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Thymic development and peripheral functional polarisation of human Vγ9Vδ2 T cells

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« Διά τὸ θαυμάζειν ἡ σοφία »

Η αρχή της σοφίας είναι η αναζήτηση, Σωκράτης

Wisdom begins in wonder, Socrates

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Abstract

Vγ9Vδ2 T cells are a subset of human T lymphocytes activated by phosphoantigens in a T cell receptor-dependent manner to fight microbial invaders or kill transformed cells. Phosphoantigens are low molecular weight nonpeptidic pyrophosphate containing metabolites produced both endogenously (upregulated in transformed cells) and by microbes. Vγ9Vδ2 T cells are the first T cells generated in the foetus and have programmed functions before encountering the post-partum environment.

In this PhD thesis, the aim was to assess the origin of Vγ9Vδ2 T cells in early versus adult life and to evaluate their T cell receptor repertoire and effector potential in the neonatal and infant period.

First, human Vγ9Vδ2 T cells were characterised coming from foetal blood and generated by the foetal thymus and then similarities and differences with adult blood Vγ9Vδ2 T cells were identified. The data showed that there is a post-natal thymic output of Vγ9Vδ2 T cells which are different from their foetal counterparts. This finding could help guide the development of cancer immunotherapy strategies aiming to improve the resistance and tenacity of Vγ9Vδ2 T cells which enter an exhaustion state after long encounter with the antigen.

Furthermore, human Vγ9Vδ2 T cells were studied early after birth regarding their T cell receptor repertoire and function. At 10 weeks after birth, Vγ9Vδ2 T cells had expanded, and a big part of the Vγ9Vδ2 T cell repertoire was foetal-derived. Additionally, Vγ9Vδ2 T cells had undergone significant functional polarisation toward potent killer effector cells. The expansion and shift in effector functions were not influenced by neonatal BCG vaccination, highlighting the role of environmental exposure upon birth.

The data gathered here highlight the unique properties of this innate-like lymphocyte population which can act as a first wave of protection in early life while conventional αβ T cells are not yet optimal. Later in life, another wave of Vγ9Vδ2 T cells arrives from the thymus to expand and populate the adult periphery, providing a possible avenue of new and robust cancer cell killers in the scope of immunotherapy.

List of abbreviations

2M3B1PP	2-methyl-3-butenyl-1-pyrophosphate
APC	antigen-presenting cell
BCG	bacilli Calmette-Guérin
BrHPP	bromohydrin pyrophosphate
BTN	butyrophilin
CAR	chimeric antigen receptor
CCL3	CC-chemokine ligand 3
CDR3	complementarity-determining-region-3
CML	chronic myelogenous leukaemia
CTL	cytotoxic T lymphocyte
CXCR3	CXC-chemokine receptor 3
D	diversity
DCs	dendritic cells
DETCs	dendritic epidermal T cells
DNA-PK	DNA-dependent protein kinase
Eomes	eomesodermin
FPP	farnesyl pyrophosphate synthase
H	helper
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSPC	hematopoietic-stem-and-precursor-cell
HTS	high-throughput sequencing
IFNγ	interferon-γ
ILC	innate lymphoid cell
IPP	isopentenyl pyrophosphate
J	joining
MAIT	mucosal-associated invariant T
MEP	2-C-methyl-D-erythritol 4-phosphate
MHC	Major histocompatibility complex
Mtb	*Mycobacterium tuberculosis*
NKG2D	natural killer group 2D

NKT	natural killer T
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PDL1	programmed cell death ligand 1
STAT	signal transducer and activator of transcription
TB	tuberculosis
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TNFα	tumour necrosis factor-α
TPST2	tyrosyl protein sulfotransferase
V	variable
WHO	World Health Organisation

I. Introduction

1. γδ T cells, the unconventional T lymphocytes

Our bodies need protection against pathogen invasion and for that we employ our immune system which is armed with an arsenal of cells and proteins. This immune network is destined to eliminate microbes and substances determined to pose significant threat to the normal functioning of our bodies by confronting viruses, bacteria, toxins, fungi and parasites, or even by battling tumoral cells. So, the immune system develops mechanisms at least equal in effectiveness and ingenuity to the infectious or transforming threat. At the same time, the maintenance of homeostasis and prevention of self-destruction is a very important function managed also by the immune system via tolerance mechanisms. This orchestration and customisation of the immune system aim in securing our best survival[1,2].

The network of immune cells is usually divided into the innate and adaptive leg (**figure I.1**). The first harbours ready-for-action cells (e.g. monocytes, neutrophils, natural killer cells) and the second highly specialised agents (T and B lymphocytes). Even though separated in categories the cross talks and cooperation between the two legs are constant and crucial. T and B lymphocytes recognise and bind a broad range of antigens through the highly diverse antigen receptors on their surface (T-cell receptor for T lymphocytes and B-cell receptor for B lymphocytes) and confer the essential aspect of immunological memory[1]. In the T spectrum, there are unconventional cases of more rapid responders such as mucosal-associated invariant T (MAIT) cells (αβ), natural killer T (NKT) cells (αβ) or the γδ T cells[3]. The focus of this work is on the human γδ T cells and especially the Vγ9Vδ2 T cell population which provide unique functional specialisation.

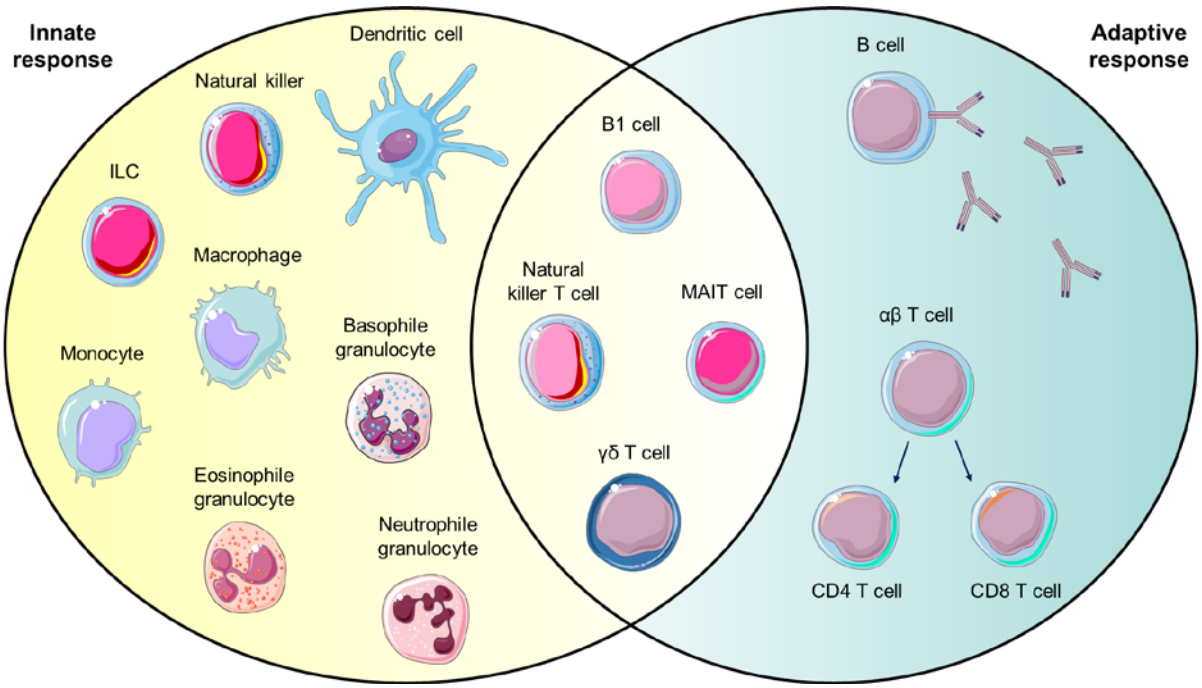


Figure I.1. Bridging innate and adaptive immunity. The innate immune response serves as first line of defence against infection. It consists of granulocytes (basophils, eosinophils and neutrophils), macrophages, dendritic cells, innate lymphoid cells (ILC) including natural killer cells etc. The adaptive immune response, consisting of antibodies, B cells, and CD4+ and CD8+ αβ T lymphocytes, is slower to develop, but displays increased antigenic specificity and memory. Natural killer T cells, MAIT cells and γδ T cells are cytotoxic lymphocytes and B1 cells are innate-like B cells, that bridge innate and adaptive immunity (inspired by[3,4], forms from[5]).

1.1. Discovery of γδ T cells and differences from αβ T cells

T lymphocytes are subdivided into αβ and γδ T cells based on the expression of the T cell receptor (TCR) on their cell surface. Together with B lymphocytes, they have been conserved seemingly since the emergence of jawed vertebrates, more than 450 million years ago and serve a multitude of roles. The αβ T cells are mostly known for their adaptive nature, comprising of the helper CD4 T cells and the cytotoxic CD8 T cells (**figure I.1**). CD4 T cells are divided based on their helper (H) functions with the interferon-γ (IFNγ) producers TH1, the IL-4, IL-5 producers TH2, the IL-17 producers TH17, as well as regulatory T cells Treg and follicular helper cells TFH[2]. They are MHC-restricted, meaning the antigenic peptide is presented to their TCR via MHC molecules (class I or II) and require time in order to develop an immune response. However, γδ T cells follow a different rather unconventional path, having properties of both the innate and adaptive immunity, are not MHC-restricted and display rapid effector functions (**table I.1**). Since the unexpected discovery of the γδ TCR in 1980's,

while characterizing the αβ TCR genes[6–10], a variety of valuable contributions to the immune system have been described for the transitional γδ T cells, including lysis of infected and stressed cells, cytokine and chemokine production, dendritic cell maturation, priming of αβ T cells via antigen presentation and other functions[11]. Their MHC-unrestricted recognition of target cells in principle would allow therapeutic application across a wide range of patients.

	αβ T cells	**γδ T cells**
Antigen	Peptide	Non-protein, protein, lipids etc.
Antigen processing	MHC I or II	No MHC restriction (or rare)
Time for response	Days - weeks	Hours
CD4, CD8 phenotype	Either CD4 or CD8	Mostly double negative (exc. CD8aa)
Frequency in blood	65-70%	1-5% (25-60% in gut)
Distribution	Blood, lymphoid tissues	Blood, epithelial & lymphoid tissues
Effector capability	CTLs (CD8+), cytokine release (Th1/Th2/Th17)	CTLs, cytokine release (Th1>Th2)
TCR diversity	Very high	Limited to high (theoretical very high)

Table I.1. Main differences between αβ and γδ T cells (based on [12]). (CTL: cytotoxic T lymphocyte; MHC: Major histocompatibility complex).

αβ T cells undergo positive and negative selection in the thymus during lymphocyte development which is indispensable for immune tolerance. This selection from within the diverse naïve immune receptor repertoire allows expansion of specific TCRs that deploy amplified responses to specific immune challenges, such as pathogen infection. αβ T cells go through clonal expansion and clonal differentiation to effectors while preserving long-lived expanded populations with immunological memory, enabling faster and stronger immune responses to secondary antigenic challenge. It is immunological memory that underpins the concept of vaccination and prevents reinfection with pathogens that have been repelled successfully by an adaptive immune response[1]. γδ T cells sometimes follow and sometimes deviate this immunological pathway as it will be discussed in the next sections.

1.2. T cell development in the thymus

γδ T cells, just like αβ T cells, (generally) develop in the thymus. Bone marrow T cell progenitors migrate into the thymus where they interact with environmental cues, such as ligands for the Notch receptor, that drive commitment to the T lineage. The thymus provides a specialized and architecturally organized microenvironment to promote TCR gene rearrangement and distinct stages of T cell development and repertoire selection. It is a primary lymphoid organ of the immune system located in the upper anterior thorax just above the heart and consists of two lobes[1] (**figure I.2**).

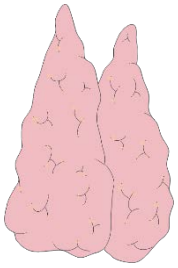


Figure I.2. Representation of the thymus[5].

Different compartments of the thymus reflect different developmental events. Main compartments are the outer thymic cortex, containing immature thymocytes, and the inner medulla, where more mature thymocytes are found. In young individuals, the thymus harbours large numbers of developing T-cell precursors embedded in a network of epithelia known as the thymic stroma[1] (**figure I.3**).

The thymus is colonized by hematopoietic stem cells on embryonic day 60 in humans[13], reaches its maximal output during early postnatal life, and declines in size and output during young adulthood and throughout adult life through the process of age-related involution. Although the onset of thymic involution has been linked to the increase of steroid hormones associated with sexual maturity, the timing and influential factors for this decline remain controversial[14]. Moreover, the progressive reduction in the size of the thymus is accompanied by a replacement of thymocytes by connective and adipose tissue[2] (**figure I.3**).

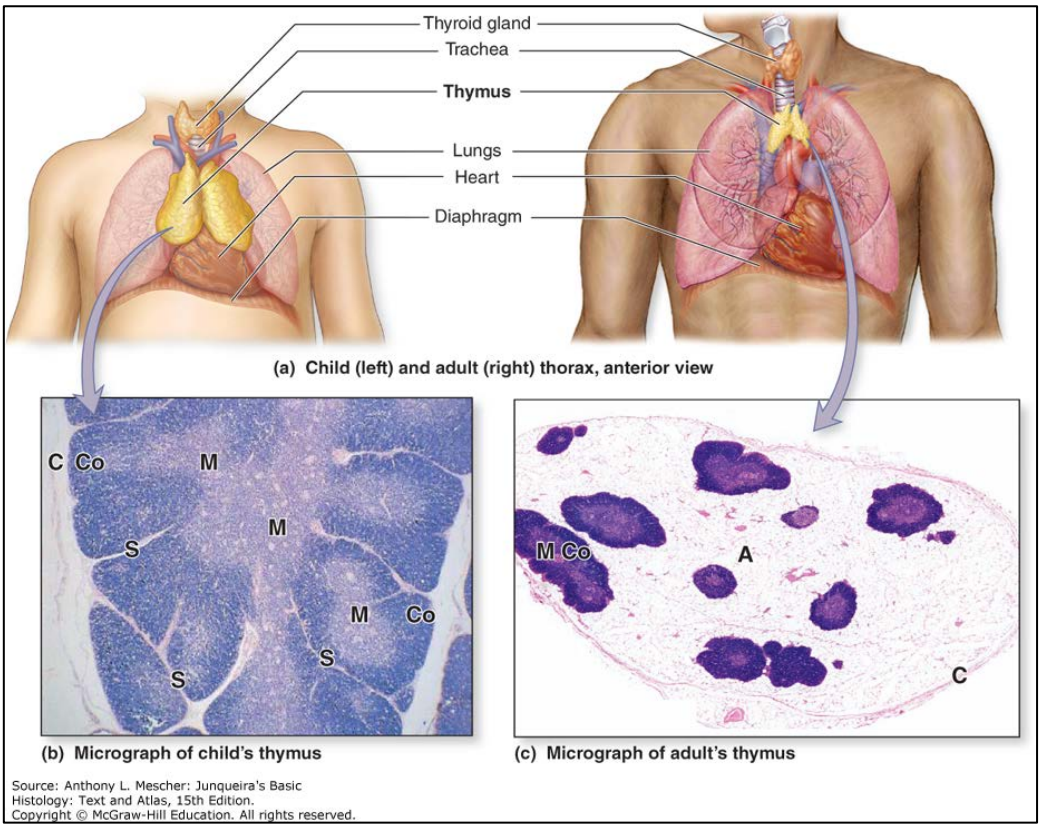


Figure I.3. Thymus involution. (a) Child (left) and adult (right) thorax view. The thymus is situated in the upper anterior thorax, just above the heart. Its size decreases in the adult. (b-c) Histology micrograph of child's and adult's thymus (M: medulla, Co: cortex, S: stroma, C: connective tissue)[15].

The origin of the term "thymus" comes from the Greek word "θύμος" which has both literal and metaphorical meanings. These include fume (literal) or mental effervescence, anger, passion, spirit, and the place of the soul situated in the heart, the "breath" of the soul. The fact that ancient Greek physicians referred to the decrease or "disappearance" of the thymus (small organ dissection was probably difficult for ancient anatomists), makes a possible link of the name of this organ with the idea of vapor and evaporation[16].

1.2.1. VDJ recombination

Vertebrates have evolved in such a way that with a relatively limited number of nucleotides assigned for the lymphocyte receptors, they can still recognise almost all pathogens. This is due to the possibility of combining different gene segments to create one final product and not by a continuous complete domain. This process of gene

rearrangement happens during the development of each lymphocyte and is common for B and T cells including γδ T cells. For T cells, it happens in the thymus, from where they get their name (thymus-dependent (T) lymphocytes: T cells)[1]. Interestingly, the production of γδ T cells predominates over αβ T cells early in development.

The TCR expressed on γδ T cells contains a pair of chains composed of a gamma and a delta chain (**figure I.4**). Each chain is the result of an assembly of a constant and a variable domain. The variable domain contains three loops that comprise three complementarity determining regions (CDRs) that determine the receptor's antigen/ligand binding site and allow for seemingly limitless diversity in specificity. In the antigen-binding region of a chain, CDR1 and CDR2 are encoded directly in the V (variable) gene segment while CDR3 is encoded by the additional DNA sequence that is created by the assembly of the V and J (joining) gene segments for the gamma chain, and the V, D (diversity), and J gene segments for the delta chain[1].

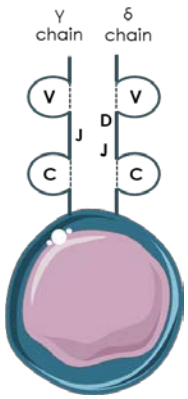


Figure I.4. Representation of a γδ T cell, expressing a γδ TCR containing a gamma and delta chain with their constant domain, C, variable domain, V, joining domain, J and diversity domain, D (D only for the delta chain) (cell form based on[5]).

Most of the diversity resides in the CDR3 loop that is generated by four sources of variability. First, the multiplicity of germline gene segments. There are various V, D and J gene segments available in the original (before rearrangement) DNA loci for both the gamma and delta chain (**figures I.5, I.6**).

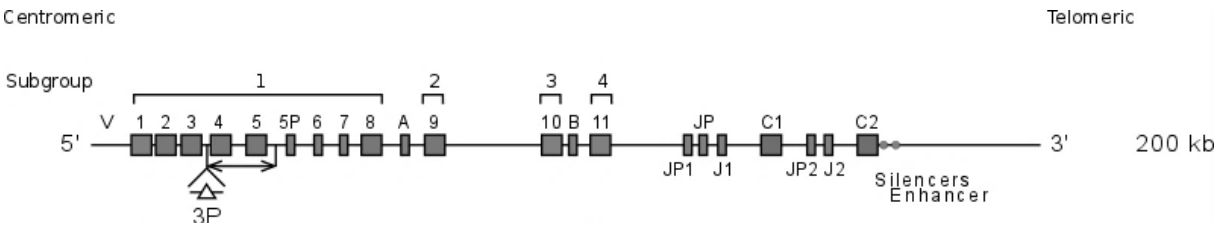


Figure I.5. Human (*Homo sapiens*) TRG locus representation with the V, J and C genes (including functional and pseudogenes) (modified from[17]).

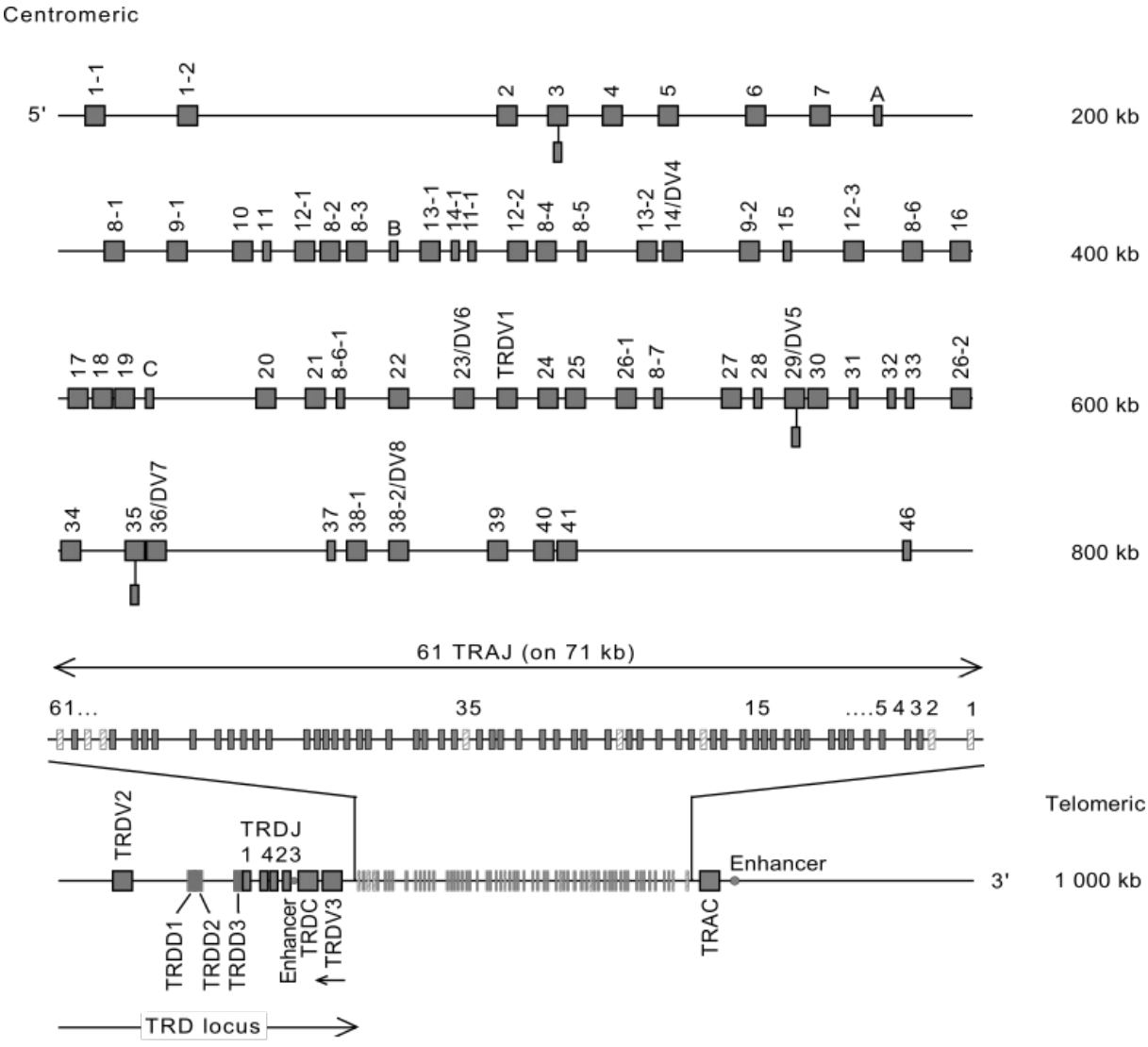


Figure I.6. Human (Homo sapiens) TRA/TRD locus representation with the V (TRDV), J (TRDJ) and C genes (TRDC) (including functional and pseudogenes) (modified from [17]).

Second, the combinatorial diversity due to the fact that single V, D, and J segments are joined to form a variable region. The variation created by different combinations in

the γδ is not as high as in the αβ rearrangements as the available gene segments are more abundant in the latter (**Table I.2**).

	Variable	*Diversity*	*Joining*
TCRα	~43		61
TCRβ	~42	2	13
TCRγ	6		5
TCRδ	7	3	4

Table I2. Number of available functional genes for the V, D and J domains of each TCR chain (from [17]).

