cells can be a potential good biomarker for the diagnose of *T. gondii* infection at birth.

γδ T cells are the top favorable immune cells in pan-cancer prognostics[430], indicating their importance in the controlling of malignant diseases. Vγ9Vδ2 T cells share many similarities with NK cells and CD8 αβ T cells, but they are unique in their own way[431,113]. Like NK cells, they show pattern recognition and fast responding, however, they are unique in strategies. NK cells rely on MHC-I molecules and the balance of stimulatory and inhibitory signals on target cells to make the killing decision, while Vγ9Vδ2 T cells sense the key metabolites needed for every living cell. Tumors are highly heterogeneous[432], they can manipulate the pathways to control their membrane receptor collections to avoid and mislead immune surveillance. But the isoprenoid pathway is key for cell survival, it is unlikely to downregulate the most important building blocks for isoprenoid synthesis during tumor evolution, this might be related

to the overall favored prognostics for γδ T cells in tumors. However, in recent years, the role of IL-17 producing γδ T cells in cancer development is disputable[433]. They are found to promote neutrophil infiltration and lead to tumor growth[434,435]. Although most of the IL-17 γδ T cells studies are in mice, attentions should be raised in γδ T cell immunotherapy. We tested IL-17a production after mistletoe stimulation at different time points. We did not observe IL-17a increase after one day of stimulation and the response was similar with HMBPP stimulation. Instead, mistletoe stimulation induces IFNγ and TNFα productions, as well as a strong cytotoxic-associated (CD107a upregulation) response. These all indicate Vγ9Vδ2 T cells may play an important role in the potential anti-tumor effects of mistletoe therapy.

6. Conclusions and Perspectives

The main findings within this study are:

(1) The human fetus generates a group of public Vγ9Vδ2 T cells that develop strong cytotoxic effector phenotypes towards congenital parasite infection; and these cells provide a potential protection to the fetus in the defense of the infection.

(2) Vγ9Vδ2 T cells may be one of the main target immune cells in non-fermented mistletoe extract drug therapy.

In the first part of this study, we expanded the current knowledge about fetal Vγ9Vδ2 T cells immune response on their phenotypes and TCR CDR3 sequences in congenital toxoplasmosis and the acquired results support for a protective effect of these Vγ9Vδ2 T cells in early life infection. However, several questions remain open to be investigated in the future. First, currently there is no *in vitro* functional information on fetal Vγ9Vδ2 T cells from congenital toxoplasmosis subjects. In order to directly compare the functioning status of Vγ9Vδ2 T cells from congenital *T. gondii* infected neonates and their age-matched counterpart, *in vitro* functioning tests can be done to explore their proliferation potency and effector functions such as cytotoxicity and cytokine productions. Second, as aging is an independent factor to shape Vγ9Vδ2 T cells, also disease progression along with anti-*T. gondii* treatment all influence the disease outcomes, comparing between symptomatic and asymptomatic congenital toxoplasmosis, different treatment strategies and durations, and the dynamics of neonatal Vγ9Vδ2 T cells in congenital *T. gondii* infection during early years would be useful for understanding Vγ9Vδ2 T cell immunity in early life and for clinical significance. Third, former studies also observed increased NK cells and CD8 αβ T cells in congenital toxoplasmosis neonates[363], thus it is interesting to investigate the contributions of Vγ9Vδ2 T cells among all those effector cells in cytokine production

and cytotoxicity, as well as the dynamics among these subsets during disease progression.

In the second part of the study, we found that non-fermented mistletoe preparation drugs induce BTN3-mediated effector functions in Vγ9Vδ2 T cells that similar to PAg *in vitro*. These observations are of potential clinical values to current Vγ9Vδ2 T cell immunotherapy and to mistletoe therapy. First, although HMBPP is the most potent Vγ9Vδ2 T cells activator, it has poor pharmacokinetic properties as it is easy to be degraded in the blood and has low membrane permeability[367]. That's why current Vγ9Vδ2 T cell immunotherapy use N-BP drugs and synthetic PAg to obtain Vγ9Vδ2 T cell expansions[422]. Though only tested in one subject, we observed a protective effect for HMBPP from apyrase digestion by the ascorbate-phosphate buffer used in the non-fermented mistletoe products. Also, we found that instead of the -20°C to -80°C preservation for HMBPP, long time (several months) preservation in 4°C does not influence the stimulation effects from these non-fermented mistletoe extracts. This might be of interest to take into consideration for the way of developing PAg related drugs, like the way to prolong the effects of the drug before injection. Second, the most common administration route for mistletoe preparation drugs is subcutaneous injection, as it is convenient for the patients to conduct at home by themselves. However, intravenous infusion and intratumor injection are also used[301]. Regarding the *in vitro* observations we have in Vγ9Vδ2 T cells, it would be of interest in the future to compare *in vivo* if there are differences on the immune responses among different administration routes, as Vγ9Vδ2 T cells are most popular in the peripheral blood. Also, it would be interesting to check after intratumor injection of the mistletoe extracts, whether Vγ9Vδ2 T cells will be attracted to the tumor area by indirectly measuring the dynamics of related chemokine receptor expressions and medical imaging[436,437]. Third, as both Vγ9Vδ2 T cell immunotherapy and mistletoe therapy are safe[8,303] and involved in several clinical trials, it would be interesting to combine these treatments. As mistletoe extract drugs may contain compounds that influence other immune cells,