The reaction that recombines V, D, and J gene segments involves both lymphocyte-specific (RAG complex) and ubiquitous DNA modifying enzymes. The RAG recombinase complex binds to appropriate gene segments to form a synapse structure and the DNA between the gene segments is lost from the genome. Then, the DNA of each segment forms a hairpin structure which is bound by the Ku protein of the DNA repair complex enzyme DNA-dependent protein kinase (DNA-PK) and are opened by nicking, presumably by Artemis. The final formation of a double-stranded DNA joint between the two segments is completed by some processing and ligation at the opened hairpins[18] (**Figure I.7**).

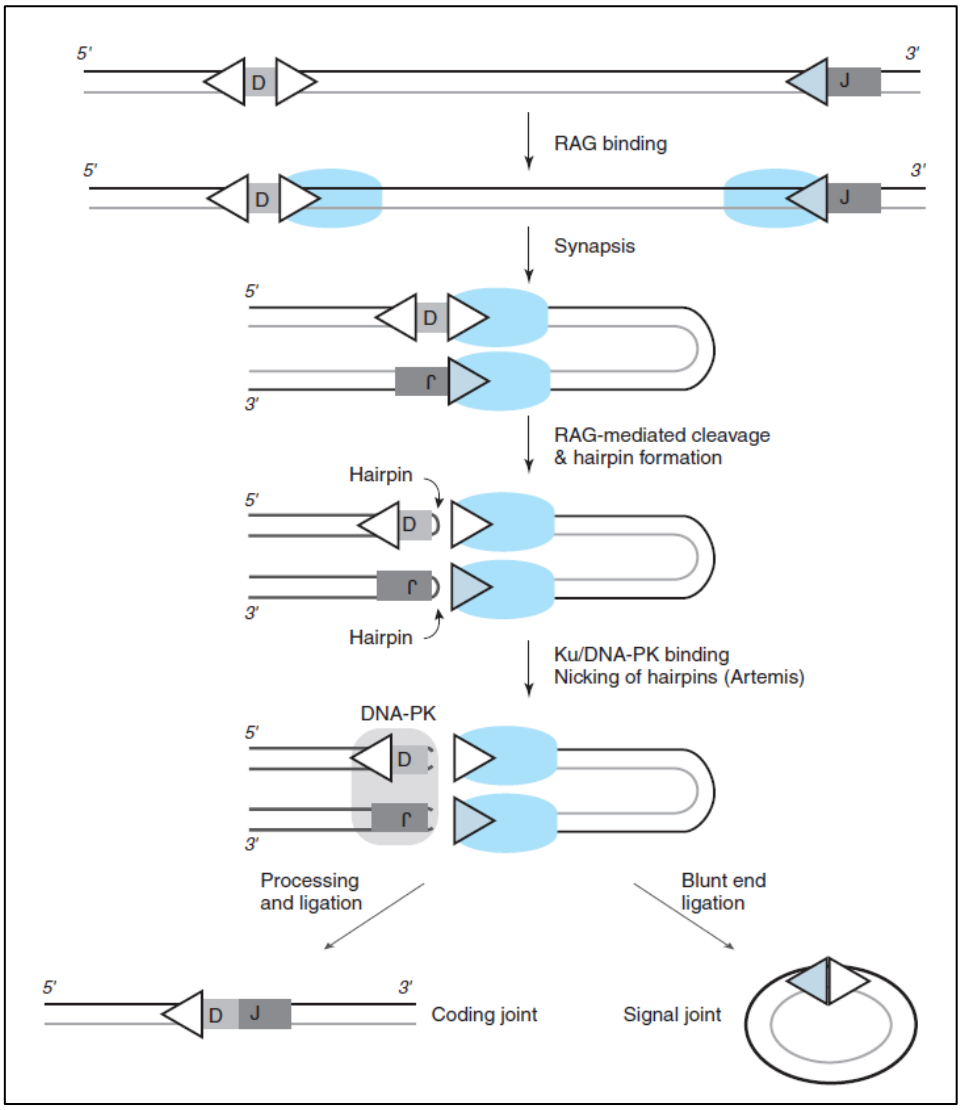


Figure I.7. An Example of a D-J joining during V(D)J Recombination. Recombination signal sequences (triangles) are recognised by the RAG recombinase complex (large blue oval) to initiate the recombination. Continuation in the text (modified from [18]).

The third source of variation stems from the fact that the joining of the V, D and J regions is not always precise. This leads to what is called junctional diversity which is introduced at the joints between the different gene segments as a result of the addition and subtraction of nucleotides by the recombination process (**Figure I.8**). During processing of the coding joint, the exonuclease cuts out nucleotides from the germline sequence causing deletions (**Figure I.8(1)**). This process can lead to a shorter rearranged DNA. In contrast, longer sequences are obtained by addition of nucleotides, either germline or random. If a synapse is cleaved at a site which is several base pairs away from the end of the hairpin structure, nucleotides are left unpaired. These nucleotides at the uneven ends can serve as templates for the incorporation of complementary nucleotides during the final processing of the coding

joint ends, leading to P additions (**Figure I.8(2)**). Finally, the activity of the enzyme terminal dideoxy transferase (TdT) leads to N additions which are non-templated nucleotides added to the ends of nicked hairpins prior to their final ligation, in a random way[18] (**Figure I.8(3)**).

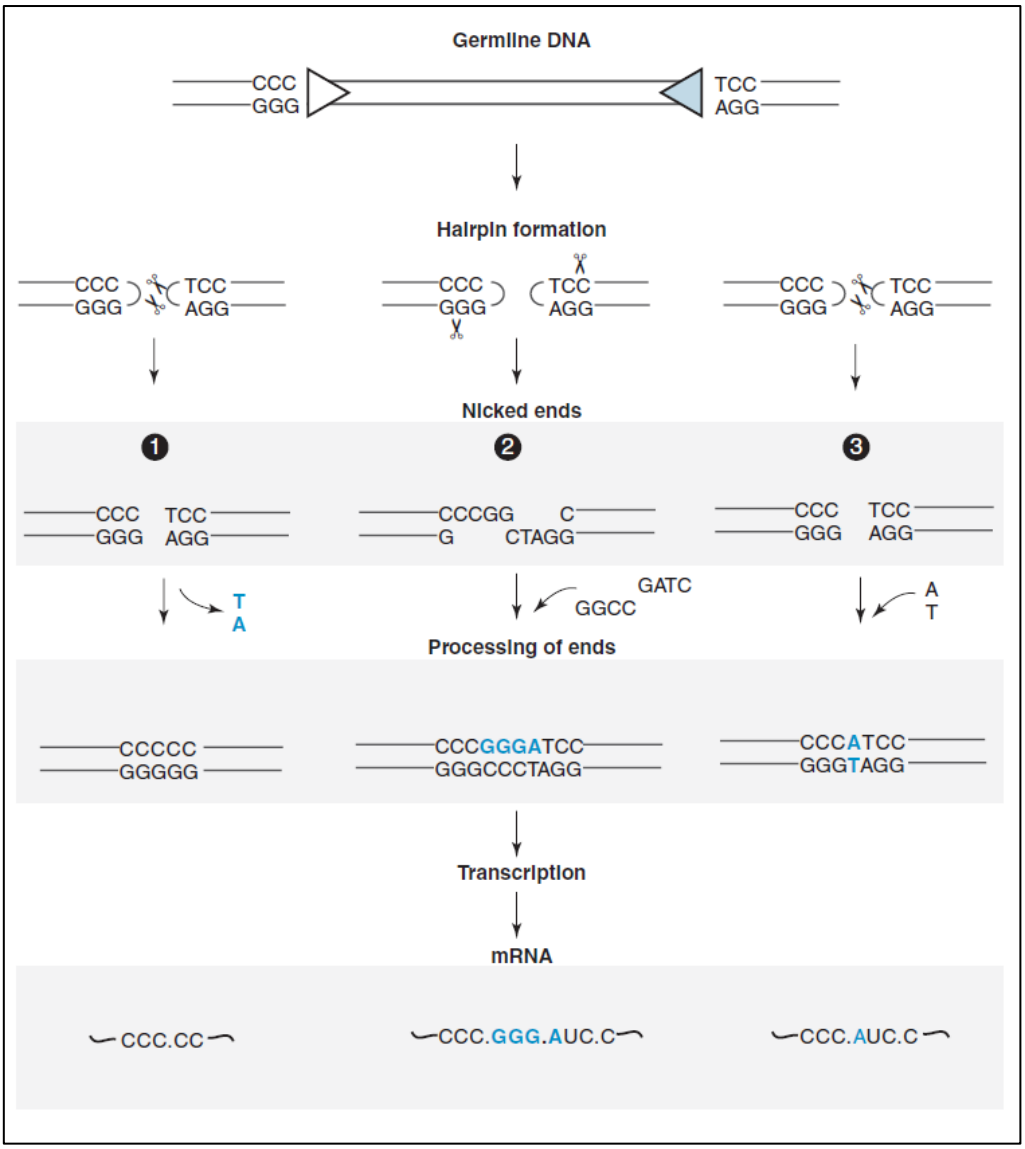


Figure I.8. Examples for the mechanism of deletions (1), P additions (2) and N additions (modified from [18]).

Finally, the overt potential in the pairing of any gamma chain with any delta chain creates diverse TCRs. Even though in theory there would be no limiting factors, there are preferential or enriched pairings. And this is true not only for the gamma and delta pairing but also in the V(D)J choices.

Interestingly, two D elements can participate simultaneously in a V-D recombination while N additions can be included in all three junctional points, the potential number of different delta chains is of the order of 10^{13}. Together with the TRG diversity of $>10^4$ possibilities, the theoretical total γδ TCR diversity is around 10^{17} [19]. Despite this vast potential diversity, parts of the γδ repertoire harbour invariant T cell populations. For example, in foetal blood, the main γδ T cell population is enriched for a particular Vγ9 chain, shared among individuals[20].

For both αβ and γδ T cells, the CDR3 region contains the junctional region providing the highest potential for diversity and is crucial for antigen recognition[1,21]. Of note, the CDR3 region of γδ T cells is often longer than of αβ T cells facilitating a direct interaction with ligands recognised by the γδ TCR, as γδ T cells are not MHC-restricted. Other characteristics of the CDR3, such as hydrophobicity of a specific residue or preferential J usage are linked to the optimal activity of a γδ TCR[21–25].

1.2.2. γδ T cell subsets

This unique process of DNA rearrangement gives rise to diverse TCRs ready to be released in the periphery. The γδ TCR sequence can be regarded as a signature or footprint to what γδ T cells will recognise, where they will reside and what will be their functions. The γδ T cells are commonly divided based on the V delta, and often on the V gamma chain they bear, creating γδ subsets with specific properties. In mouse, the subset distribution is generally based on the gamma chain, but here the focus will be on the human subsets.

In human, a first division is made based on the V delta chain giving rise to the Vδ2+ and Vδ2- cells. The Vδ2 chain paired with the Vγ9 chain, creates the Vγ9Vδ2 T cell subset (nomenclature according to Lefranc & Rabbitts[26] – annex 1, also termed Vγ2Vδ2[27,28]) which is the main γδ population in the adult peripheral blood. Vδ2 pairing with other V gamma chains is often rare in the adult blood but more common in early life or during infection[29–31].

Vγ9Vδ2 T cells appear to be the prototypic innate γδ T cell subset in human expressing a semi-invariant TCR which acts as a pattern-recognition receptor[30,32]. This is especially depicted by a selection for public (shared among individuals) Vγ9 sequences with multiple individuals showing broad overlap in their repertoire. This type

of selection implies the presence of a monomorphic antigen presenting molecule as is the case for Vγ9Vδ2 T cells (described in section 2.1). Selecting and amplifying public T cell receptors may be key in developing an anticipatory TCR repertoire with accelerated kinetics of response to pathogens and beneficial impact to the survival of the host[33].

Vδ2- cells consist mainly of Vδ1 and Vδ3 chains pairing with various V gamma chains. Each of these Vδ2- subsets recognise different antigenic targets, though they are only minimally elucidated[34]. Vδ1 cells are the predominant tissue-associated γδ T cells being most abundant in the gut epithelia, dermis, spleen and liver, in contrast to the blood enriched Vδ2[35]. Vδ1 T cells respond to cellular dysregulation such as in viral infection. They show adaptive characteristics with a diverse and private repertoire (different in each individual) which becomes focused after preferential expansion of reactive clonotypes[36,37] (**Figure I.9**). There is one characteristic exception in the Vδ1 repertoire with the specific public Vγ8Vδ1 TCR enriched during congenital CMV infection[29].

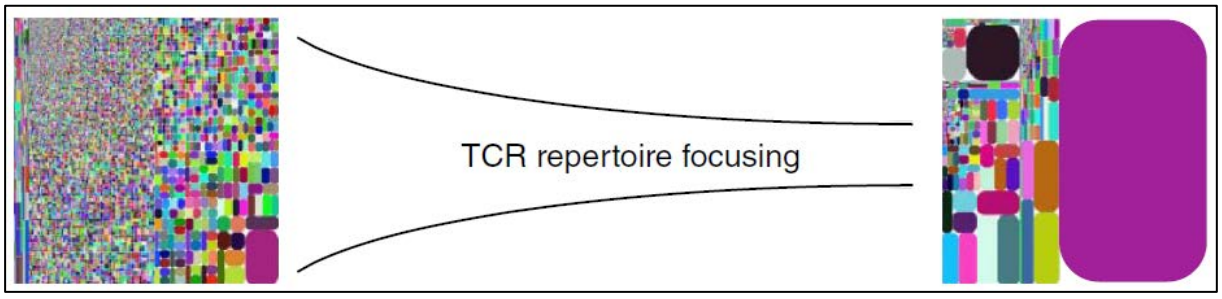


Figure I.9. Tree-maps show CDR3 clonotype usage in relation to repertoire size (rectangle colours are chosen randomly and do not match between plots). Following exposure to a relevant stress stimulus (e.g., viral infection), select clonotypes within the TCR-diverse γδ T naive cell compartment (left panel) go through TCR-dependent activation and subsequent clonal expansion alongside concomitant differentiation into a T effector cell state (right panel) (from [38]).

1.2.3. Development scheme in mouse and human

γδ T cells are remarkably conserved across vertebrate species and they are the first T-cells to develop in all species studied to date[11]. Although γδ T cells arise from the same progenitors as αβ T cells, a big part of the γδ T cells are components of the innate rather than the adaptive immune system.

Even though γδ T cells exist in mouse, there is not a complete parallel with human γδ T cell subsets based on TCR usage, with no obvious homologies between human and mouse V gene sequences[39,40]. For example, the abundant Vγ9Vδ2 T cell population does not exist in rodents, while the well-studied innate-like γδ T cells found in the mouse skin epidermis (Vγ5Vδ1, nomenclature according to Heilig and Tonegawa[41] – annex 2) do not exist in human[27,42]. Nonetheless, studies in mouse models bring insight in the development and function of this enigmatic lymphocyte population.

When the maturation of mouse γδ T cells is complete in the thymus, they have acquired a defined effector function that can be rapidly employed upon activation. After emigration from the thymus, most γδ T cells home to mucosal and epithelial sites in the body and become resident[1]. However, human postnatal γδ thymocytes have been described as functionally immature[43], while recently, we showed that the foetal thymus harbours invariant effector γδ thymocytes[44]. The fate of these invariant foetal γδ populations and their possible tissue homing is not yet established.

Regarding the development of γδ T cells, it has been previously shown in experimental mouse models that it occurs in waves[12]. Early innate γδ T cells express semi-invariant γδ TCRs, while further in development the TCRs possess polyclonal CDR3 sequences. Typical example of invariant TCR is the first foetal wave of γδ T cells in the mouse, the Vγ5Vδ1 subset[41]. These cells have very limited junctional diversity and emigrate from the thymus to the skin epidermis where they become dendritic epidermal T cells and are maintained until adulthood by clonal self-renewal[12,45]. The next wave of mouse γδ T cells is the Vγ6Vδ1 T cells, also of limited TCR diversity and then more diverse γδ T cell populations using Vγ4, Vγ1, and Vγ7 chains arise postnatally[12]. Some of these γδ populations undergo intrathymic and others extrathymic selection events[35].

In human development, the Vγ9 and Vδ2 gene segments are the first γδ chains to undergo rearrangement, detected in foetal liver from as early as 5–6 weeks gestation[46], and in foetal thymus after 8 weeks of gestation[47]. By mid-gestation (<30 weeks), semi-invariant Vγ9Vδ2 T cells dominate the blood γδ repertoire[20]. These semi-invariant Vγ9Vδ2 T cells use a single, identical germline TRG rearrangement of Vγ9 and JγP without any N additions (the amino acid sequence CALWEVQELGKKIKVF). This germline Vγ9-JγP sequence seems to be shared among all individuals until adulthood[30,48,49]. Towards gestation term, Vδ1 T cells seem to take over the blood γδ peripheral repertoire[20]. Interestingly, postnatal thymus has been shown to produce almost only Vδ2- (mainly Vδ1) γδ T cells[43,50,51]. This would suggest that Vγ9Vδ2 T

cells, the main innate γδ population, which has been described as similar between cord and adult blood[30] follows a foetal wave of generation like innate murine γδ T cells. Microbial exposure would lead to postnatal expansion of Vγ9Vδ2 T cells to ultimately take over the peripheral γδ T cell repertoire during childhood. However, the possibility that a postnatal wave of Vγ9Vδ2 T cells contributes to the adult repertoire is not excluded[35] (**Figure I.10**).

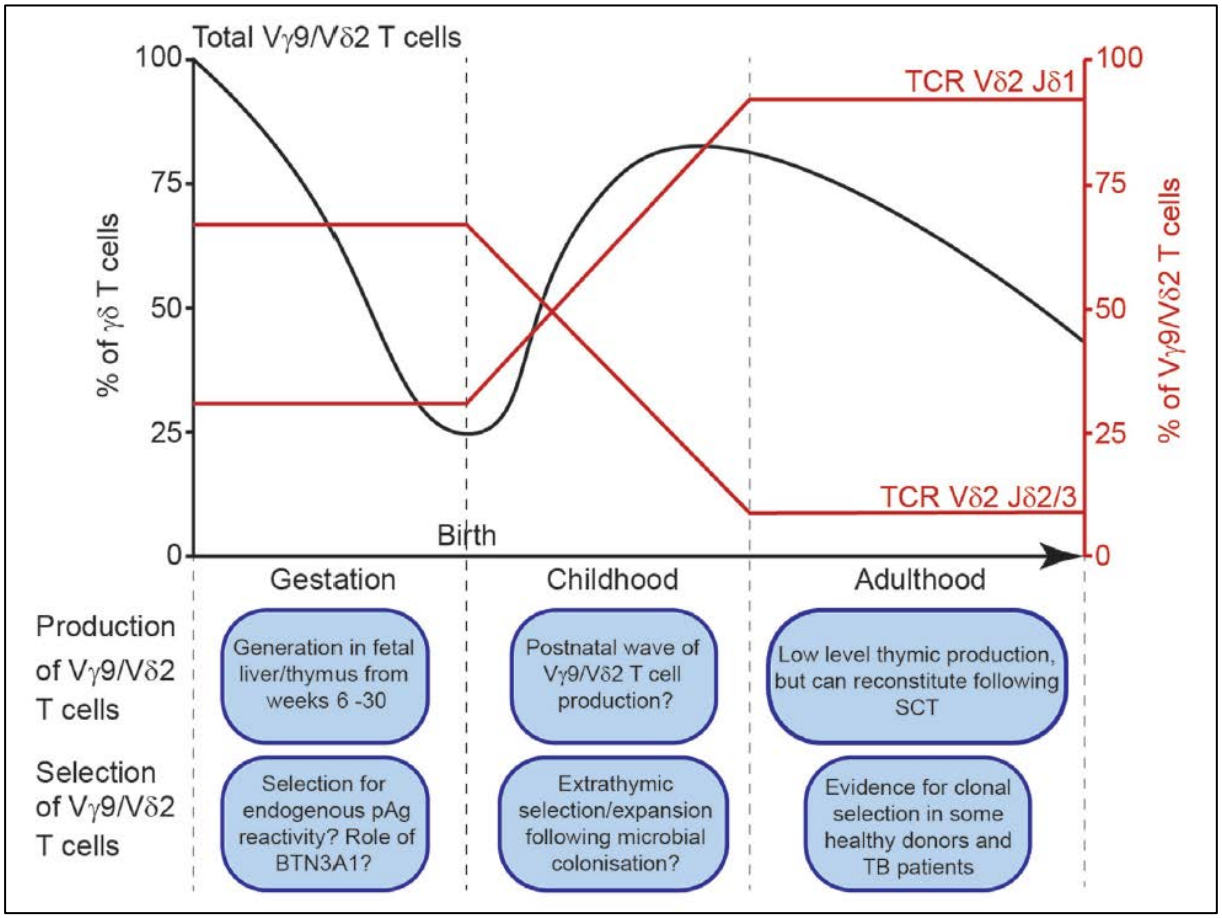


Figure I.10. Schematic depiction of Vγ9Vδ2 T cell generation and selection throughout life. Vγ9Vδ2 T cells as a percentage of total peripheral blood γδ T cells throughout life (black line, left axis). Jδ usage among Vγ9Vδ2 T cells (red lines, right axis) throughout life (from [35]).

Concurrent with the public (shared among individuals) Vγ9 TCR repertoire, the Vδ2 TCR repertoire is rather diverse and private[30], and differences between neonatal and adult Vδ2 repertoires imply selection events throughout life. The TRDJ3 region is more prevalent in cord blood Vδ2 T cells while in the adult the TRDJ1 takes over (**figure I.10**). On the one hand, the Vδ2-Jδ1 (TRDV2-TRDJ1) TCRs may be selected from rare rearrangements in cord blood following microbial exposure. On the other hand, newly

generated Vγ9Vδ2 T cells could arrive from the postnatal thymus[35]. These different hypotheses elaborated regarding the generation and selection of the Vγ9Vδ2 T cells throughout life were addressed in this work where we distinguished the development of foetal and adult Vγ9Vδ2 T cells (section III.3).

2. Vγ9Vδ2 T cells, activation and signalling

The Vγ9Vδ2 T cells are the major γδ T cell population in human peripheral adult and mid-gestation foetal blood[20,50,52]. Specific activation and expansion of Vγ9Vδ2 T cells is observed after infection with a broad range of microbial pathogens and considered crucial partakers in the immune response against diseases like tuberculosis or malaria[52]. But what mediates this response?

2.1. TCR activation by (phospho)antigens

Vγ9Vδ2 T cells are activated in a TCR-dependent manner by low molecular weight nonpeptidic pyrophosphate containing metabolites called phosphoantigens[52,53] (**figure I.11**).

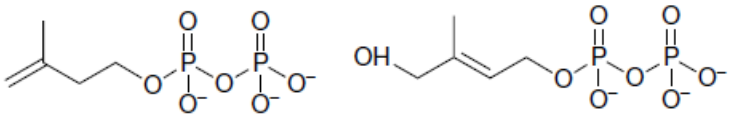


Figure I.11. Phosphoantigens: isopentenyl pyrophosphate, IPP (left), (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, HMB-PP (right).

The phosphoantigens are derived from the isoprenoid metabolic pathway either endogenously, such as the isopentenyl pyrophosphate (IPP), an intermediate of the mevalonate pathway in mammalian cells, or by microbes, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a microbial intermediate of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway[54] (**figure I.12**).

Isoprenoids are key compounds in cellular metabolism and in the synthesis of various products, such as cholesterol, steroid hormones, rubber, ubiquinones, dolichols, and several vitamins (**figure I.12**). The mevalonate pathway is found in Archaebacteria, eukaryotes, and the cytoplasm of plants, while the MEP pathway is found in most Eubacteria, including microbiota[55], apicomplexan protozoa (ex. *Toxoplasma gondii*), and chloroplasts[52].

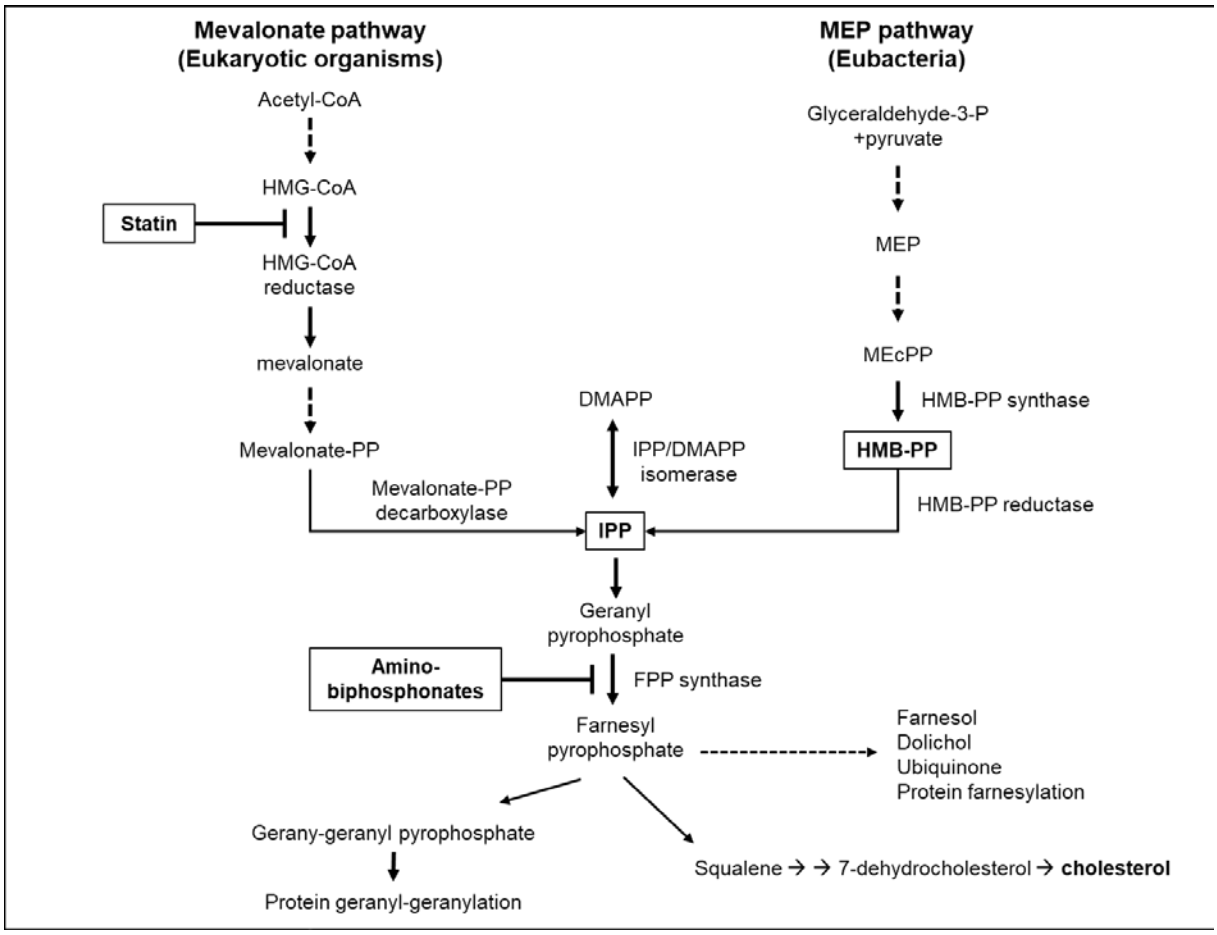


Figure I.12. Isoprenoid biosynthesis in *L. monocytogenes* via the classical mevalonate pathway (left) and the alternative MEP pathway (right) (dashed arrows for omitted intermediate steps) (FPP: farnesyl pyrophosphate synthase) (based on [56]).

IPP was isolated from *Mycobacterium smegmatis* as the first 'natural ligand' for Vγ9Vδ2 T cells[57]. However, the quantity of IPP present in bacteria was not adequate to induce T cell activation[58]. It was a bit later, when HMB-PP was found as a novel metabolite in *Escherichia coli*, that they observed that even traces of HMB-PP were sufficient to elicit Vγ9Vδ2 T cell responses[59]. HMB-PP turned out to be approximately 10000 times more potent in stimulating Vγ9Vδ2 T cells than IPP. HMB-PP, abundant in plenty of microbes, is produced by the *Mycobacterium tuberculosis* and is also present in the commonly administered BCG vaccine (attenuated *Mycobacterium bovis*, see also section 5.2)[57,60,61].

Phosphoantigen recognition by Vγ9Vδ2 T cells drives their broad reactivity toward not only infected cells but also transformed cells. The enhanced production of the weak agonist IPP in transformed cells, resulting from increased cell metabolism and

cholesterol biosynthesis, underlies the tumour cell recognition by Vγ9Vδ2 T cells. Intracellular accumulation of IPP and subsequent tumour cell recognition by Vγ9Vδ2 T cells can be intentionally exacerbated in the presence of aminobisphosphonates such as pamidronate and zoledronate (**Figure I.13**). They are potent inhibitors of the farnesyl pyrophosphate synthase, an IPP downstream enzyme of the mevalonate pathway[62] (**Figure I.12**). Accordingly, pharmacologic inhibitors of the mevalonate pathway that down-regulate IPP production such as statins (**Figure I.12**), decrease the antitumor Vγ9Vδ2 T-cell responses[62].

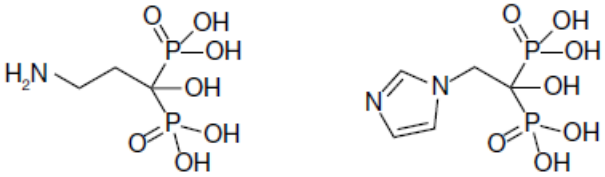


Figure I.13. Aminobisphosphonates: Pamidronate (left), Zoledronate (right). Commonly used as first-line treatment in postmenopausal osteoporosis[63] and to treat bone metastases[64,65].

The recognition of phosphoantigens leads to various anti-microbial and anti-tumoral properties of the Vγ9Vδ2 T cells, increasing their importance in the immune surveillance in various settings. We will further discuss their anti-microbial role and potential implications in immunotherapy in sections 4,5.

2.1.1. Butyrophilins, sensor of phosphoantigens

Key for the recognition of the phosphoantigens by the Vγ9Vδ2 T cells are the ubiquitous butyrophilin 3A1 (BTN3A1)[66,67] and 2A1 (BTN2A1)[68] proteins (**figure I.14**). They are considered to be members of the B7 family of costimulatory receptors and belong to the human immunoglobulin superfamily receptor proteins of the butyrophilin. The BTN3A1 and BTN2A1 genes, just as the Vγ9 and Vδ2 genes, are conserved among a range of vertebrates but are absent in rodents[42,69].

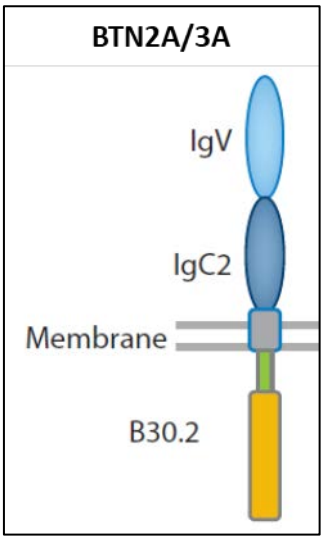


Figure I.14. Protein domain structure of butyrophilins BTN2A/3A. The defining feature of the butyrophilin family is IgV and IgC2 immunoglobulin domains. Both BTN protein families BTN2A and BTN3A have identical structures. BTN2A and BTN3A molecules contain a cytosolic B30.2 domain (modified from [69]).

The mechanism of recognition of phosphoantigens by a Vγ9Vδ2 T cell does not follow a classical antigen presentation in an MHC-restricted manner. Since the identification of BTN3A1 as a required player in phosphoantigen-mediated Vγ9Vδ2 activation, various and contradicting studies have investigated how this recognition is mediated. The current understanding supports the hypothesis of "inside-out signalling": phosphoantigens are sensed by the Vγ9Vδ2 TCR, most probably after the phosphoantigen binds in the intracellular B30.2 domain of the BTN3A1, provoking changes in the conformation of the extracellular domain leading to the activation of the Vγ9Vδ2 T cells[66,70,71]. This comes in contrast to another study reporting direct interaction of the BTN3A1 ectodomain with a phosphoantigen responsive TCR and direct presentation of phosphoantigens by the BTN3A1 immunoglobulin V domain[67], which has been later questioned[70,72]. Based on the first model of recognition, different pathways have been proposed as figured below (**figure I.15**) whilst the complete mechanism remains undefined.

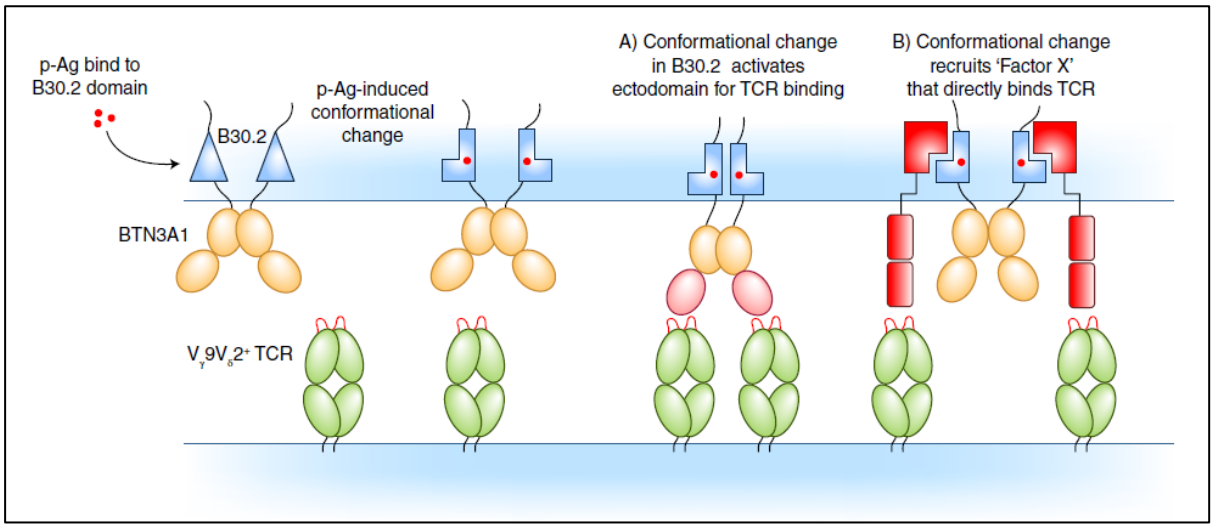


Figure I.15. Potential role of BTN3A1 in the sensing of phosphoantigen by Vγ9Vδ2 T cells. BTN3A1 acts as a sensor of phosphoantigen either A) by inducing a conformational change in the BTN3A1 ectodomain[73], or B) by facilitating the recruitment of a direct ligand of the Vγ9Vδ2TCR ("factor X") (from [38]).

BTN3A1 was necessary but not enough for inducing optimal phosphoantigen responses of Vγ9Vδ2 T cells[74]. It was found that BTN3A1 functions as a pairing, with its subcellular trafficking and optimal activity regulated by BTN3A2[75]. Very recently, Rigau and colleagues demonstrated the involvement of the BTN2A1 molecule in the activation of the Vγ9Vδ2 T cells by phosphoantigens[68]. BTN2A1 acts as a ligand that binds to the Vγ9 chain of the TCR (**figure I.16**). BTN2A1 associates with BTN3A1, which act in tandem to instigate responses to phosphoantigens. Binding of a second ligand, possibly BTN3A1, to the Vδ2 chain is also required[68].

Even though the activation of Vγ9Vδ2 T cells is TCR mediated, their activators, phosphoantigens do not bind directly to the TCR but to the endogenous butyrophilins. The recognition mediated by an endogenous molecule would allow for invariant TCR repertoire. Indeed, some features of the CDR3 repertoire indicate limited diversity (gamma chain) or specific requirements for the pairing of Vγ9 to Vδ2 chain and position-related hydrophobicity (delta chain)[21–25]. Detailed analysis of the CDR3 repertoire of both gamma and delta chain featured in this work.

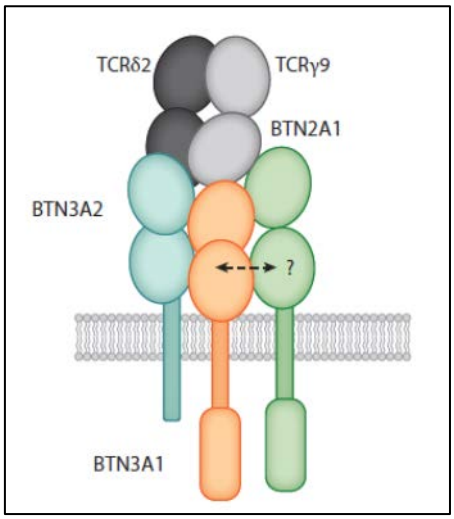


Figure I.16. Mode of reactivity of Vγ9Vδ2 TCR. Hypothetical simultaneous engagement of BTN3A1/BTN3A2/BTN2A1 by phosphoantigen-reactive Vγ9Vδ2 TCR. The dashed arrow and question mark indicate potential interactions between the BTN3 complex and BTN2A1 (from [74]).

As the activation of γδ T cells does not require antigen processing or MHC molecules but relies on cell-cell contact with antigen presenting cells (APCs)[76,77], stimulated γδ T cells themselves seem to serve as APCs. However, the "self" presentation is not as effective as the optimal stimulation by monocytes or tumour cells[77] indicating that TCR recognition of phosphoantigens requires antigen presentation molecules on APCs and/or co-activation molecules.

2.2. Co-stimulation and signalling

Apart from the recognition of phosphoantigens through interaction with the BTN molecules, additional signalling pathways have been shown to guide the Vγ9Vδ2 T cell activity.

Previous studies have reported bystander activation of Vγ9Vδ2 T cells through natural killer group 2D (NKG2D) receptor with no involvement of the TCR[78,79]. NKG2D is an activating receptor, traditionally expressed on NK cells which bind to MIC and ULBP proteins and initiate cytotoxicity (e.g. stress-induced molecule MICA)[80]. In a similar manner to NK cells, NKG2D-expressing Vγ9Vδ2 T cells released cytotoxic mediators and produced tumour necrosis factor-α (TNFα) after NKG2D triggering[78]. Interestingly, NKG2D related upregulation of activation markers (CD69 and CD25) was observed in

NK cells and Vγ9Vδ2 T cells but not in CD8 T cells. Another study examined the NKG2D role in the lysis of tumour cells with two cases of TCR-independent lysis of carcinoma and melanoma by Vγ9Vδ2 T cells[79]. However, NKG2D MICA interaction alone failed to mediate Vγ9Vδ2 cytolytic function in a different study[81].

An additive effect of TCR and NKG2D signalling pathways in the activation of cytotoxic effector function by Vγ9Vδ2 T cells has been suggested by Wrobel and colleagues[79]. This was in line with other studies showing enhancement of cytolytic functions of Vγ9Vδ2 T cells with the NKG2D acting as a costimulatory receptor, accessory to the TCR[81,82]. Indeed, in other studies, NKG2D blockade was linked to reduced capacity of Vγ9Vδ2 T cells to target cancer cells with the involvement of binding proteins ULBP1 or ULBP4[83,84]. Overall, apart from TCR-dependent sensing of intracellular phosphoantigen accumulation, NKG2D expressed on the cell surface of most Vγ9Vδ2 T cells helps in the discrimination between tumour and healthy cells by Vγ9Vδ2 T cells[85].

NKG2D is not the only costimulatory molecule in the activation of Vγ9Vδ2 T cells. Others include the typical immunoglobulin superfamily co-stimulatory receptors (e.g. CD28) and tumour necrosis factor receptor (CD27). CD27 is a protein constitutively expressed on naive T cells that binds CD70 on dendritic cells and delivers a potent co-stimulatory signal to T cells early in the activation process. Both co-stimulatory receptors CD27 and CD28 are expressed on naïve T cells and receive signals additional to those received through the TCR, to lead to the full activation of the lymphocyte. This dual requirement for stimulation helps prevent naive T cells from responding to self-antigens on tissue cells, which lack co-stimulatory activity. Once a T cell has differentiated into an effector cell, encounter with its specific antigen results in immune attack without the need for co-stimulation[1]. Interestingly, it has been shown that Vγ9Vδ2 T cell survival and proliferation upon activation are determined by independent and nonredundant CD27 and CD28 costimulatory signals[86].

Expansion and differentiation of naive T cells involve not only co-stimulatory signals that promote the survival and expansion of the T cells, but also cytokines that direct T-cell differentiation into one of the different subsets of effector T cells. Of note, CD28 constitutively expressed on lymphoid γδ T cells, promotes survival and proliferation via IL-2 production. IL-2, together with other interleukins, are key determinants of T-cell survival, proliferation, and differentiation, which is essential for lymphocyte development and homeostasis[87]. IL-2 and IL-15 play key roles in the peripheral

expansion of Vγ9Vδ2 T cells in response to microbial phosphoantigens or synthetic drugs like bisphosphonates[88,89]. That is why, combination of these cytokines with TCR agonists provide optimal effector responses in the context of therapeutic applications of Vγ9Vδ2 T cells[90] (more in section 4).

Important in the regulation of proliferation, cytokine release and cytotoxicity of γδ T cells is also the surface expression of the NKG2A receptor. This inhibitory CD94-associated receptor is translocated to the Vγ9Vδ2 cell surface upon activation by phosphoantigens[91,92].

3. Effector functions of the Vγ9Vδ2 T cells

Once the Vγ9Vδ2 T cells get activated, they are set to perform various effector functions. These functions include induction of cell death of infected or transformed cells and release of immunomodulatory cytokines resembling a CD8 and Th1 immune response but with the advantage of immediate defence[27].

3.1. Cytotoxicity – the granule exocytosis pathway

Once the effector Vγ9Vδ2 T cells, just like other cytolytic cells such as CD8 αβ T cells and NK cells, recognise a microbially infected or tumoral cell, they employ molecules able to induce apoptosis at the target cell in a serial-killer manner. These molecules are cytotoxic proteins such as granzymes (e.g. A and B) and pore-forming proteins (e.g. perforin) and are stored in the cytosol of the killer lymphocyte in "granules". The granules are released by the killer lymphocyte in order to kill a target cell via the granule exocytosis pathway (**figure I.17**). This process can be initiated within minutes after the target recognition and does not cause any surrounding inflammation or bystander death[93,94].

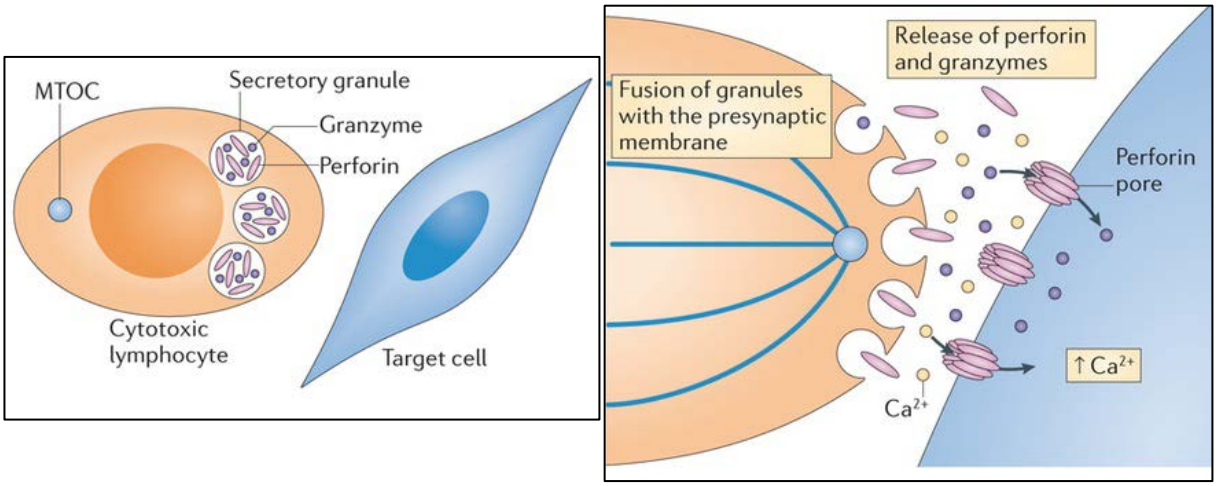


Figure I.17. Interaction of a cytotoxic lymphocyte with a target cell. An immunological synapse is formed once the cytotoxic lymphocyte recognizes its target cell (left panel). The secretory granules containing the cytotoxic mediators traffic towards the presynaptic membrane before they fuse with the presynaptic membrane to release perforin and granzymes into the synaptic cleft (right panel). At the postsynaptic membrane, perforin forms large transmembrane pores that enable the delivery of granzymes into the cytosol of the target cell. Once the granzymes initiate apoptosis of

the target cell, the cytotoxic lymphocyte detaches from the dying cell being ready to interact with another target cell to carry out serial killing (from [94]).

Granzyme B is a serine protease cleaving after aspartic acid residues. It induces target cell apoptosis by activating the caspase. Human granzyme B, but not the mouse enzyme, also activates cell death by directly cleaving the key caspase substrates to activate the same mitochondrial and DNA damage pathways as the caspases. Granzyme B together with perforin are the most potent and well-known killing proteins. In the absence of granzyme B, other granzymes which have similar or different regular roles will take over. Perforin has a very important role in this death inducing process and its impaired delivery or function leads to a range of human immune-mediated disorders called perforinopathies[94].

Granzyme A is a tryptase that induces caspase independent cell death. While perforin is mostly used by all granzymes in order to access the target cell, Vγ9Vδ2 T cells were able to inhibit the growth of intracellular mycobacteria with the help of granzyme A via a perforin-independent mechanism[95]. In addition, granzyme A has been shown to possess alternative properties, other than direct killing of target cells. There have been described extracellular activities of granzyme A including proinflammatory effect, degrading extracellular matrix proteins, including heparin sulphate proteoglycans, collagen type IV, and fibronectin, as well as mediating the release of growth factors (reviewed in [96]).

Granulysin is a small, saposin-like, antibacterial toxin which is expressed in cytotoxic cells of most mammals but not rodents. The delivery of granulysin into infected cells can be perforin-dependent like granzymes. Pathogenic bacteria that escape from the endolysosomal compartment of the dying target cells are killed after infusion of granulysin through the perforin pores[94]. However, granulysin also directly kills intracellular bacteria, such as *Mycobacterium tuberculosis*, by rupturing their membranes. It acts as a pore-forming protein like perforin but with a cholesterol sensitivity enabling a more efficient microbial membrane permeabilization compared to mammalian (cholesterol expressing) membrane[97].