combined treatment might magnify the antitumor reaction of Vγ9Vδ2 T cells. Also, the stimulation of mistletoe extract drugs is mediated by BTN3 molecules. Thus, combination with therapies that target BTN molecules might also be of interest. A recent study found BTN3A1 is abundantly expressed on malignant cells and inhibits αβ T cell activation in ovarian cancer[438]. Combined treatment of γδ T cells, tumor-specific αβ T cells, and anti-BTN3A1 antibody achieved the maximum antitumor effects in human ovarian tumor-bearing mice[438]. This indicates multiple targeting treatment is a promising way in cancer therapy.

Overall, current mistletoe therapy studies are rarely focused on γδ T cells[310]. The *in vitro* observations we have in this work may provoke future *in vivo* studies to look at the immune responses of Vγ9Vδ2 T cells in patients receiving mistletoe therapy.

7. References

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8. Annex

Table III B from McVay L. D., 1998[13]

DNA sequence analysis of TRDV2-DD-DJ-DC receptors from 6-9 weeks liver (L), gut (G), and thorax/thymus (T) of the same donor

C	DV2 D	T		DD1		DD2		DD3		Code no.:	No. seqs. 6-01		8.7-691		
TGT	GAC	ACC	N/P	GAAATAGT	N/P	CCTTCCTAC	N/P	ACTGGGGGATACG	N/P	DJ	L	G	T	L	G
TGT	GAC							ACTGGGGGATAC		ACC GAT (1) (+)					3
TGT	GAC		GT					ACTGGGGG		AC ACC GAT (1) (+)		2	3	2	18
TGT	GAC	AC						ACTGGGGGAT		ACC GAT (1) (+)			1		
TGT	GAC	ACC	G					CTGGGGGATAC		ACC GAT (1) (+)		1			
TGT	GAC	ACC	TT					ACTGGGGG	CTTCC	AC ACC GAT (1) (−)			1		
TGT	GAC	ACC						TGGGGGATAC		C GAT (1) (−)		1			
												4	5	2	21
TGT	GAC	ACC						TGGGGGATACG		C TCC TGG (3) (+)	8		4	21	1
TGT	GAC		GATAT					GGGATAC		C TGG (3) (−)		1			1
TGT	GAC	A	T					ACTGGGGGATACG		C TCC TGG (3) (−)	1				
TGT	GAC	A	T					ACTGGGGGAT	CCC	C TCC TGG (3) (−)	1				
TGT	GAC	ACC						TGGGGGATAC	CCGT	C TGG (3) (+)			1		
TGT	GAC	ACC	G					CTGGGGGATAC		TCC TGG (3) (+)			1		
TGT	GAC	AC	GGGTATT					ACTGG		C TCC TGG (3) (+)			1		
TGT	GAC	ACC						GGGGG	TCTTAAG	C TCC TGG (3) (−)			1		
TGT	GAC	AC	ACT					CTGGGGGATA	G	C TCC TGG (3) (−)			1		
TGT	GAC	ACC						TGGGG	TAG	C TCC TGG (3) (+)			1		
TGT	GAC	ACC								TGG (3) (+)			2		
TGT	GAC	ACC						TGGGGGATA		GCA (2) (+)			2		
TGT	GAC	ACC													
TGT	GAC	ACC						GGGGGATAC		TTG ACA (2) (+)			1		
TGT	GAC		GT					ACTGGGGGATAC		A GCA (2) (+)			1		
TGT	GAC	A	AGG					CTGGGGGATACG	CGAG	CTC (2) (−)			1		
											10	5	22	23	23

In-frame joints are labeled (+), while out-of-frame joints are labeled (-). The identity of the DJ gene segment is indicated by the number (1), (2), (3), or (4). No. seqs. is the frequency with which a particular sequence was found among cDNA clones. Code no. 6-01 is of 6 weeks subject, 8.7-691 is of 8 weeks subject. Red arrow indicates the top shared VLGD sequence among congenital *T. gondii* infected infants[13].