3.2. Cytokine production

Coordination of host immune responses plays a key role in host protection. Vγ9Vδ2 T cells are known to produce immunomodulatory cytokines that are involved in protective immunity against viruses and intracellular pathogens like TNFα and IFNγ[98].

IFNγ is produced by γδ T cells and other innate-like lymphocytes such as group 1 innate lymphoid cells (ILC1s) as well as adaptive lymphocytes, including TH1 cells and CTLs, in response to cytokine and antigen stimulation. IFNγ acts on its receptor to induce quick and ephemeral Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling and interferon-stimulated gene induction. Progressively, the cellular IFNγ response affects the expression and function of numerous enzymes and regulators of metabolism, chromatin and transcription to induce a reprogrammed cellular landscape. IFNγ has general effects on various cells such as increased antigen presentation, antimicrobial responses and chemokine production as well as decreased proliferation. It exerts homeostatic and protective roles (**table I.3**) as well as pathological effects in case of high levels[99].

Homeostatic and protective roles of IFNγ
Promotes host defence
Vascular remodelling
Decreased tissue damage
Decreased fibrosis
Social behaviour
Tumour immunosurveillance

Table I.3. Homeostatic and protective roles of IFNγ (from [99]).

IFNγ can directly block viral replication and promote the expression of proteins involved in peptide loading in infected cells. The latter process facilitates the recognition of infected cells as targets by cytotoxic CD8 αβ T cells. Moreover, IFNγ initiates macrophages to sites of infection, where they act as effectors as well as antigen presenting cells. IFNγ can synergize with TNFα in macrophage activation and can kill some target cells through their interaction with TNF-receptors (II and I respectively),

which can induce apoptosis[1]. In general, TNFα is a pleiotropic cytokine that displays both homeostatic (**table I.4**) and pathogenic effects[100].

Homeostatic functions of TNFα
Defence against pathogens
Organogenesis and development: lymphoid organ architecture
Tissue regeneration: neuronal remyelination; cardiac remodelling; cartilage regeneration
Immunoregulation: tolerization of macrophages, apoptosis of inflammatory cells
Inhibition of tumorigenesis: necrosis, senescence (cytostatic effect)

Table I.4. Homeostatic functions of TNFα (From [100]).

IFNγ produced by phosphoantigen-stimulated Vγ9Vδ2 T cells promotes the development of TH1 response by αβ T cells[101]. Furthermore, Vγ9Vδ2 T cells induce rapid differentiation of monocytes into inflammatory dendritic cells (DCs), which elicits, under the influence of further microbial stimuli, polarised development of T cells expressing IFNγ or Il-17[102]. Vγ9Vδ2 T cells seem to tackle a dual role in the early microbial response by killing infected monocytes promptly[103] while promoting the survival of non-infected monocytes and their differentiation into inflammatory DCs[104].

3.3. Regulation of the immune response

The cytotoxic and immunomodulatory functions described above are either programmed or initiated after antigen contact following a series of controlling events. Main orchestrators of these events are the transcription factors which control the development and differentiation of immune cell lineages by activating or repressing genes crucial to cellular identity, proliferation, migration and effector functions. A main subset of transcription factors influencing the IFNγ and granzyme expression are the T-box transcription factors: T-bet and eomesodermin (Eomes)[105].

T-bet is a TH1-specific transcription factor, inducing IFNγ production and promoting CD4 TH1 development through binding to the promoter of the gene for this cytokine[106]. T-bet, as a lineage-specifying transcription factor, can also bind to the promoter region

of other genes related to TH1 cell activity and at the same time block TH2 and TH17 responses[107] (**figure I.18**).

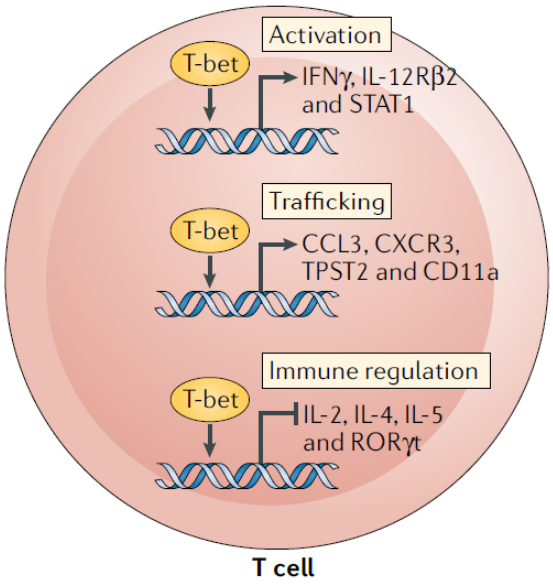


Figure I.18. The diverse target genes of T-bet. After T-bet is induced, it binds to genes that exert diverse functions such as activation (via IFNγ, IL12Rβ2 and STAT1), cellular trafficking (via CC-chemokine ligand 3 (CCL3), CXC-chemokine receptor 3 (CXCR3), tyrosyl protein sulfotransferase 2 (TPST2) and CD11a) and immune regulation (via IL-2, IL-4, IL-5 and RORγt) (from [107]).

The highly homologous transcription factor Eomes is important for full effector differentiation of CD8+ T cells including IFNγ, perforin and granzyme B expression[108]. These two T-box transcription factors, T-bet and Eomes, are considered as complementary in governing cellular immunity and follow different mechanisms of induction, unique functions and possibly antagonistic activities, in case of competition for DNA-binding sites or transcriptional modifiers[107]. Besides, multiple transcription factors act in a combinatorial manner to promote the acquisition of effector function, for example granzymes A and K and perforin (genes GZMA, GZMK, Prf1) are regulated by both Blimp-1 and T-bet in a partially redundant manner in cytotoxic CD8 αβ T cells[109].

It has been previously shown that the transcription factor T-bet, not expressed in naive γδ T cells, is upregulated upon TCR signalling and is directly correlated with IFNγ secretion in splenic mouse γδ T cells. Genetic depletion of T-bet leads to significant impairment of TH1 differentiation, with 50% reduction in the percentage of IFNγ-

positive cells, associated with higher IL-4 production, suggesting that T-bet promotes IFNγ and inhibits IL-4 expression in γδ T cells[110]. In human peripheral blood γδ T cells, both T-bet and Eomes are expressed at 60% and 40% respectively, indicating a role of these transcription factors in human γδ T cell function[111].

4. Vγ9Vδ2 T cells in cancer immunotherapy

Cancer is a state where cells break the rules governing multicellularity including controls that govern cell division, cellular longevity and localisation. It represents a spectrum of conditions rather than a single disease that is mainly defined by the tissue of origin. Cancer arises from a series of genetic mutations that progressively lead to a transformed state[2]. Even though, therapeutic strategies such as surgery, chemotherapy and irradiation are widely used, cancer remains one of the leading causes of death worldwide. An estimated 14.1 million people are expected to develop cancer annually based on the World Cancer report of 2014[112] with a predicted increase to 22.2 million by 2030[113]. The fact that immune surveillance can operate against immunogenic tumours has led to the development of immunotherapy strategies making use of the patient's own immune system in the fight against cancer, as a second line treatment[2].

Cancer immunotherapy approaches include passive immunotherapy with monoclonal antibodies targeting antigens elevated on tumour cells (ex. HER2), monoclonal antibodies targeted against negative regulators of T-cell activation (e.g. PD-1), cancer vaccines (for virus-induced cancers), adoptive transfer and other. Adoptive transfer refers to either of *ex vivo* expanded T, NK or dendritic cells or of *ex vivo* generated chimeric antigen receptor (CAR) T cells[2]. Adoptive immunotherapy is gaining momentum to fight malignancies with γδ T cells receiving more and more attention as an alternative cell source as to natural killer cells and αβ T cells[114].

In general, γδ T cells do not rely on the recognition of classic MHC molecules and sensing of infection or cancer depends on more ubiquitous changes observed across many individuals. Therefore, γδ T cell immune systems can be transferred more easily between individuals[27]. Tumour-infiltrating γδ T cells have been demonstrated to be the most favourable prognostic immune population among many cancer types[115]. The antitumor function of γδ T cells is most generally associated with their production of both IFNγ and TNFα, and/or with their cytotoxic potential. Different functional subsets of γδ T cells might have different functions within tumours, and even the same subset can undergo different functional polarizations. For example, Vδ1 and Vγ9Vδ2 T cells express distinct chemokine receptors and cell adhesion molecules implying different homing mechanisms in tumours that can be specifically targeted for

immunotherapy[116,117]. Indeed, in patients with colorectal cancer, the 5-year disease-free survival probability was significantly higher when the number of tumour-infiltrating Vγ9Vδ2 T cells was high[118], while Vδ1 γδ T cells showed pro-tumour activity via IL-17 production[119].

4.1. Strategies for therapeutic manipulation of Vγ9Vδ2 T cells

Vγ9Vδ2 T cells have the capacity to recognize cancer cells due to an increased intracellular abundance of IPP within tumour cells generated by overstimulation of the mevalonate pathway[57,62] as discussed in section 2.1. Clinical studies have been exploring the *in vitro* expansion or the adoptive transfer of Vγ9Vδ2 T cells as an immune-therapeutic strategy in the context of cancer (**Figure I.19**). Furthermore, γδ T cells can be redirected to kill cancer cells using a CAR made from an antibody that targets a tumour-specific molecule at the cancer cell surface[120,121] (**Figure I.19)**. In recent clinical strategies, chemical compounds have been used to enhance or mimic the expression of phosphoantigens to stimulate Vγ9Vδ2 T cells directly. Some strategies have used aminobisphosphonates or synthetic phosphoantigen analogues such as bromohydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), which enhance the expression of endogenous phosphoantigens both on the side of antigen presenting cells, such as monocytes, and on the tumour side[122] (see also section 2.1).

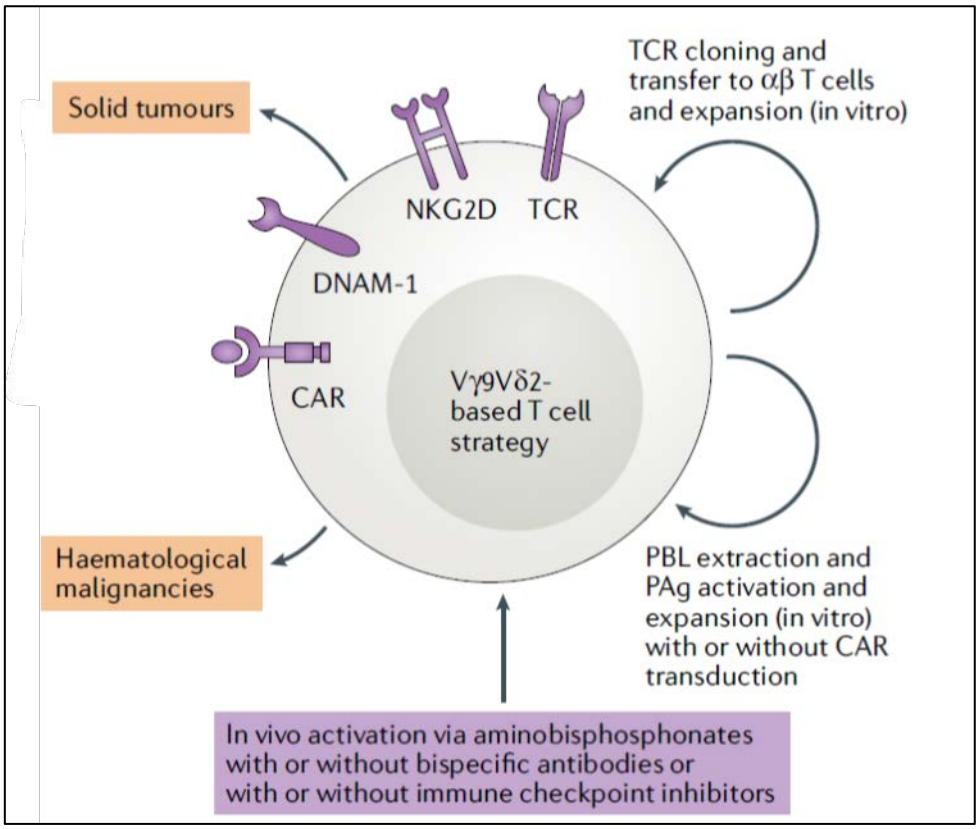


Figure I.19. Current strategies for therapeutic manipulation of human Vδ2 γδ T cells: peripheral blood extraction and *in vitro* activation with phosphoantigens; generation of T cells engineered with defined γδ TCRs (TEGs), which consists of the cloning and transfer of Vγ9Vδ2 TCRs into αβ T cells (PBL, peripheral blood lymphocyte) (from [123]).

Administration of Vγ9Vδ2 T cells at suitable intervals after chemotherapy and zoledronate treatment increased the cytotoxic function and IFN-γ production by γδ T cells followed by a complete lysis of tumour cells in different malignancies[124]. Different studies also reported the activation of Vγ9Vδ2 T cells after phosphoantigen or aminobiphosphonate injection, with improvement of the cytotoxicity of γδ T cells particularly in presence of tumour-targeting antibody[125]. In melanoma, tumour-infiltrating Vδ2 T cell lines showed cytotoxic capability which was improved by pre-treatment of tumour target cells with zoledronate, while higher levels of Vδ2 T cells correlated with early stage of development of melanoma and absence of metastasis[126]. In the context of chronic myelogenous leukaemia (CML), the activation of Vγ9Vδ2 T cells by zoledronate enhanced anti-leukaemia activities in a NKG2D-dependent manner, notably in CML-resistant patients[127,128].

4.2. Obstacles and how to overcome them

It's been a few years since the first γδ-focused clinical trials were conducted with often unexpected results. Various clinical trials have made attempts of *in vivo* stimulation of autologous Vγ9Vδ2 T cells in patients by using aminobisphosphonates, and even though there was no severe toxicity, there was no significant antitumoral activity either (reviewed in [122]). Lack of efficacy was also reported in clinical trials that explored the transfer of *ex vivo*-expanded autologous or allogeneic Vγ9Vδ2 T cells, alone or in combination with stimulating agents. Thus, the activation or transfer of polyclonal γδ T cells outside the context of allogeneic stem cell transplantation for the control of tumours has been rather disappointing (reviewed in [122]).

The unsatisfactory outcome of different trials applying γδ T cell-targeted strategies is under investigation. On the one hand, an important heterogeneity in the effector repertoire of Vγ9Vδ2 T cells with part of clones not responding to cancer cells could explain the failure observed in clinical trials[122]. On the other hand, induced anergy and exhaustion has been reported with the tumour cells seemingly winning the immunosuppressive battle (**Figure I.20**).

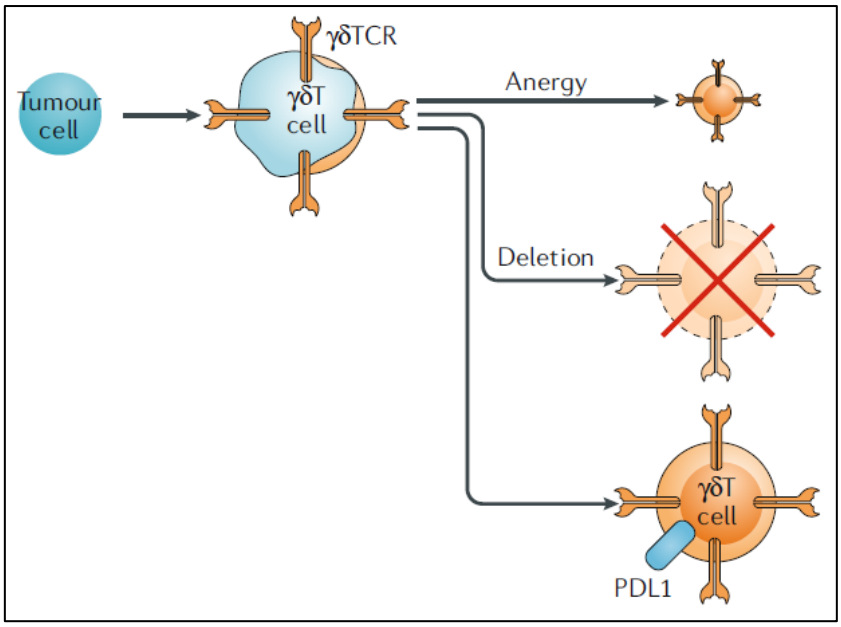


Figure I.20. Potential causes for failure of clinical trials utilising natural γδ T cells. Tumour- driven mechanisms of tolerance operating in γδ T cells from cancer patients. Tolerance can be driven by avoiding the activity of tumour-reactive γδ T cells through the induction of anergy or deletion of tumour-reactive γδ T cells, most likely mediated by expression of the immunosuppressive molecule programmed cell death ligand 1

(PDL1) by tumour cells. The arrows indicate these different functional fates of healthy γδ T cells after tumour engagement (modified from [122]).

Other speculations include the potential insufficient phosphoantigen levels leading to suboptimal recognition of *ex vivo* tumour cells resulting to limited antitumor efficacy of adoptively transferred Vδ2 T cells and active immunotherapy trials using Vδ2 agonists[128].

Regardless of the hurdles met until now, the natural contribution in tumour immunosurveillance and the effector functions of Vγ9Vδ2 T cells hold significant advantages that have to be better exploited alone or in combination with other therapies, as in current clinical trials targeting different types of cancer[122] (**table I.5**).

Current clinical trials, Diseases (non-exhaustive list)
Bladder cancer
Breast cancer
Bone metastases
Neuroblastoma
Liver cancer
Lung cancer
Pancreatic cancer
Kidney cancer
Leukaemia

Table I.5. Diseases for which clinical trials utilising γδ T cells are ongoing (modified from [122]).

In an effort to bring insight and solve the problems encountered, different approaches could be followed. First, monitoring the γδ TCR repertoires at the molecular level. Presuming that tumour-reactive γδ T cells become activated and proliferate when their γδ TCR recognizes specific signals emitted by transformed cells, repertoire analysis will be instructive for the identification of novel tumour-reactive γδ TCR variants. In addition, elucidating the exact mechanism of interaction between the antigen and the TCR receptor will facilitate the optimisation of the recognition. It is also crucial to understand the impact that different CDR3 regions of the Vγ9Vδ2 TCR have on the

functional consequences regarding their ligands and epigenetic regulation, including the regulatory mechanism for each individual subset. Characterisation of human Vγ9Vδ2 T cell subsets and the engaged mechanisms in individual cancers, especially the stage of differentiation, the activation status, and the immune checkpoint-ligand expression could also be instructive[122,128]. Finally, deeper understanding of the γδ T cell generation and the stages of TCR development could guide the design of cancer immunotherapy-related studies. All the knowledge on the work mode of tumour reactive Vγ9Vδ2 T cells and their repertoire shaping could serve to irreversibly convert them towards an antitumor function for efficient immunotherapy.

5. Immune response of Vγ9Vδ2 T cells to *Mycobacterium*

The phosphoantigen-reactive Vγ9Vδ2 T cells have showed expansion following various infections in humans including bacterial, like *salmonella* infection[129] and infection by *Francisella tularensi*[130], and protozoan, like *Plasmodium falciparum*[131] and *Toxoplasma gondii*[132] infections. Proliferation and effector function of this subset is studied in order to assess and improve the immune response during infection or for prevention (vaccination).

One of the most studied bacterial infections in the context of γδ T cell response is the one caused by the *Mycobacterium tuberculosis* (Mtb). Particularly, infection with Mtb leads to proliferation and accumulation of phosphoantigen reactive Vγ9Vδ2 T cells at mucosal epithelial tissues such as the lungs[133–135].

5.1. Mycobacterium tuberculosis

Mycobacterium tuberculosis is the causing agent of tuberculosis (TB) which is a communicable disease and a major cause of ill health[136]. Although it has affected humans for thousands of years, its cause, the bacillus Mtb, was discovered by Dr Robert Koch only in 1882. It is spread when people who are sick with TB expel bacteria into the air, for example, by coughing. It typically affects the lungs (pulmonary TB) but can also affect other sites (extrapulmonary TB). According to the 2019 report of the World Health Organisation (WHO), about a quarter of the world's population is infected with M. tuberculosis while around 5-10% of them will develop TB disease during their lifetime. The probability of developing TB disease is much higher among people living with HIV; it is also higher among people affected by risk factors such as undernutrition, diabetes, smoking and alcohol consumption[136].

TB is the leading cause of death from a single infectious agent (ranking above HIV/AIDS) and one of the top ten causes of death worldwide. Without treatment, the mortality rate from TB is high. With a timely diagnosis and treatment with first-line antibiotics for 6 months, most people who develop TB can be cured and onward transmission of infection can be restricted. The currently recommended treatment for

cases of drug-susceptible TB disease is a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide[136].

Infection begins when Mtb enters the lungs via inhalation and reaches the resident alveolar macrophages (**Figure I.21.a**). If they do not succeed in eliminating the bacteria, Mtb invades the lung interstitial tissue, either by the bacteria directly infecting the alveolar epithelium or the infected alveolar macrophages migrating to the lung parenchyma. Then, inflammatory monocytes or dendritic cells carry Mtb to pulmonary lymph nodes for T cell priming. The subsequent recruitment of immune cells (such as T and B cells) to the lung parenchyma leads to the formation of granulomas. This phase is called latent infection (**Figure I.21.a**). Within the granulomas, bacteria replicate and may disseminate to other organs (e.g. brain). The bacteria can now enter the blood circulation or re-enter the respiratory tract to be released making the infected host infectious and symptomatic. This phase is the active TB disease (**Figure I.21.b**).

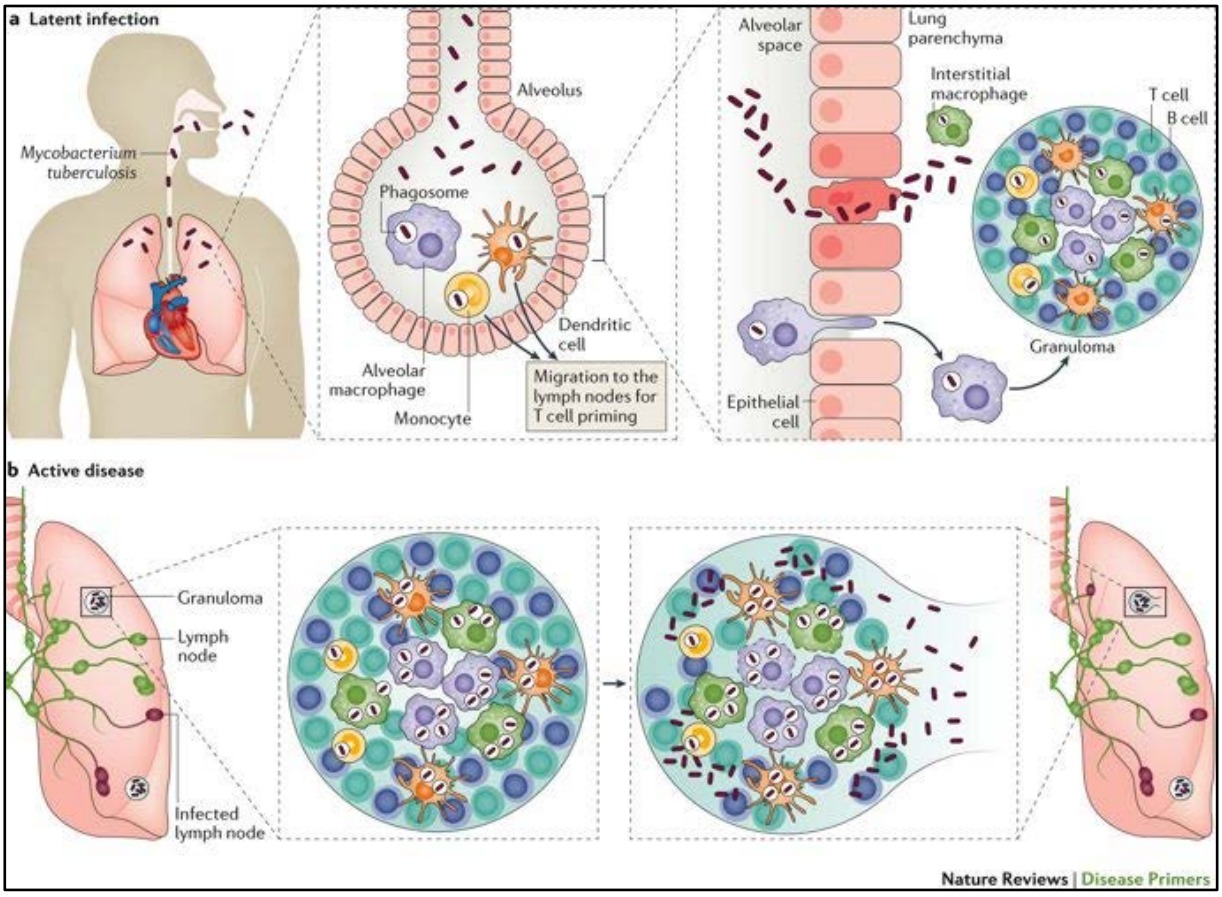


Figure I.21. Mycobacterium tuberculosis infection. a. Latent infection. a. Active disease (more explanation in text above) (from [137]).

Mtb is an intracellular pathogen for which Th1 cells and macrophages are major players of the immune response[2] (**Figure I.22**). IL-12– and IL-23–dependent signalling pathways play critical roles both individually and cooperatively in human antimycobacterial immunity via the induction of IFNγ[138].The importance of studying the IL-12 family of cytokines was heightened by the discovery of IL-12p40 deficiencies that predispose the host to tuberculosis[139].

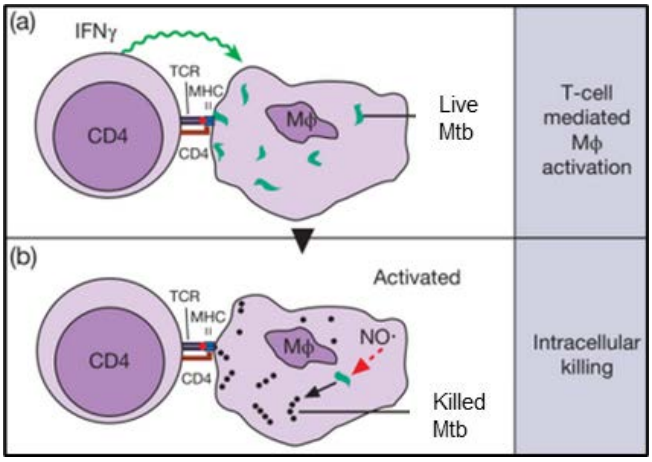


Figure I.22. The « cytokine connection » : nonspecific macrophage killing of intracellular bacteria triggered by a specific T-cell-mediated immunity reaction. (a) Specific CD4 TH1 cell recognises mycobacterial peptide associated with MHC class II and releases macrophage (Mφ) activating IFNγ. (b) The activated Mφ kills the intracellular Mtb, mainly through generation of toxic $NO^{\bullet}$ (modified from [2]).

Several *in vitro* studies have put forward that Vγ9Vδ2 T cells may mediate protection from Mtb. These cells appear to be capable of directly killing extracellular Mtb via release of granulysin and intracellular Mtb via granulysin and perforin[140]. Moreover, mycobacteria reactive Vγ9Vδ2 T cells from individuals positive for the tuberculosis skin test also produce granzyme A, which indirectly leads to Mtb destruction by stimulating TNFα production by infected macrophages[95].

Various studies have investigated the Vγ9Vδ2 function in the context of Mtb in nonhuman primate models (**Figure I.23**). Expanded Vγ9Vδ2 T cells express IFNγ as well as perforin and granulysin and are able to inhibit intracellular Mtb growth. Moreover, they intensify pulmonary responses of peptide-specific CD4+/CD8+ T cells, which is associated with the ability of effector Vγ9Vδ2 T cells to produce IL-12[141]. Interestingly, Vγ9Vδ2 T cells accumulated in tissues in TB granulomas express

granzyme B[142]. Finally, Vγ9Vδ2 T cells show secondary memory-like response in re-infection with Mtb which correlates with protection[143].

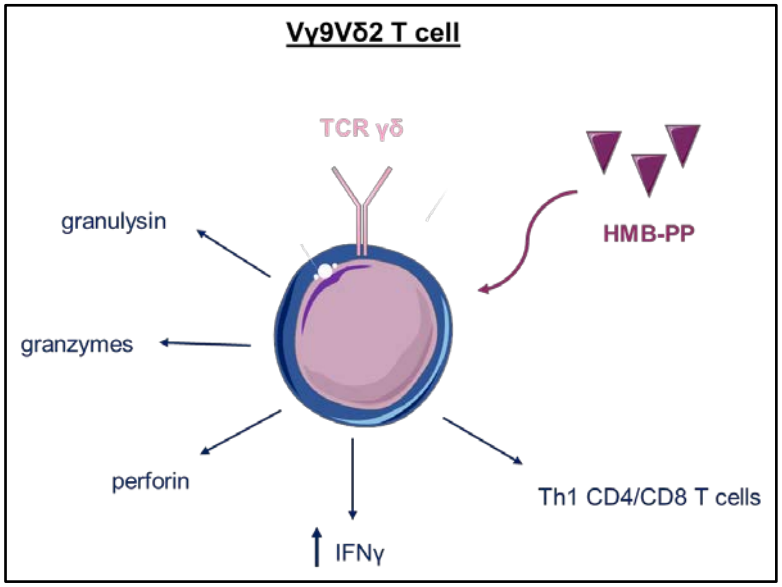


Figure I.23. In response to Mtb infection, Vγ9Vδ2 T cells produce inflammatory cytokines and exert cytotoxicity on infected cells (inspired by [144], forms from[5]).

5.2. Attenuated *Mycobacterium bovis*

Previous studies have also evoked the role of γδ T cells in immunity induced by the live attenuated *Mycobacterium bovis*, the BCG vaccine[145–147]. BCG (bacilli Calmette-Guérin) is the only licensed vaccine for prevention of TB disease, was developed around 100 years ago and prevents severe forms of TB (TB meningitis and miliary TB) in infants and young children[136]. BCG vaccination is recommended as part of national childhood immunization programs according to a country's TB epidemiology. In high TB burden countries, a single dose of the BCG vaccine should be administered to all as soon as possible after birth, while in countries with low TB incidence rates, BCG vaccination may be limited to neonates and infants in recognized high-risk groups. There is currently no vaccine that is effective in preventing TB disease in adults, either before or after exposure to TB infection, although there are promising results from current clinical trials (e.g. Phase II trial of the M72/AS01E candidate)[136].

BCG, such as other live vaccines (measles vaccine, smallpox vaccine and oral polio vaccine) reduces mortality more than can be explained by prevention of the targeted

infection(s), referred to as “nonspecific vaccine effects”[148] (see also section I.6.2). BCG offers heterologous protection in the context of cancer as well. BCG immunotherapy is the gold-standard treatment for high-risk non-muscle-invasive bladder cancer to prevent disease recurrence and progression[149]. Although BCG has been used for the treatment of bladder cancer for more than 40 years, many questions on how it achieves its therapeutic effect remain under investigation[150].

BCG contains lower levels of phosphoantigens compared to Mtb[60], yet, γδ T cells expand and produce IFNγ in response to BCG vaccination. In adults, Vδ2+ γδ T cells from BCG vaccinated individuals expanded more than cells from non-vaccinated individuals in response to *in vitro* Mtb restimulation[147]. Noteworthy, the helper functions from mycobacteria specific memory CD4+ T cells could not fully explain the memory-like phenotype at restimulation[147]. Neonatal BCG vaccination was accounted as the trigger of the γδ response in 10-week-old[146] or 7-month-old[145] infants. Based on these data, BCG seemed to elicit proliferation and effector function in the phosphoantigen reactive γδ T cells even in early life. However, in those studies on neonatal BCG vaccination[145,146], age-matched controls were missing. The influence of BCG vaccination in early life Vγ9Vδ2 T cells, on top of age-related and environmental effects, was a subject tackled in this work (section IV.3.6).

6. Immune system in neonate and infant

6.1. Bridge between the tolerant foetal and vigorous adult immune system

The foetal immune system is characterised by a dynamic capacity to handle opposing demands such as preservation of semi allogeneic existence in utero while protecting against a multitude of potentially infectious microbes. The sudden antigen encounter of a new-born occurs post-partum after abandoning the relatively protected intra-uterine environment of the mother (**Figure I.24**). There is this abrupt shift from a regulatory landscape, where the foetus should not be recognised as an allograft to the mother, towards post-natal life, facing prominent environmental cues where microbes, including pathogens, can be directly invasive. This transition at birth is considered the most dramatic life event for our mammalian immune system and leads from principally immune regulation to a fully effective and robust immune response somewhere early after birth. Early immune responses have been described as sub-optimal with neonates and infants being susceptible to infections and mounting weaker responses to vaccines than older children or adults[151–153].

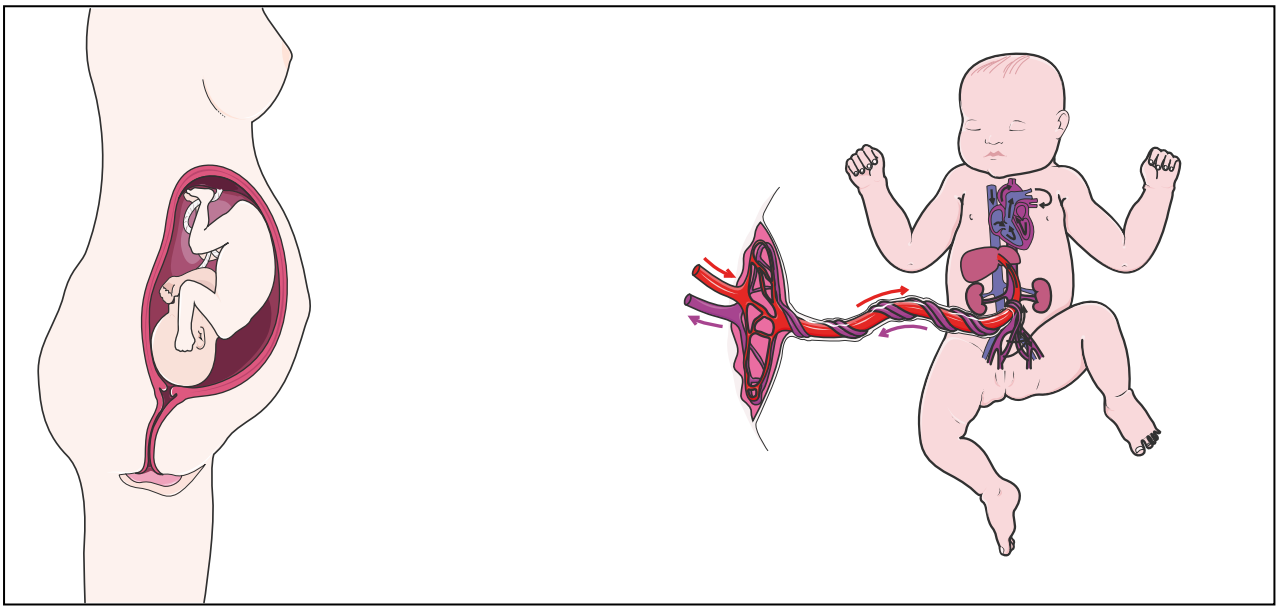


Figure I.24. Pregnancy and foetal circulation[5]. The semi allogeneic state of the mother-foetus requires suppression of rejection.

This impairment in immune response has been ascribed (among others) to defects in αβ T lymphocytes and APCs in early life[154]. Particularly, an increased risk for severe infection with intracellular pathogens requiring TH1 protective responses for effective host defence, including bacteria (e.g., L. monocytogenes, Salmonella spp.), mycobacteria, and viruses is observed in new-borns and young infants[155]. Such defects in conventional T-cell responses have fuelled the hypothesis that γδ T cells, which are the first T-cells to develop, may contribute to immune protection and regulation, conceivably compensating for a weaker αβ T cell response.

More specifically, a key limitation is the weak IFNγ production by conventional αβ T cells. Since Vδ2 γδ T cells are the first T lymphocytes to be generated and expand variably during childhood[50], they were thought of counterbalancing in part the immune response. Previous studies have shown remarkable IFNγ production by γδ T cells in young children and expression of perforin by Vδ2 γδ T cells[156,157]. Compared to αβ T cells, neonatal γδ T cells convey stronger effector functions without IFNγ deficits. γδ T cells are able to express effector molecules before birth (e.g IFNγ, granzyme A), in contrast to αβ T cells[20]. Notwithstanding, neonatal γδ T cells remain hyporesponsive compared to their adult counterparts in relation to a series of phenotypic and functional markers, including cytotoxicity, cytokine production, differentiation and activation status, as described in previous studies[31,158].

Of relevance, a recent system biology study demonstrated that cord blood is not representative of postnatal immunity[159]. Olin and colleagues followed new-borns from birth until 3 months of age and profiled their immune cells[159]. Although γδ T cells were not investigated (due to technical issues), substantial changes were observed in the immune components after birth which could not be predicted from cord blood measurements[159]. Dynamic parameters involving microbial interactions seem to guide the early immune development while microbial dysbiosis in early life is associated with the development of asthma and hindered immune system development[159,160]. This leaves the open question of how γδ T cells develop in early life and how much they differ from their cord blood counterparts. The shift in response with increasing age and child growth and whether extra-uterine exposure influences the development and evolution of Vγ9Vδ2 T cells were main aspects addressed in this work (section IV).

6.2. Intervening in the neonatal host defence

The postnatal transposition towards a stable microbiome and immune maturation has critical impact on lifelong health and disease susceptibility[155,159]. The early life immune system is not in a fixed state of ‘‘immaturity’’, but rather rapidly adapts to environmental cues. It is thus feasible to boost protection from infection by providing broadly active immune modulatory stimuli during early life such as vaccines and probiotics[155] (**Figure I.25**).

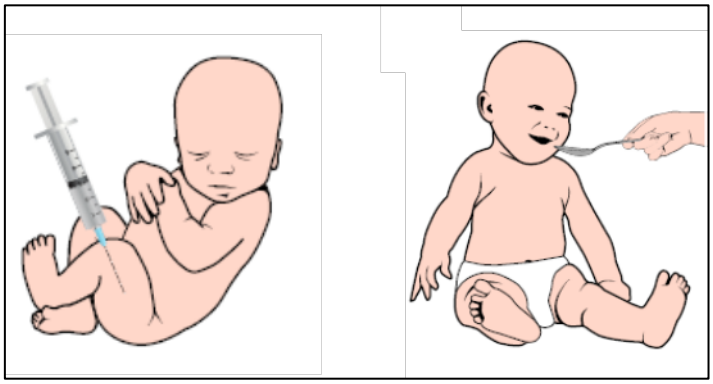


Figure I.25. Interventions that broadly enhance host defence against infectious disease in early life. New-born and infant immunisation (left), administration of probiotics (right) (From [155]).

There are key windows of opportunity during early life to enhance host resistance and reduce risk for infections via homologous, classic antigen specific responses, as well as broadly protective heterologous (‘‘non-specific’’) responses. The traditional pathogen-centric approach has led to the successful development of childhood vaccines that prevent an estimated two to three million deaths each year worldwide[155,161]. Protective heterologous effects of vaccines have been demonstrated for live attenuated vaccines such as BCG[148,162,163]. These non-specific effects can be mediated by ‘‘trained immunity’’. Trained immunity refers to long-term functional upregulation of innate immune cells through epigenetic and metabolic reprogramming[164]. For example, peripheral blood mononuclear cells (PBMC) isolated from BCG-vaccinated individuals produced increased levels of pro-inflammatory cytokines TNFα and IL-1β in response to stimulation with unrelated pathogens such as Staphylococcus aureus and C. albicans. These effects were maintained at three months after vaccination and declined one year later. These responses were partly

mediated by epigenetic changes in monocytes, including histone modifications as they can influence long-term transcriptional regulation. Three months after BCG vaccination, H3K4 trimethylation was found to be significantly increased at the level of cytokine and Toll-like receptor 4 promoters in circulating monocytes[165].

Probiotics are live microorganisms that confer a health benefit when consumed in adequate amounts[166]. They can positively modulate or restore the gut microbiota resulting in amelioration or prevention of gut inflammation and other intestinal or systemic disease phenotypes. Administration of probiotics reduces infection and can enhance immune mediated homeostasis[160]. Certain enteral probiotics reduced the risk of necrotising enterocolitis in prematurely born infants and infection-related mortality in humans[167].

The same concept of an age-restricted ‘‘window of opportunity’’ seems to apply on commensal microbes. Colonization with commensal microbes of germ-free or antibiotic-treated neonatal, but not adult, mice promotes normal immune function of mucosal iNKT cells[168]. Moreover, it has been shown, in other unconventional T cells, that early-life microbial exposure imprints the abundance of MAIT cells, and subsequent interactions with the microbiota modulate their function[169]. Hence, it is reasonable to consider a critical perinatal period where the interaction of the host with the microbiome leads to optimal immune development and exposure to specific microbes, including probiotics, might enhance immune mediated homeostasis[160]. The influence of the microbiota composition on early life Vγ9Vδ2 T cells is not yet elucidated, while various bacterial species are potential Vγ9Vδ2 T cell activators (see also section 2.1).

It is a suitable approach to broadly boost protection from infection and disease through immune modulation. However, optimal design and implementation of immunomodulatory interventions require an in-depth understanding of the developmental changes happening in the neonatal immune system at the cellular and molecular levels. The phenotype and function of Vγ9Vδ2 T cells during early post-natal life and after neonatal BCG vaccination were studied in this work (section IV).

II. Objectives

Interest in γδ T cells grows rapidly with Vγ9Vδ2 T cells being key players in immune surveillance. Their contributions are especially valuable when their unconventional nature provides critical advantages compared to αβ T cells. Although a lot of their unique properties are well known, as well as their anti-microbial capacity and anticancer potential due to their activation by phosphoantigens, several aspects of their biology and development remain to be elucidated.

Therefore, this work had three main objectives:

- To define the ontogeny of foetal and adult blood Vγ9Vδ2 T cells
- To assess the repertoire and function of Vγ9Vδ2 T cells early after birth
- To evaluate the influence of neonatal vaccination in infant Vγ9Vδ2 T cells

To achieve these objectives, the TCR repertoire was analysed after high-throughput sequencing of sorted Vγ9Vδ2 T cells and extensive phenotypic analysis and *in vitro* stimulation assays were performed.

To address the first aim, the TCR repertoire of Vγ9Vδ2 T cells was compared between foetal blood (mid-gestation and term) and adult blood and the differences observed were then identified between foetal and post-natal thymus. *In vitro* phosphoantigen stimulation of foetal blood Vγ9Vδ2 T cells and post-natal thymocytes was performed to identify the origin of adult-like Vγ9Vδ2 T cells.

To tackle the second and third objective, Vγ9Vδ2 T cells at 10 weeks after birth were studied on their TCR repertoire as well as their phenotypical and functional profiles with comparison to cord and adult blood. The potential influence of the phosphoantigen containing BCG vaccine was assessed between 10-week-old infants that were immunised at birth and 10-week-old infants that did not receive BCG vaccination at birth.

The findings of this work could guide cancer immunotherapy strategies as well as immune-related interventions in early life.

III. T cell receptor sequencing reveals the distinct development of foetal and adult human Vγ9Vδ2 T cells

Abstract

Phosphoantigen-reactive Vγ9Vδ2 T cells represent the main innate human γδ T cell subset and dominate the foetal and adult peripheral blood γδ T cell repertoire. It has been hypothesised that adult blood Vγ9Vδ2 T cells find their origin in the foetus like it is established for mouse innate γδ T cells. In order to address this issue, we analysed the complementarity-determining-region-3 (CDR3) of the T cell receptor (TCR) of human blood and thymic Vγ9Vδ2 T cells from foetal until adult life. We first identified key differences in the CDR3 repertoire of foetal and adult blood Vγ9Vδ2 T cells, including in CDR3 features important for phosphoantigen-reactivity. Next, we showed that most of these key adult CDR3 features were already present in the postnatal thymus and were further enhanced upon selection *in vitro* by the microbial-derived phosphoantigen HMB-PP. Finally, we demonstrated that the generation of a foetal-type or adult-type Vγ9Vδ2 CDR3 repertoire is determined by the foetal and post-natal nature of the hematopoietic-stem-and-precursor-cell (HSPC). Thus, our data indicate that foetal blood Vγ9Vδ2 T cells find their origin in the foetal thymus while adult blood Vγ9Vδ2 T cells are generated to a large degree independently after birth.

1. Introduction

Since the emergence of jawed vertebrates more than 450 million years ago, γδ T cells have been conserved, and can play an important role in anti-microbial and anti-tumour immunity[11,27,170,171]. γδ T cells, like αβ T cells and B cells, use V(D)J gene rearrangement with the potential to generate a set of highly diverse receptors to recognize antigens. This diversity is generated mainly in the complementary-determining region 3 (CDR3) of the T cell receptor (TCR) via combinatorial and junctional diversity[172,173]. The junctional diversity is generated during the V(D)J gene segment joining process by: (i) asymmetrical opening of the hairpinned coding ends allowing incorporation of palindromic sequences ('P nucleotides'); (ii) the introduction of additional nucleotides ('N additions') in the junction by the terminal deoxynucleotidyl transferase (TdT); (iii) exonucleolytic cleavage that results in the trimming of nucleotides at the boundary between the two coding ends[173].

Vγ9Vδ2 T cells express a TCR containing the γ-chain variable region 9 (Vγ9, TRGV9) and the δ-chain variable region 2 (Vδ2, TRDV2) and are the dominant population of γδ T cells in the blood circulation of human adults. They are activated and expanded in a TCR-dependent manner by microbe- and host-derived phosphorylated prenyl metabolites (phosphorylated antigens or 'phosphoantigens'), derived from the isoprenoid metabolic pathway[54,174]. Prototypical examples of phosphoantigens include the microbial HMB-PP produced by the MEP or 'non-mevalonate' pathway of isoprenoid synthesis, and host isopentenyl pyrophosphate (IPP) generated via the mevalonate pathway. These phosphoantigens do not bind directly the Vγ9Vδ2 TCR but have been recently shown to be sensed by the butyrophilin BTN3A1 and, via a mechanism that is yet to be fully understood, activate indirectly the Vγ9Vδ2 T cells[34,174]. The recognition of phosphoantigens allows Vγ9Vδ2 T cells to develop potent antimicrobial immune responses or to promote the killing of transformed host cells that up-regulate IPP production, which has led to the development of clinical trials targeting Vγ9Vδ2 T cells as a cancer immunotherapy approach[171,175–177].

In the mouse model, it is long established that innate γδ T cells develop in waves during foetal development[12,32]. These early innate γδ T cells express (semi-)invariant γδ TCRs, whereas later on in development the TCRs possess polyclonal CDR3

sequences[32]. Representing the prime example of such an invariant TCR, the first γδ T cells to emerge in the murine foetal thymus express a fixed TCR composed of invariant TRGV5-TRGJ1 and invariant TRDV1-TRDD2-TRDJ2 CDR3 chains (Vγ5Vδ1 in short; nomenclature according to Heilig and Tonegawa[41]) and migrate to the skin epidermis where they become dendritic epidermal T cells (DETCs). Here they are maintained until adulthood by clonal self-renewal[12,32,45,178,179]. Among human γδ T cells, Vγ9Vδ2 T cells appear to be the prototypic innate γδ T cell subset since, in contrast to other subsets (such as Vδ1+ and Vγ9-Vδ2+), they express a semi-invariant TCR[20,30,36,37,48]. We have shown that the human mid-gestational foetal peripheral blood is highly enriched for Vγ9Vδ2 T cells expressing such a semi-invariant TCR[20]. Combined with the relative absence of the Vδ2 chain in the postnatal thymus[43,50,51] and the described similarities between the cord and adult blood Vγ9Vδ2 TCR repertoire[30], this suggests a foetal wave of Vγ9Vδ2 T cells that, as is the case for mouse innate γδ T cells, persist in adulthood by clonal expansion. However, other scenarios involving a post-natal thymic output as a source of the adult blood Vγ9Vδ2 T cells cannot be ruled out[35].

To track the lineage relationship of foetal and adult phosphoantigen-reactive Vγ9Vδ2 T cells, we analysed in detail the CDR3 repertoire by high-throughput sequencing (HTS) of *ex vivo* sorted blood and thymus Vγ9+Vδ2+ γδ T cells from postnatal (birth until adult) and foetal subjects. This was complemented with the CDR3 repertoire analysis of HMB-PP-expanded Vγ9Vδ2 thymocytes and of Vγ9Vδ2 T cells generated by hematopoietic-stem-and-precursor-cells (HSPC) *in vitro*. We found that adult blood Vγ9Vδ2 T cells are derived from the small number of Vγ9Vδ2 T cells generated in the postnatal thymus and, surprisingly, not from the abundant foetal population.

2. Materials and Methods

2.1. Human cell material

Human peripheral blood, thymus and liver samples were collected from the following sources where all participants (when apply mothers/parents) gave written informed consent in accordance with the Declaration of Helsinki. Foetal blood because of interruption of pregnancy (22-30 weeks of gestation), approved by the Hôpital Erasme ethics committee; umbilical cord blood after delivery (vaginal) (39-41 weeks term delivery) with the approval of the University Hospital Centre Saint-Pierre; adult peripheral blood (>18years, 26-64 years), approved by the Ethics committee of the CHU Tivoli, La Louvière; foetal thymus and foetal liver (15-21 weeks of estimated gestational age), approved by the Centralised Institutional Research Board of the Singapore Health Services in Singapore; post-natal thymus obtained from children (4 months to 8 years old) who underwent cardiac surgery and mobilized peripheral blood samples of adult donors (for the OP9DL1 co-culture), approved by Medical Ethical Committee of Ghent University Hospital.

Blood was layered over lymphoprep™ (Axis-Shield) with resulting PBMC cryopreserved for subsequent experiments. (Lymphoprep™ is a density gradient medium for the isolation of mononuclear cells from peripheral blood after centrifugation[180]). Cell suspensions from foetal thymus and liver and post-natal thymus samples were obtained as previously described[181,182].

2.2. Flow cytometry, sorting and cell cultures

For flow cytometry and associated cell sorting, cells were thawed in complete medium, washed twice and labelled with Zombie NIR™ dye (Biolegend), and the cells were then subsequently stained for cell surface antigens with antibodies directed against CD3 (clone UCHT1, BD), TCR-γδ (11F2, BD), TCR-Vγ9 (IMMU360; Beckman Coulter), TCR-Vδ2 (IMMU389; Beckman Coulter). CD3+γδTCR+Vγ9+Vδ2+ were sorted for “Vγ9Vδ2” (mean purity 98.0% of CD3+γδ+) and the rest CD3+ γδTCR+ non(Vγ9Vδ2) were sorted for “nonVγ9Vδ2” γδ T cells (mean purity 98.6 % of CD3+γδ+) on a FACS Aria III (BD). Gating strategy: FS singlets → alive cells (zombie negative) → SSC-FSC

lymphocyte/thymocyte gate → CD3+γδTCR+ → Vγ9 – Vδ2, Vγ9+Vδ2+ or non(Vγ9Vδ2). Data were analysed using FlowJo software (Tree Star).

For Vγ9Vδ2 T cell expansion experiments, post-natal thymocytes enriched for untouched γδ T cells with the TCRγ/δ+ T Cell Isolation Kit (130-092-892, Miltenyi Biotec) were cultured for 10 days at 37 °C, 5% CO_2 in 14-mL polypropylene, round-bottom tubes (Falcon; BD) at a final concentration of 1 $\times 10^6$ cells/mL, in presence of HMB-PP 10nM (Echelon Bioscience) and IL-2 100U/ml ((Proleukin, Chiron/Novartis) which was added every 3 days. Culture medium consisted of 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). Irradiated PBMC (30 min at 2400cGy) were used as feeders at the ratio of 1/1 in the cultures of thymocytes. After expansion, Vγ9Vδ2 T cells were sorted as described above.

OP9DL1 cells were obtained from Dr. J. C. Zúñiga-Pflücker (University of Toronto, Canada)[183]. Isolated CD34+ cells were seeded in a 6-well culture plate (5x105 cells/well) containing a monolayer of OP9DL1 cells in MEMα (Minimum Essential Medium α) (Gibco, Life Technologies) supplemented with 20% foetal bovine serum (Sigma), 1% NEAA, 1% Glutamine, 1% Pen/Strep (Lonza) in the presence of 10 ng/ml IL-7 (R&D Systems), 10 ng/ml Flt3L (PeproTech) and 5 ng/ml SCF (PeproTech). Every 4-5 days cells were harvested by vigorous pipetting and transferred in a new 6-well plate (between $5x10^5$-$1x10^6$ cells/well)[182]. After ca. 30 days in co-culture with OP9-DL1 cells, HPSC-derived cells were snap frozen in liquid nitrogen and stored at -80 C for later RNA extraction.

2.3. TCRγ and TCRδ NGS

RNA was isolated from sorted cells (usually 5000-15000 Vγ9Vδ2 T cells) with the RNeasy Micro Kit (Qiagen) or from OP9DL1 co-cultures with the RNeasy Mini Kit (Qiagen). cDNA was generated performing a template switch anchored RT-PCR. RNA was reverse transcribed via a template-switch cDNA reaction using TRCG (5'-CAAGAAGACAAAGGTATGTTCCAG) and TRDC (5'-GTAGAATTCCTTCACCAGACAAG) specific primers in the same reaction tube, a template-switch adaptor (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG) and the Superscript II RT enzyme (Invitrogen). The TRCG primer binds both TRCG1 and TRCG2. 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 TRCG1 and TRCG2 5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*AATAGTGGGCTTGGGGGAA ACATCTGCAT, adapter in italic) and a specific TRDC primer (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*ACGGATGGTTTGGTATGAG GCTGACTTCT, adapter in italic) and a primer complementary to the template-switch adapter (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*AAGCAGTGGTATCAACGCAG, adapter in italic) 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 centre, University of Liège, Belgium].

Raw sequencing reads from fastq files (read 1 and read 2) 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 2.1.12[184]. Default parameters were used except to assemble TRDD gene segment where three instead of five consecutive nucleotides were applied as assemble parameter. CDR3 sequences were then exported and analysed using VDJtools software version 1.2.1 using default settings[185]. Sequences out of frame and containing stop codons were excluded from the analysis. The CDR3 repertoire data shown are filtered for TRGV9 and TRDV2 sequences. Note that the nucleotype lengths generated by VDJtools include the C and Vends of the CDR3 clonotypes. For OP9DL1 cultures, the CDR3 repertoire was examined on total culture cells (and not on sorted Vg9Vd2 T cells), but we focused on the TRGV9–TRGJP repertoire, as this TRGV–TRGJ combination is associated with phosphoantigen reactivity[22,23]. Tree maps were created using the Treemap Package (https://CRAN.R-project.org/package=treemap). Conservation of TRGV9 and TRGJP sequences among primates was investigated by using the genome browser at https://genome-euro.ucsc.edu/ (human assembly Dec. 2013 GRCh38/hg38) and its comparative genomics tool; the figure illustrating the conservation of sequences was generated with the GeneRunner software.

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6. Parametric tests were used after verifying the normality of the data using the Kolmogorov-Smirnov normality test. Differences between groups were analysed using Kruskal–Wallis ANOVA and Dunn's post-tests for non-parametric data. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3. Results

3.1. Adult and foetal blood Vγ9Vδ2 T cells show a different CDR3 repertoire

To verify whether adult blood Vγ9Vδ2 T cells can be derived from their counterparts in the foetal blood circulation, we compared in detail the CDR3 repertoire of sorted adult, cord and foetal blood Vγ9Vδ2 T cells by HTS. We used foetal blood derived from gestation ages 22-30 weeks when the Vγ9Vδ2 T cells are highly enriched, cord blood from term delivery (39-41 weeks), by which time the Vδ1+ T cells become the major γδ T cell population[20] and adult blood from healthy volunteers (18-65 years old).
First, we quantified the level of N additions in the CDR3 repertoire. Adult blood Vγ9Vδ2 T cells used clearly many more N additions than foetal blood Vγ9Vδ2 T cells, both in their CDR3γ and CDR3δ repertoire; cord blood Vγ9Vδ2 T cells used an intermediate number of N additions (**Fig. III.1a, supplementary Fig. III.1a**). The mean CDR3 lengths were longer in adult compared to foetal Vγ9Vδ2 T cells (**Fig. III.1b**). The distribution of the CDR3γ was highly restricted towards the length corresponding to 14 aa +/-1 (or 48 nt +/-3, including C and F ends), matching phosphoantigen-reactive CDR3γ lengths[23], both in foetal and adult blood Vγ9Vδ2 T cells (**supplementary Fig. III.1b, top panel**). Since the differences in CDR3 length were not as high as the differences in N additions would suggest (**compare Fig. III.1b with Fig. III.1a**), we wondered whether differential J segment usage could contribute to the minimization of the CDR3 length differences. For CDR3δ, we could indeed identify an enriched usage of TRDJ3 and TRDJ2 in foetal blood Vγ9Vδ2 T cells which are 8 and 3 nucleotides longer than the adult prevalent TRDJ1 (**Fig. III.1c, bottom panel**). The differences in N additions were maintained when only sequences using ‘adult-type’ TRDJ1 or ‘foetal-type’ TRDJ3 were analysed (**supplementary Fig. III.1c**). There was also a slight increase in the usage of shorter TRDD1 (8 nucleotides) and TRDD2 (9 nucleotides) in adult compared to foetal Vγ9Vδ2 T cells at the expense of the longer (13 nucleotides) TRDD3 (**supplementary Fig. III.2a**). In the CDR3γ repertoire however, the vast majority of the TRGV9 sequences in both foetal and adult blood Vδ2+ (**Fig. III.1c top panel**) but not of Vδ2- (**supplementary Fig. III.2b**) γδ T cells used TRGJP, consistent with the importance of this J gene segment for phosphoantigen-reactivity[22,23]. It seems that an increase of nucleotide deletion (trimming) in the adult at the end of the TRGV9

gene segment (**supplementary Fig. III.2c**) is more likely to contribute to the conservation of the CDR3γ length (**Fig. III.1b, supplementary Fig. III.1b**).

The TRGV9 CDR3 repertoire was more focused than the TRDV2 CDR3 repertoire for all groups (**Fig. III.1 d,e**). While there was some variability, the diversity of the foetal/cord and adult CDR3 TRDV2 repertoire was broadly similar (**Fig. III.1d-e**) as described previously[30]. However, there was a significantly higher CDR3 overlap within pairs of foetal compared to pairs of postnatal subjects (**Fig. III.1f**). The higher sharing within the foetal TRGV9 repertoire was in great part due to the high prevalence of the germline-encoded nucleotype 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3' (**Fig. III.1g**), encoding the clonotype CALWEVQELGKKIKVF at amino acid level, giving rise to a phosphoantigen-reactive receptor[20]. Furthermore, the CALWEVQELGKKIKF clonotype in the adult blood Vγ9Vδ2 T cells was also encoded by alternative nucleotypes that included N additions, which could not be detected in foetal blood Vγ9Vδ2 T cells (**Fig. III.1f, green bar**). While CDR3δ sequence and length can be variable in Vγ9Vδ2 T cells, it has been shown that the presence of a hydrophobic amino acid at position 5 of the CDR3δ is important for phosphoantigen reactivity, both in cord and adult blood-derived Vγ9Vδ2 T cells[22,23,25]. The percentage of Vγ9Vδ2 T cells expressing a hydrophobic residue (VILWFMC, mainly V and L) at this position 5 was high in both foetal and adult blood Vγ9Vδ2 T cells (**Fig. III.1h**), independent of their TRDJ usage (**supplementary Fig. III.2d**). However, analysis of the CDR3δ at the nucleotype level revealed that in foetal blood Vγ9Vδ2 T cells the vast majority of nucleotypes use a germline codon to encode this hydrophobic residue (**Fig. III.1h, black bars**), while in more than half of adult blood Vγ9Vδ2 T cells that particular codon contains N insertions (**Fig. III.1h, green bars**).

In summary, both the CDR3γ and CDR3δ repertoire of foetal and blood Vγ9Vδ2 T cells show major differences, including in how the features important for phosphoantigen reactivity are encoded.

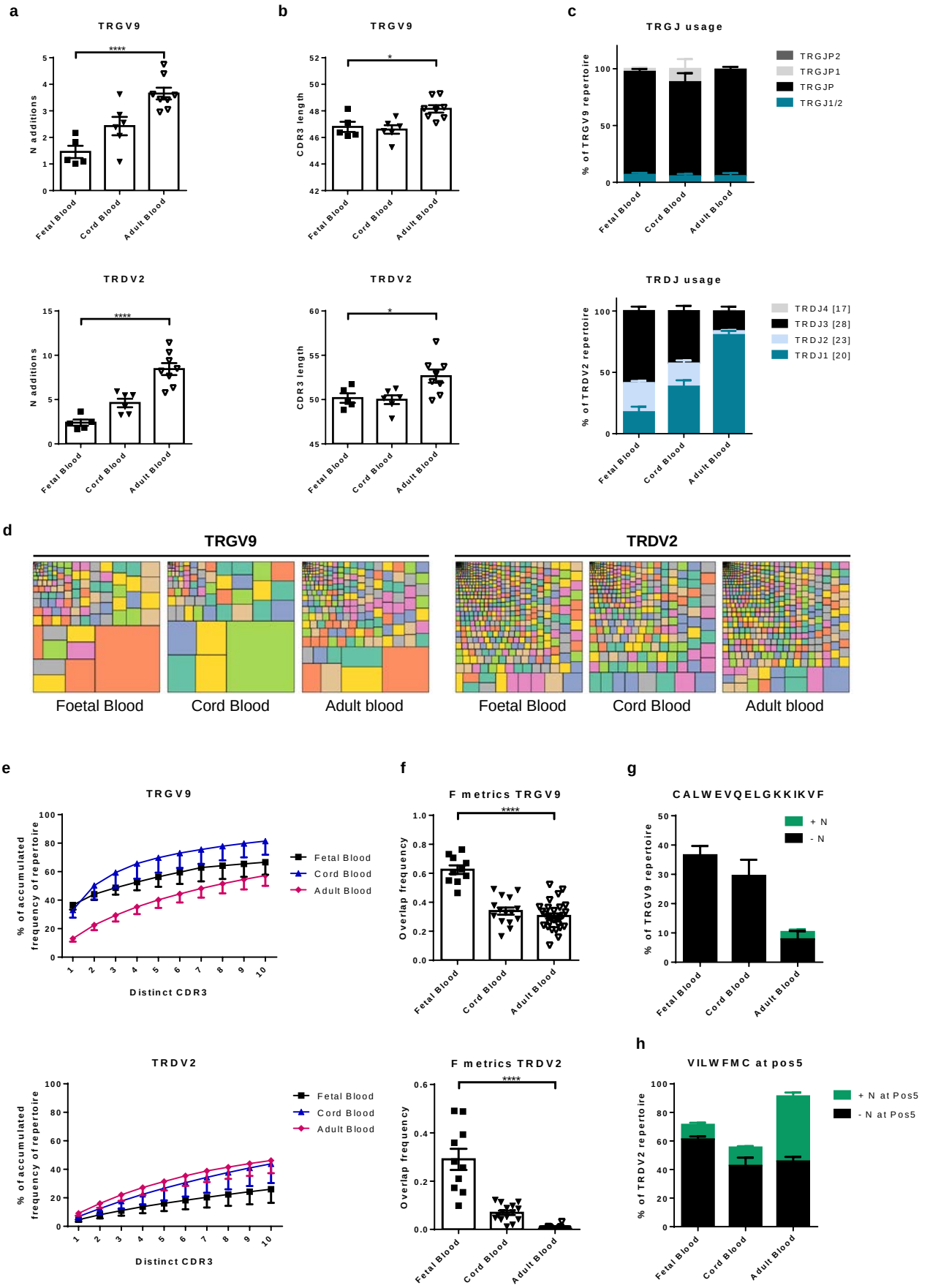
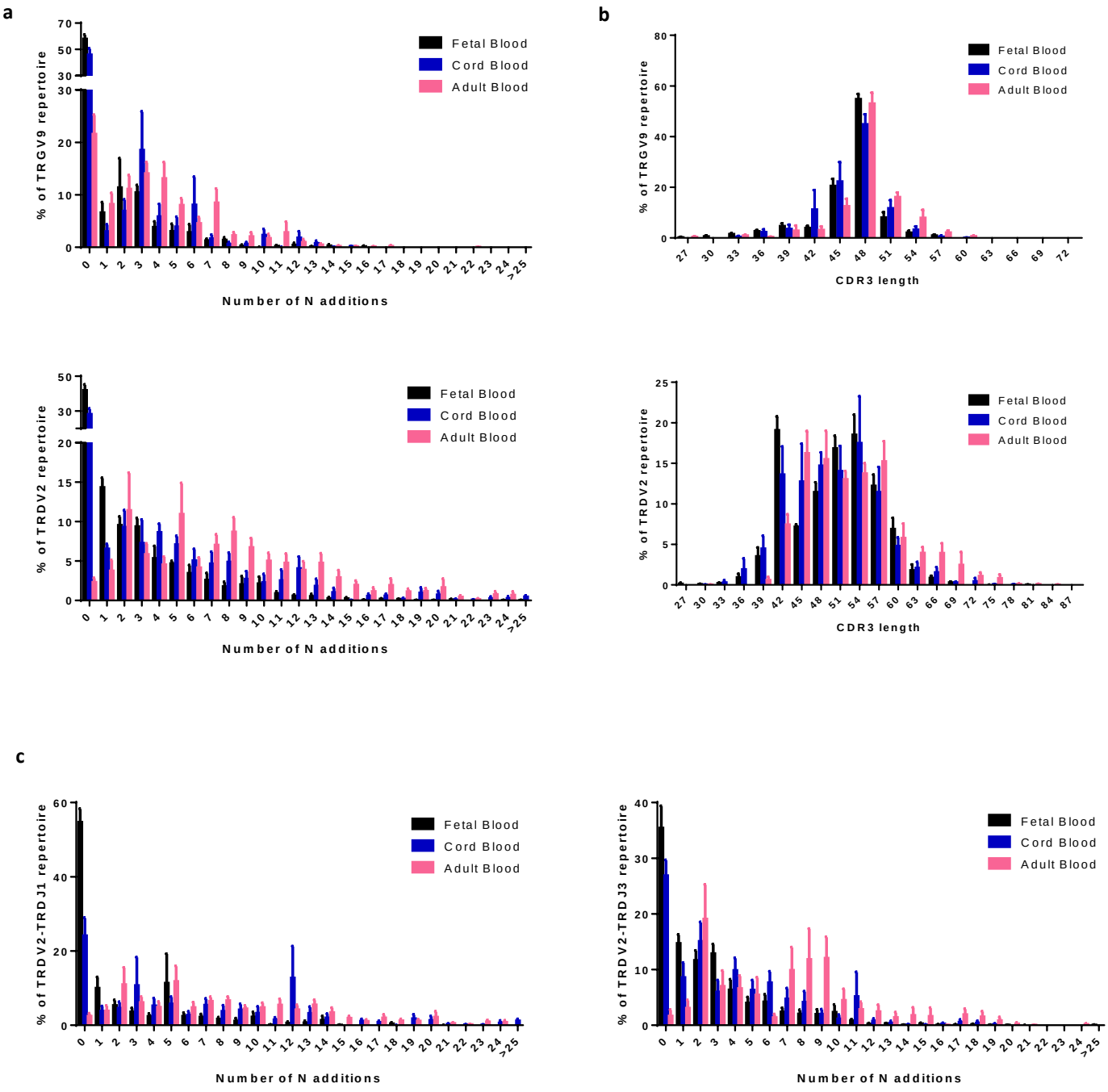
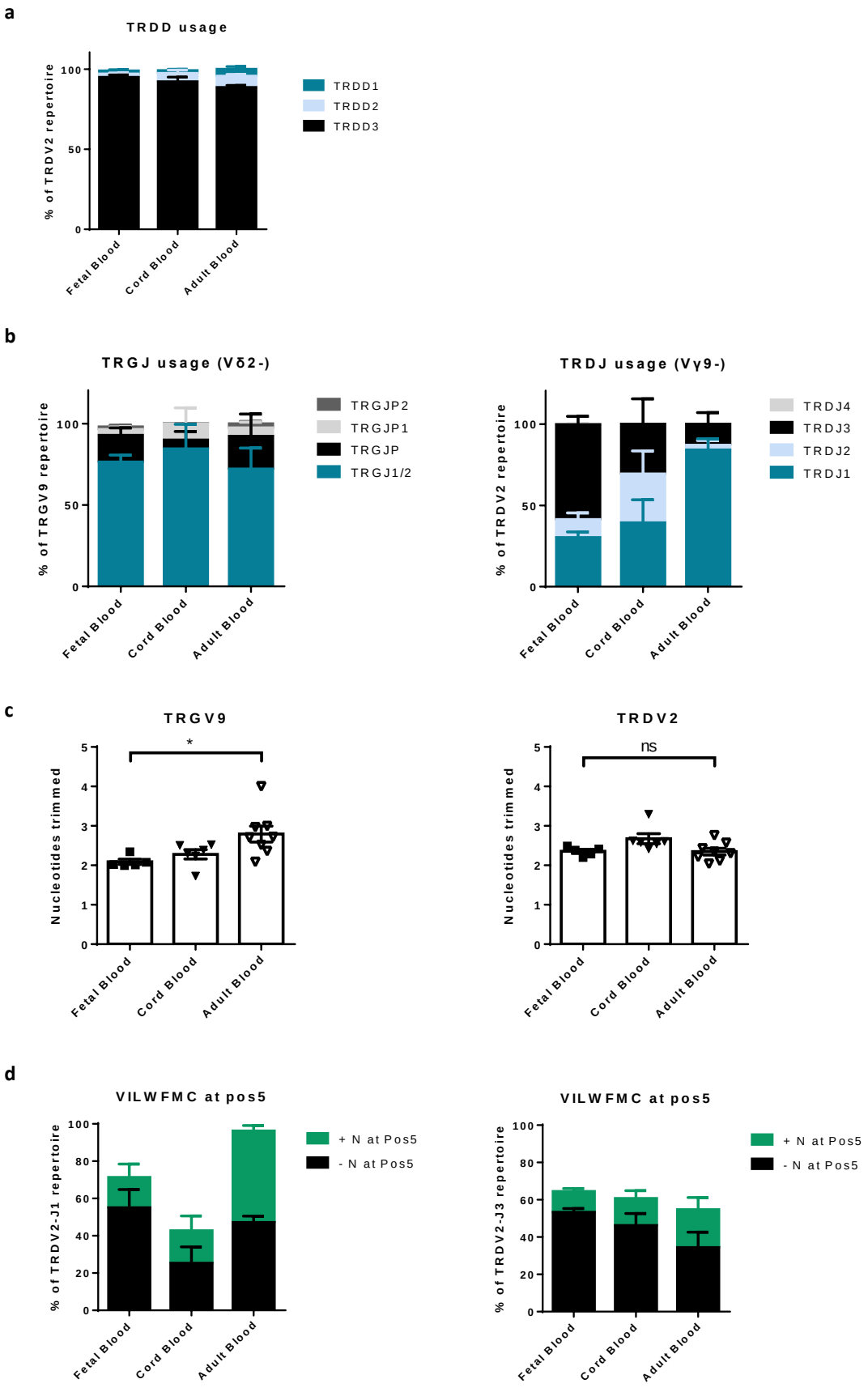

a
TRGV9
N additions
****
Fetal Blood
Cord Blood
Adult Blood
b
TRGV9
CDR3 length
*
c
TRGJ usage
% of TRGV9 repertoire
TRGJP2
TRGJP1
TRGJP
TRGJ1/2
TRDV2
TRDJ usage
% of TRDV2 repertoire
TRDJ4 [17]
TRDJ3 [28]
TRDJ2 [23]
TRDJ1 [20]
d
TRGV9
TRDV2
Foetal Blood
Cord Blood
Adult blood
e
TRGV9
% of accumulated frequency of repertoire
Distinct CDR3
Fetal Blood
Cord Blood
Adult Blood
TRDV2
f
F metrics TRGV9
Overlap frequency
F metrics TRDV2
g
CALWEVQELGKKIKVF
+ N
- N
% of TRGV9 repertoire
h
VILWFMC at pos5
+ N at Pos5
- N at Pos5
% of TRDV2 repertoire

Fig. III.1. Adult and foetal blood Vγ9Vδ2 T cells show major differences in their CDR3γ and CDR3δ repertoire. **a-c** Comparison of the CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire of sorted Vγ9Vδ2 T cells derived from foetal, cord and adult peripheral blood: **a** Number of N additions, each dot represents the weighted mean of an individual sample, **b** CDR3 length in nucleotides (including the codons for C-start and F-end residues), each dot represents the weighted mean of an individual sample, **c** J gene segment usage distribution. **d** Treemaps show CDR3 clonotype usage in relation to TRGV9 (left) and TRDV2 (right) repertoire size in sorted Vγ9Vδ2 T cells from one representative foetal, cord and adult blood subject (rectangle colours are chosen randomly and do not match between plots). **e-h** Comparison of the CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire of sorted Vγ9Vδ2 T cells from foetal, cord and adult peripheral blood: **e** Accumulated frequency curves generated from the 10 most prevalent clonotypes, **f** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of foetal, of cord and of adult blood subjects, each dot represents the F value of a pair of samples; **g** Comparison of prevalence of the TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N additions in black (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') or encoded with N additions in green, and **h** Percentage of the TRDV2 repertoire containing at position 5 of the CDR3δ a highly hydrophobic residue (V, I, L, W, F, M or C): residue encoded without N additions in black or by N addition(s) in green. Data shown from independent subjects, sorted Vγ9Vδ2 T cells from foetal (n=5), cord (n=6) and adult peripheral blood (n=8). Error bars indicate means ± SEM.



Supplementary Fig. III.1. CDR3 repertoire of foetal, cord and adult blood Vγ9Vδ2 T cells. a Frequency of TRGV9 (top) and TRDV2 (bottom) CDR3 repertoire per number of N additions. **b** Frequency of TRGV9 (top) and TRDV2 (bottom) CDR3 repertoire per CDR3 length (nucleotide count including the C-start and F-end of the clonotypes). **c** Frequency of the TRDV2-TRDJ1 (left) and TRDV2-TRDJ3 (right) CDR3 repertoire per number of N additions. Data shown from independent subjects, sorted Vγ9Vδ2 T cells from foetal (n=5), cord (n=6) and adult (n=8) blood. Error bars indicate means ± SEM.



Supplementary Fig. III.2. CDR3 repertoire of foetal, cord and adult blood γδ T cells. **a** TRDD usage distribution of the TRDV2 repertoire of sorted Vγ9Vδ2 T cells. **b** J usage distribution of the TRGV9 (left) and TRDV2 (right) of sorted γδ T cells nonVγ9Vδ2 (foetal blood n=4, cord blood n=5, adult blood n=6). **c** Number of

nucleotides trimmed at the Vend of the CDR3 of the TRGV9 (left) and TRDV2 (right) repertoire; each dot represents the weighted mean of an individual sample. **d** Percentage of the TRDV2-TRDJ1 (left) and TRDV2-TRDJ3 (right) repertoire containing at position 5 of the CDR3δ a highly hydrophobic residue (V, I, L, W, F, M or C): residue encoded without N additions in black or encoded by N addition(s) in green, of sorted Vγ9Vδ2 T cells. (Sorted Vγ9Vδ2 T cells in a, c and d: Foetal Blood n=5, Cord Blood n=6, Adult Blood n=8.) Error bars indicate means ± SEM. *p < 0.05; ns: non-significant.

3.2. Expansion of phosphoantigen-reactive foetal blood Vγ9Vδ2 T cells does not lead to an adult-type CDR3 repertoire

To verify a possible selection upon microbial phosphoantigen exposure after birth, we expanded *in vitro* foetal blood Vγ9Vδ2 T cells with the microbial-derived phosphoantigen HMB-PP[20] and studied the changes in the CDR3 repertoire. Despite a strong Vγ9Vδ2 T cell expansion[20], 'foetal-type' TRDJ3 was still the major TRDJ gene segment and no preferential expansion of the 'adult-type' TRDJ1 usage could be observed (**Fig. III.2a**). Note that a shift towards a TRDJ1 usage could already be seen before birth (compare foetal (<30w gestation) and cord (>37w gestation) in **Fig. III.1c**, **bottom panel**) which is in line with a shift of TRDJ usage that is independent of microbial exposure after birth. Furthermore, the percentage of the 'foetal' nucleotype 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3' (encoding the clonotype CALWEVQELGKKIKVF) remained stable upon *in vitro* expansion with HMB-PP (**Fig. III.2b**) and there was no shift towards a more adult-type CDR3 repertoire at the level of N insertions or sharing (**Fig. III.2c, 2d**).

Based on these data, we conclude that it is unlikely that the adult blood Vγ9Vδ2 T cells are derived from foetal blood Vγ9Vδ2 T cells expanded upon microbial exposure after birth.

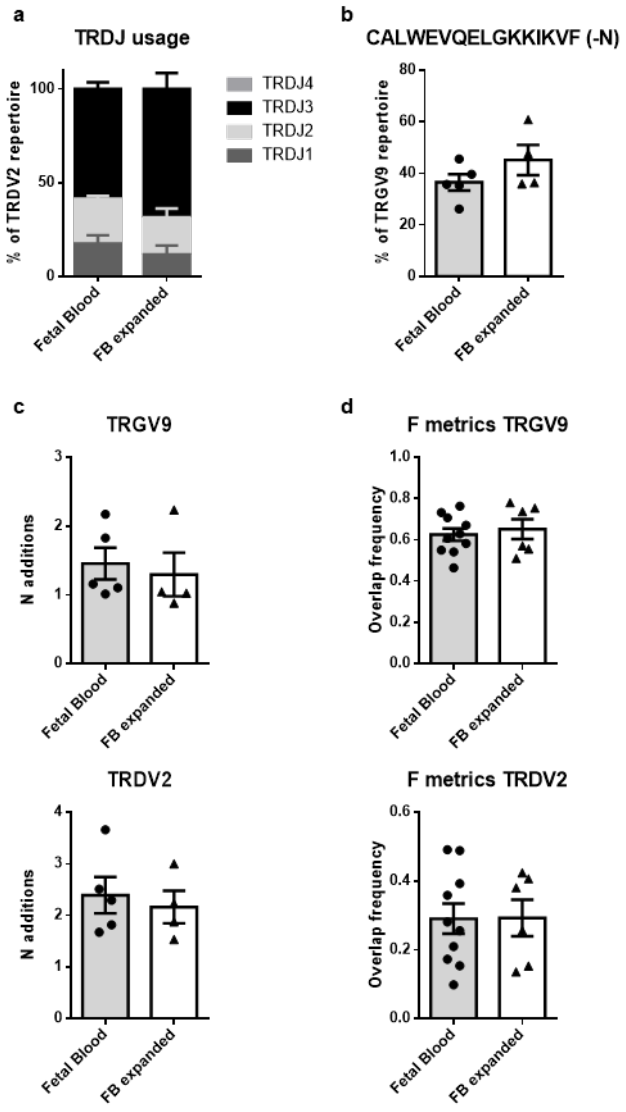


Fig. III.2. Expansion of phosphoantigen-reactive foetal blood Vγ9Vδ2 T cells does not lead to an adult-type CDR3 repertoire. **a-d** Comparison of the CDR3 repertoire of Vγ9Vδ2 T cells from *ex-vivo* foetal blood (n=5) or from expanded with HMB-PP (10 days) foetal blood (FB expanded, n=4): **a** TRDJ usage distribution in the TRDV2 repertoire, **b** Prevalence of the public TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N additions (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') in the TRGV9 repertoire, **c** Number of N additions, each dot represents the weighted mean of an individual sample and **d** Overlap frequencies of pairs of *ex-vivo or* expanded foetal blood in the TRGV9 (top) and TRDV2 (bottom) repertoire. Data shown from independent subjects (*ex-vivo* foetal blood shown also in Fig. III.1). Error bars indicate means ± SEM.

3.3. Foetal and post-natal Vγ9Vδ2 thymocytes express a different CDR3 repertoire

In an effort to determine the origin of the adult blood Vγ9Vδ2 T cells, we carefully investigated the presence of Vγ9+Vδ2+ cells within post-natal paediatric thymuses. It has been described previously that Vδ2+ thymocytes are either absent or only present at a very low frequency, which contributed to the notion that (adult) blood Vγ9Vδ2 T cells are derived from the foetal thymus[32,43,47,50,51,186,187]. Indeed, we found that the frequency of γδ thymocytes expressing the Vδ2 chain in the post-natal thymus is significantly lower compared to the foetal thymus, but they are clearly present (**supplementary Fig. III.3a, Fig. III.3a, bottom panel**). Importantly, a large fraction of the post-natal Vδ2+ thymocytes co-expressed the Vγ9 chain (**supplementary Fig. III.3a**) resulting in about 6% of the post-natal γδ thymocytes being Vγ9+Vδ2+ (**Fig. III.3a**). Taking into account the more than 20-fold increase in size of the organ between foetal week 20 and the age of 5 and the 5-fold reduction in percentage, it can be assumed that both the foetal and post-natal thymus produce and export Vγ9+Vδ2+ T cells. Thus, we sorted foetal and post-natal Vγ9+Vδ2+ thymocytes and compared their CDR3 repertoire.

Like in the foetal versus adult blood Vγ9Vδ2 comparison, the post-natal thymic Vγ9Vδ2 repertoire, compared to the foetal counterpart, contained significantly more N additions (**Fig. III.3b, supplementary Fig. III.3b**), had longer CDR3δ (**Fig. III.3b bottom panel, supplementary Fig. III.3c**), showed more trimming at the TRGV9 end (**supplementary Fig. III.3d top panel**) and preferentially used TRDJ1 and TRDD1/TRDD2 (**Fig.3d bottom panel; supplementary Fig. III.3e**). Like in the blood Vγ9Vδ2 repertoire, the CDR3γ length distribution was more focused than the CDR3δ repertoire, both in the foetal and post-natal Vγ9Vδ2 thymocytes (**supplementary Fig. III.3c**). Of note, TRGJP was the main TRGJ gene segment used both by foetal and post-natal thymic Vγ9Vδ2 T cells (**Fig. III.3d top panel**) but the percentage was lower than in their peripheral blood counterparts (**compare Fig. III.3d with Fig. III.1c**), as observed previously in thymic versus blood Vγ9Vδ2 T cell clones[25]. In contrast, the TRGV9 chain of Vγ9+Vδ2- thymocytes was mainly combined with the TRGJ1/2 gene segment (**supplementary Fig. III.3f, top panel**), like in the periphery (**supplementary Fig. III.2b, left panel**). The higher diversity of the post-natal Vγ9Vδ2 thymocytes (**Fig. III.3e, f**) contributed to a much lower repertoire overlap within post-natal subjects compared to the overlap within foetal subjects (**Fig. III.3g**). The high sharing within the foetal thymic TRGV9 repertoire was in great part due to the high prevalence of the

public canonical germline-encoded nucleotype (**Fig. III.3g right top panel**). In the post-natal Vγ9Vδ2 thymocytes, this public nucleotype was also present but it was less abundant in subjects older than 4 months (**Fig. III.3h**). Moreover, the hydrophobic amino acid at position 5 was more encoded by N-containing codons in the post-natal compared to the foetal Vγ9Vδ2 thymocytes (**Fig. III.3i**). Of note, both the foetal and post-natal Vγ9Vδ2 thymocytes contained a lower percentage of TRDV2-containing CDR3δ possessing a hydrophobic amino acid at position 5 (**Fig. III.3i**) compared to their blood counterparts (**Fig. III.1h**).

In summary, the post-natal thymic Vγ9Vδ2 TCR repertoire differs from its foetal counterpart in all parameters that differed in blood derived Vγ9Vδ2 T cells and resembles the adult blood Vγ9Vδ2 repertoire.

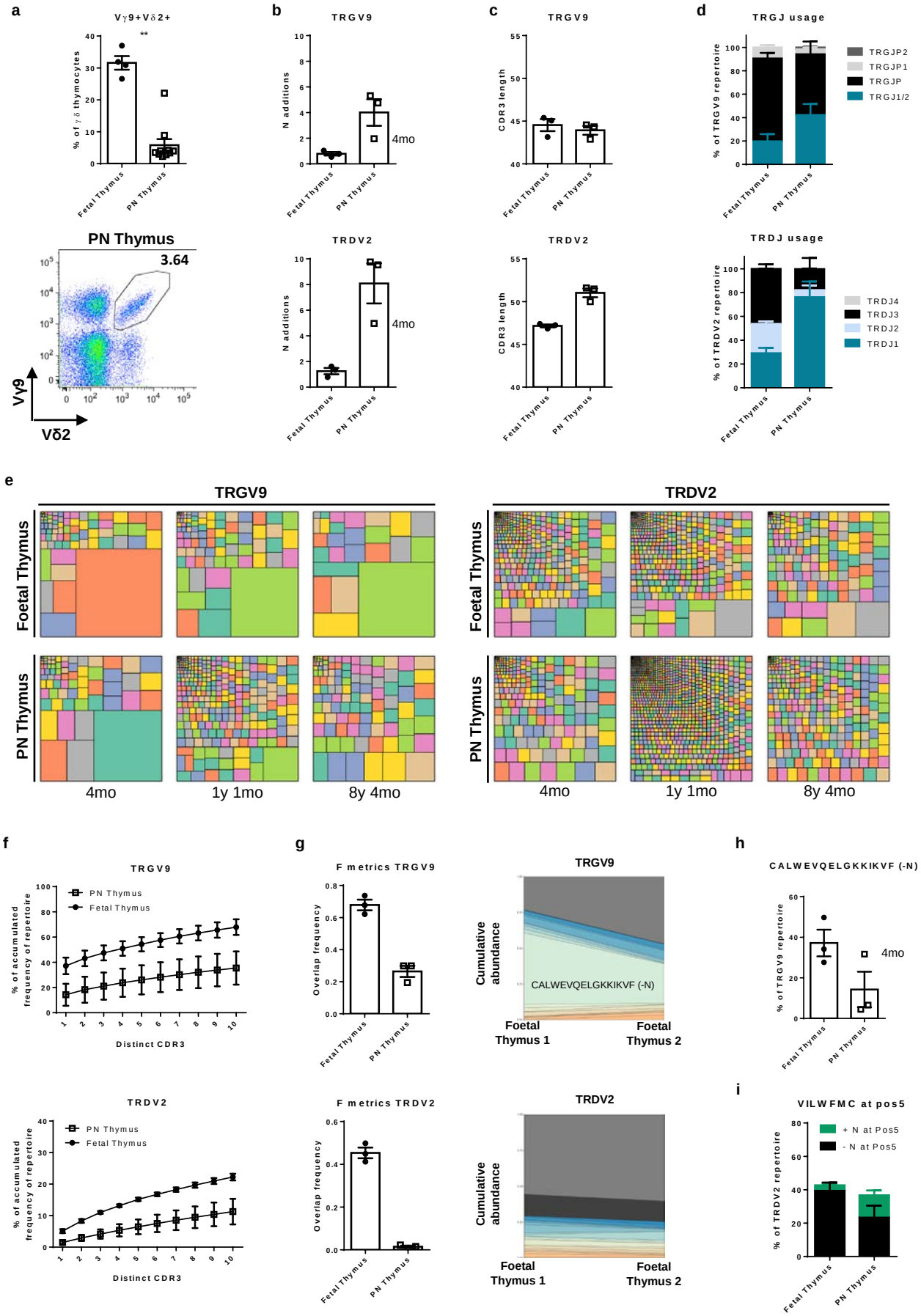
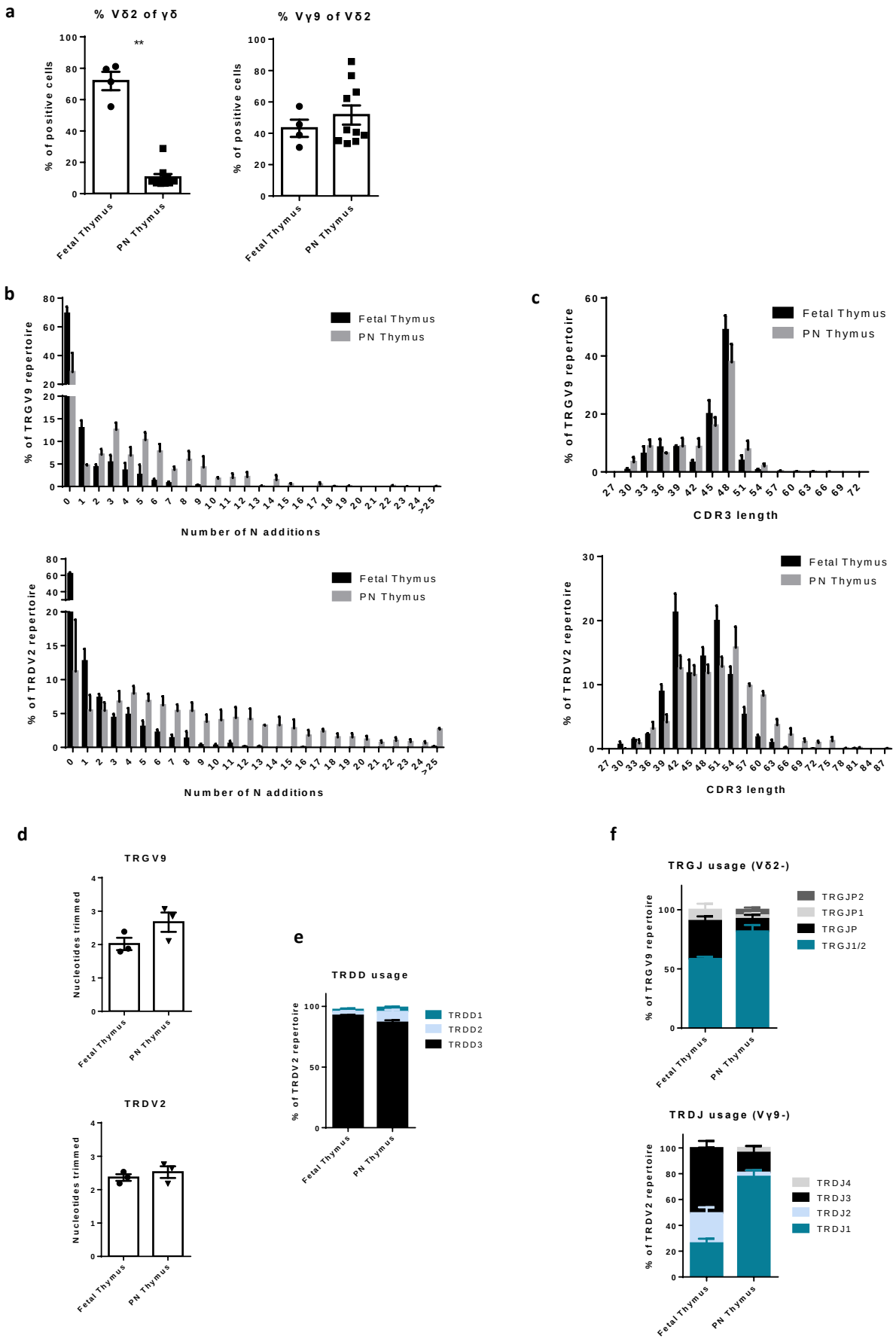

a
Vγ9+Vδ2+
% of γδ thymocytes
Fetal Thymus
PN Thymus
PN Thymus
3.64
Vγ9
Vδ2
b
TRGV9
N additions
4mo
TRDV2
c
TRGV9
CDR3 length
TRDV2
d
TRGJ usage
% of TRGV9 repertoire
TRGJP2
TRGJP1
TRGJP
TRGJ1/2
TRDJ usage
% of TRDV2 repertoire
TRDJ4
TRDJ3
TRDJ2
TRDJ1
e
TRGV9
TRDV2
Foetal Thymus
PN Thymus
4mo
1y 1mo
8y 4mo
f
TRGV9
PN Thymus
Fetal Thymus
% of accumulated frequency of repertoire
Distinct CDR3
TRDV2
g
F metrics TRGV9
Overlap frequency
F metrics TRDV2
TRGV9
Cumulative abundance
CALWEVQELGKKIKVF (-N)
Foetal Thymus 1
Foetal Thymus 2
TRDV2
h
CALWEVQELGKKIKVF (-N)
% of TRGV9 repertoire
4mo
i
VILWFMC at pos5
+ N at Pos5
- N at Pos5
% of TRDV2 repertoire

Fig. III.3. Foetal and post-natal Vγ9Vδ2 thymocytes express a different CDR3 repertoire. **a** Percentage of Vγ9+Vδ2+ cells in γδ+ thymocytes in foetal (n=4) and post-natal thymus (PN Thymus, n=10) (top); representative flow cytometry plot of post-natal γδ thymocytes (bottom). **b-d:** Comparison of the CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire of sorted Vγ9Vδ2 T cells in Foetal Thymus (n=3) and PN Thymus (n=3): **b** Number of N additions, each dot represents the weighted mean of an individual sample, **c** CDR3 length in nucleotides (including the C-start and F-end residues), each dot represents the weighted mean of an individual sample, and **d** J gene segment usage distribution. **e** Tree-maps of CDR3 TRGV9 (left) and TRDV2 (right) repertoire of foetal (top) and post-natal (bottom) Vγ9+Vδ2+ thymocytes (rectangle colours are chosen randomly and do not match between plots). **f-i** Comparison of the CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire of sorted Vγ9Vδ2 T cells in Foetal Thymus (n=3) and PN Thymus (n=3): **f** Accumulated frequency curves generated from the 10 most prevalent clonotypes, **g** Geometric mean of relative overlap frequencies (F metrics of VDJ tools) within pairs of foetal thymus and of post-natal thymus (left), each dot represents the F value of a pair of samples, and shared clonotype abundance plots (right) for two foetal thymus samples (top 20 clonotypes shared in distinct colours, collapsed in dark grey and non-overlapping in light grey), **h** Percentage of the TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N additions (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') in the TRGV9 repertoire. **i** Percentage of the TRDV2 repertoire containing at position 5 of the CDR3δ chain a highly hydrophobic residue (V, I, L, W, F, M or C): residue encoded without N additions in black or encoded by N addition(s) in green. Data shown from independent subjects. Error bars indicate means ± SEM.


a
% Vδ2 of γδ
% Vγ9 of Vδ2
% of positive cells
**
Fetal Thymus
PN Thymus
b
% of TRGV9 repertoire
% of TRDV2 repertoire
Fetal Thymus
PN Thymus
Number of N additions
c
% of TRGV9 repertoire
% of TRDV2 repertoire
CDR3 length
d
TRGV9
TRDV2
Nucleotides trimmed
e
TRDD usage
% of TRDV2 repertoire
TRDD1
TRDD2
TRDD3
f
TRGJ usage (Vδ2-)
% of TRGV9 repertoire
TRGJP2
TRGJP1
TRGJP
TRGJ1/2
TRDJ usage (Vγ9-)
% of TRDV2 repertoire
TRDJ4
TRDJ3
TRDJ2
TRDJ1

Supplementary Fig. III.3. Foetal versus post-natal thymus. **a** Flow cytometry results on the prevalence of Vδ2 of γδ+ (left) and Vγ9 of Vδ2+ (right) thymocytes in foetal (n=4) and post-natal (n=10) thymus. **b-e** Comparison of the CDR3 TRGV9 (top) and TRDV2 (bottom) repertoire of sorted foetal (n=3) and post-natal (n=3) Vγ9Vδ2 thymocytes: **b** Frequency of CDR3 repertoire per number of N additions, **c** Frequency of repertoire per CDR3 length, **d** Number of nucleotides trimmed at the Vend of the CDR3; each dot represents the weighted mean of an individual sample, **e** TRDD usage distribution. **f** TRGJ usage distribution in TRGV9 (top) and TRDV2 (bottom) CDR3 repertoire of sorted nonVγ9Vδ2 γδ+ thymocytes in foetal (n=3) and post-natal (PN) (n=3) thymus. Error bars indicate means ± SEM. **p < 0.01

3.4. Recombination of the germline-encoded public TRGV9-TRJP CDR3 sequence

The high abundance of the public germline-encoded invariant CDR3γ sequence 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3' in both the foetal blood and foetal thymus Vγ9Vδ2 T cells triggered us to investigate the mechanism of its rearrangement. The lack of N additions in the foetal repertoire may provide a favourable setting for the usage of short-homology repeats to generate invariant CDR3 as described for mouse innate γδ T cells[188,189]. Furthermore, P nucleotides can be involved in such a mechanism of invariant CDR3 generation[188]. We found that the addition of two P nucleotides at the end of the TRGV9 region generates a GCA sequence which is also found in the TRGJP region, and only in this TRGJ region (**Fig. III.4a-b**). This model explains the preferential recombination of the 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3' to encode CALWEVQELGKKIKVF in the absence of N additions in the foetus. In later life, when N additions are involved, the public TRGV9-TRGJP amino acid sequence CALWEVQELGKKIKVF can still be produced by other nucleotypes (**Fig. III.4c**)[35] but its prevalence is much lower, as previously discussed (**Fig. III.1g**). Strikingly, the nucleotides involved in this short-homology recombination are highly conserved among a series of non-human primate species (**Fig. III.4d**), except for the TRGJP gene of orangutan, which is a pseudogene[40].

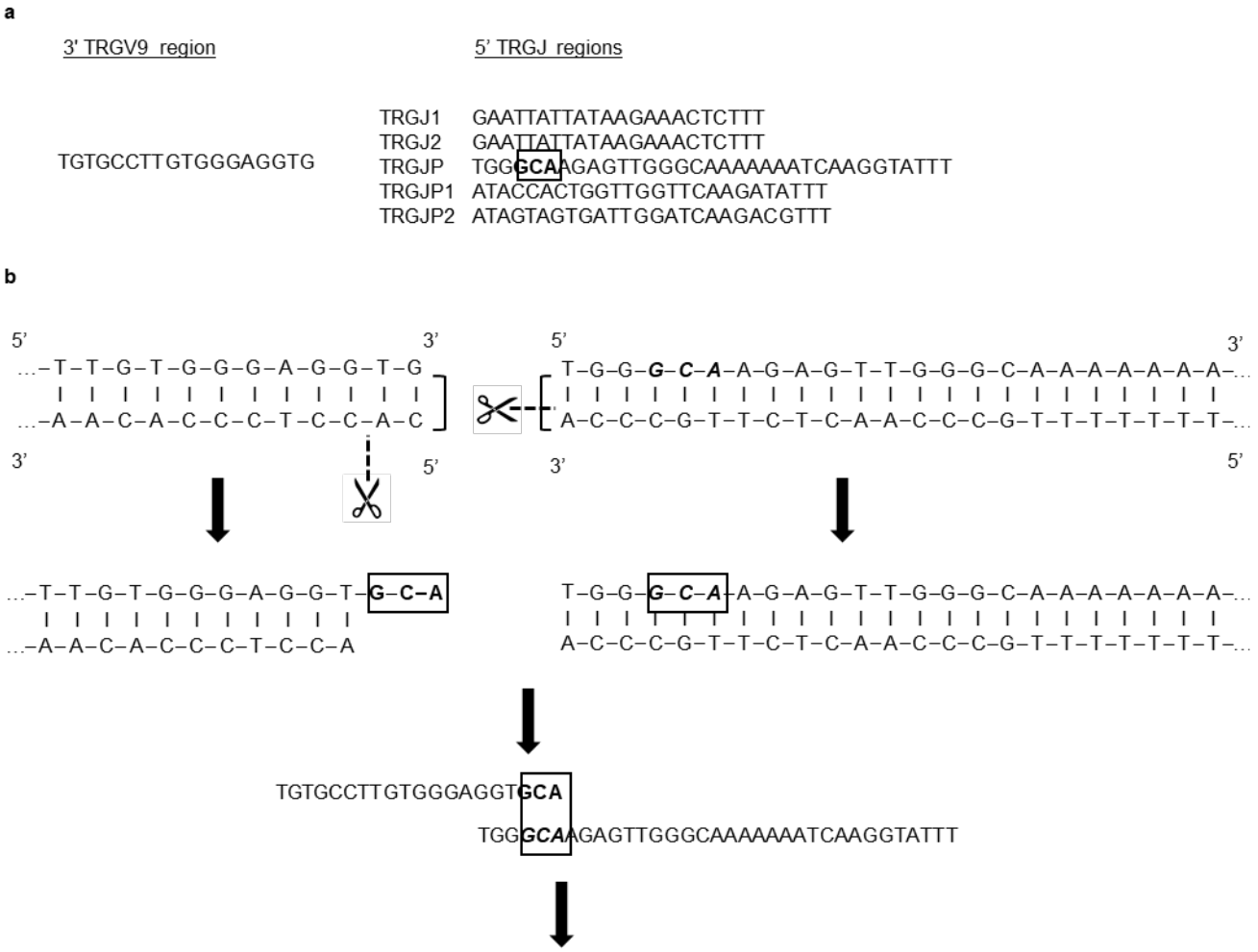


Fig. III.4. Recombination of the germline-encoded public TRGV9-TRGJP CDR3 sequence via the short-homology repeat GCA. **a** Sequence of the 3' end of TRGV9

and all possible TRGJ 5’ regions. **b** Short-homology repeats (GCA) direct the site of recombination when TRGV9 joins TRGJP, to form the highly prevalent 5’-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3’ clonotype in absence of N additions (scissors: endonuclease activity; AA: amino acid). **c** Nucleotypes containing nucleotide insertions (N underlined, P in italics) encoding the TRGV9-TRGJP clonotype CALWEVQELGKKIKVF. **d** Conservation of the nucleotide sequence GCA in primates: the great apes *Pan troglodyte*s (chimp) and *Pongo pygmaeus* (orangutan, here TRGJP is a pseudogene), the new-world monkeys *Macaca mulatta* (rhesus) and *Macaca fascicularis* (crab-eating macaque) and the old-world monkeys *Aotus nancymaae* (ma’s night monkey) and *Callithrix jacchus* (marmoset).

3.5. HMB-PP-expanded post-natal Vγ9Vδ2 thymocytes express an adult blood-type CDR3 repertoire

The post-natal thymic Vγ9Vδ2 TCR repertoire (**Fig. III.3**) resembled the adult blood Vγ9Vδ2 repertoire (**Fig. III.1**), suggesting that the adult blood Vγ9Vδ2 T cells are post-natal thymus-derived and expand in the periphery upon microbial phosphoantigen exposure after birth. In order to test this hypothesis, we expanded post-natal Vγ9Vδ2 thymocytes with the microbial-derived phosphoantigen HMB-PP and analysed their CDR3 repertoire comparing it to the adult blood repertoire. We observed high specific expansion of the Vγ9Vδ2 thymocytes upon stimulation with HMB-PP (**Fig. III.5a**), as previously observed with exposure towards heat-killed *Mycobacterium tuberculosis*[190]. The level of mean N additions did not change upon expansion (**Fig. III.5b**). In contrast, there was an increase in the TRGJP usage till adult blood-type levels accounting at the same time for an increase of the TRGV9 CDR3 length compared to *ex vivo* thymocytes (**Fig. III.5c, d**). Notably, the CDR3γ and CDR3δ diversity of the expanded Vγ9Vδ2 thymocytes became very similar to the adult blood repertoire (**Fig. III.5e, f**) consistent with the focusing of the repertoire in the periphery.

No major differences were observed upon HMB-PP-induced expansion regarding the overlap frequency of the repertoire and the abundance of the public TRGV9 clonotype (CALWEVQELGKKIKVF) which was already similar between *ex vivo* Vγ9Vδ2 thymocytes and adult blood Vγ9Vδ2 T cells (**Fig. III.5g, h**). In sharp contrast, HMB-PP-mediated expansion increased the percentage of the TRDV2 repertoire using at position 5 a hydrophobic residue, a feature important for phosphoantigen-reactivity[22,23,25], approaching adult blood-type levels (**Fig. III.5i**).

In summary, post-natal Vγ9Vδ2 thymocytes are able to expand upon phosphoantigen exposure and acquire a CDR3 repertoire that closely resembles that of adult blood Vγ9Vδ2 T cells unlike the expanded foetal blood Vγ9Vδ2 T cells.

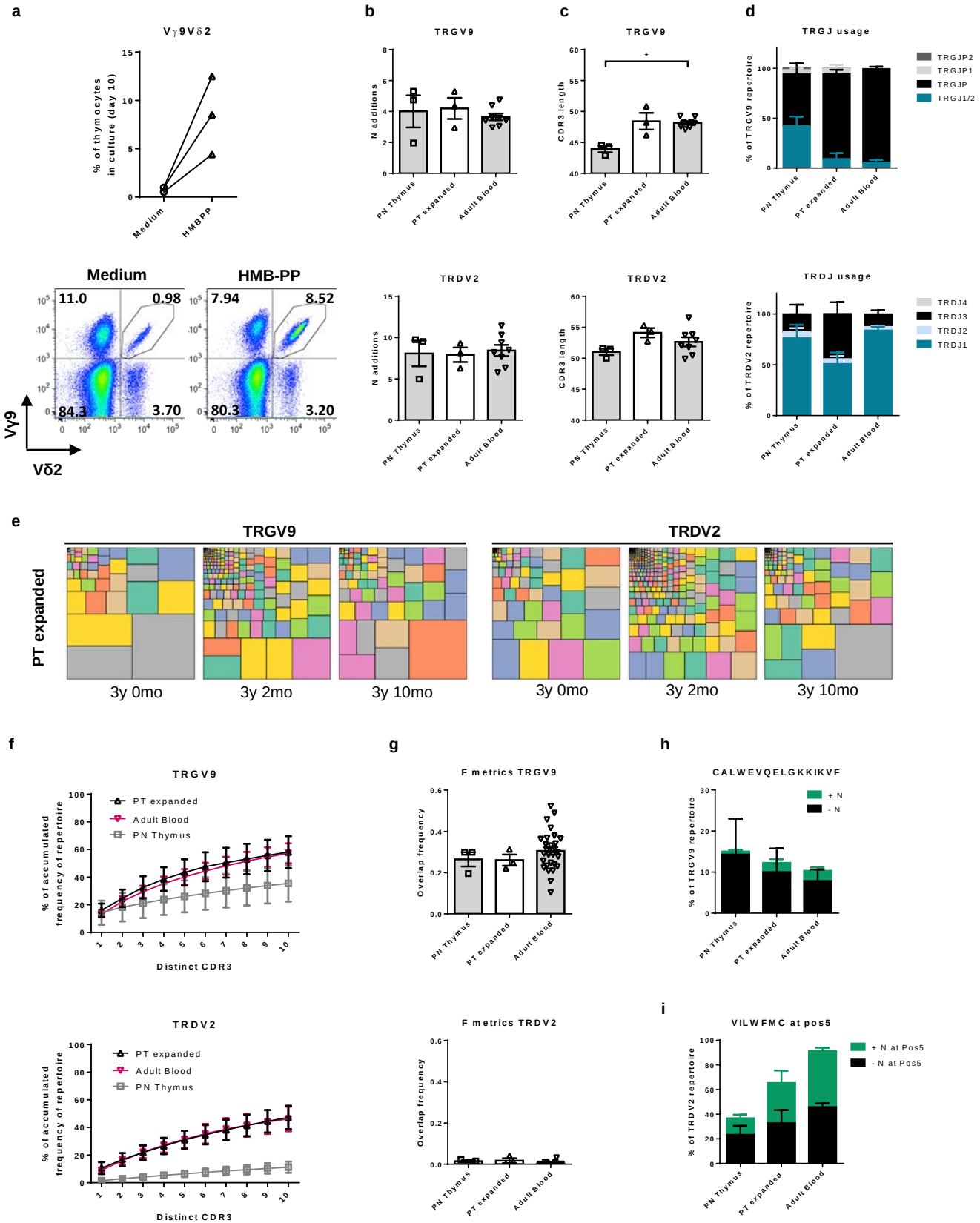

a
Vγ9Vδ2
% of thymocytes in culture (day 10)
Medium
HMBPP
Medium
HMB-PP
11.0
0.98
84.3
3.70
7.94
8.52
80.3
3.20
Vγ9
Vδ2
b
TRGV9
N additions
PN Thymus
PT expanded
Adult Blood
TRDV2
c
TRGV9
CDR3 length
TRDV2
d
TRGJ usage
% of TRGV9 repertoire
TRGJP2
TRGJP1
TRGJP
TRGJ1/2
TRDJ usage
% of TRDV2 repertoire
TRDJ4
TRDJ3
TRDJ2
TRDJ1
e
TRGV9
TRDV2
PT expanded
3y 0mo
3y 2mo
3y 10mo
f
TRGV9
% of accumulated frequency of repertoire
PT expanded
Adult Blood
PN Thymus
Distinct CDR3
TRDV2
g
F metrics TRGV9
Overlap frequency
F metrics TRDV2
h
CALWEVQELGKKIKVF
% of TRGV9 repertoire
+ N
- N
i
VILWFMC at pos5
% of TRDV2 repertoire
+ N at Pos5
- N at Pos5

Fig. III.5. HMB-PP-expanded post-natal Vγ9Vδ2 thymocytes express an adult blood-type CDR3 repertoire. **a** Expansion of post-natal Vγ9Vδ2 thymocytes after exposure to HMB-PP (10nM) in presence of IL-2 (100U/ml) for 10 days. Graph shows the percentage of Vγ9+Vδ2+ cells of total thymocytes in culture, in medium control (+IL-2) and HMB-PP (+IL-2) at day 10. Flow cytometry plots representative of 3 subjects. **b-d** CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire analysis in expanded Vγ9Vδ2 thymocytes compared to *ex-vivo* post-natal thymic and adult blood Vγ9Vδ2: **b** Number of N additions, each dot represents the weighted mean of an individual sample, **c** CDR3 length (nucleotide count including the C-start and F-end residues), each dot represents the weighted mean of an individual sample, and **d** J usage distribution. **e** Treemaps show CDR3 clonotype usage in relation to TRGV9 (left) and TRDV2 (right) repertoire size in sorted expanded post-natal Vγ9Vδ2 thymocytes (PT expanded) (rectangle colours are chosen randomly and do not match between plots). **f-i** CDR3 TRGV9 (top row) and TRDV2 (bottom row) repertoire analysis in expanded Vγ9Vδ2 thymocytes compared to *ex-vivo* post-natal thymic and adult blood Vγ9Vδ2: **f** Accumulated frequency curves generated from the 10 most prevalent clonotypes, **g** Geometric mean of relative overlap frequencies (F metrics of VDJ tools) within pairs of PN thymus, PT expanded and adult blood, each dot represents the F value of a pair of samples, **h** Percentage of the public TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N additions in black (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') or encoded with N additions in green and **i,** Percentage of the TRDV2 repertoire containing at the position 5 of the CDR3δ a highly hydrophobic residue (V, I, L, W, F, M, C): residue encoded without N additions in black or encoded by N addition(s) in green. Data shown from independent subjects, sorted Vγ9Vδ2 from post-natal thymus after HMB-PP expansion "PT expanded" n=3 , ex-vivo post-natal thymus "PN Thymus" n=3 (results also shown in Fig. III.4), ex-vivo adult blood "Adult Blood" n=8 (results also shown in Fig. III.1). Error bars indicate means ± SEM.

3.6. The generation of foetal-like or adult-like Vγ9Vδ2 T cells is HSPC-dependent

To investigate whether the differences between the foetal versus post-natal Vγ9Vδ2 thymocyte repertoire were caused by different properties of the foetal and postnatal hematopoietic precursor cells or rather by extrinsic factors such as intrathymic exposure to phosphoantigens, we cultured hematopoietic precursor cells from a spectrum of sources (foetal liver, foetal blood, cord blood, and adult blood) in the OP9DL1 *in vitro* T cell development system to generate γδ T cells[182]. Although at a low level, Vγ9+Vδ2+ γδ T cells could be generated using this system and their prevalence was inversely proportional to the age of the HSPC source (**Fig. III.6a**). Strikingly, the mean number of N additions within the TRGV9-TRGJP repertoire increased from almost zero, when using foetal liver as the HSPC source, to 2-4 N insertions, when cord or adult blood HPSC were used (**Fig. III.6b**). These numbers are very similar to what is observed in *ex vivo* sorted Vγ9Vδ2 thymocytes (**Fig. III.3b; supplementary Fig. III.3b**). Furthermore, the public canonical TRGV9-TRGJP nucleotype was generated very efficiently by foetal-derived HSPC (foetal liver and foetal blood) but only poorly by adult-derived HSPC; cord blood-derived HSPC generated intermediate percentages (**Fig. III.6c**). Likewise, in the adult/cord HSPC-derived CDR3δ repertoire, there were more TRDV2-containing CDR3δ sequences that used N-additions to encode the hydrophobic amino acid at position 5 (**Fig. III.6d**). Taken together, these data indicate that the HSPC source, foetal or later life, drives the generation of the Vγ9Vδ2 T cells towards a foetal- or adult-type repertoire.

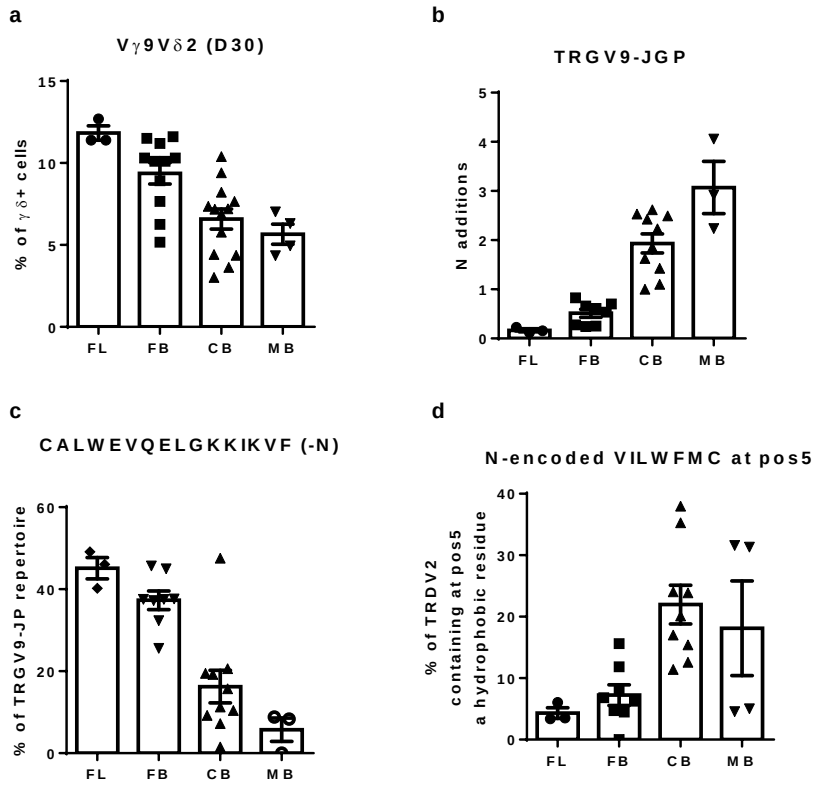


Fig. III.6. The generation of foetal- versus adult-like Vγ9Vδ2 T cells is HSPC-dependent. **a**. Percentage of Vγ9+Vδ2+ of γδ T cells produced in OP9DL1 cultures by foetal liver (FL, n=3), foetal blood (FB, n=8), cord blood (CB, n=10) and adult blood (MB, n=3) HSPC at day 30 of culture. **b-d** Comparison of the CDR3 TRGV9 and TRDV2 repertoire from the OP9DL1 cultures: **b** Number of N additions, each dot represents the weighted mean of an individual sample and, **c** Prevalence of the public TRGV9-TRGJP clonotype CALWEVQELGKKIKVF encoded without N additions (5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3') within the TRGV9-TRGJP repertoire; **d** Percentage of repertoire where the residue at position 5 was N-encoded out of the TRDV2 repertoire containing at position a highly hydrophobic residue (V, I, L, W, F, M or C). Error bars indicate means ± SEM.

4. Discussion

In the last few years, fate-mapping in mouse models has revealed the developmental origins of various immune cell types, including the DETC innate γδ T cell subset[45,191,192]. However, such fate-mapping approach is not possible for the innate phosphoantigen-reactive Vγ9Vδ2 T cells since these cells do not exist in rodents[42]. In this study, we used CDR3 HTS to track human Vγ9Vδ2 T cells and discovered that, unlike adult mouse innate γδ T cells which are generated in a single foetal wave, the development of Vγ9Vδ2 T cells continues after birth resulting in the adult Vγ9Vδ2 TCR repertoire.

Our data contrast with the hypothesis that the adult blood Vγ9Vδ2 repertoire is derived by selection after birth from the foetal-generated Vγ9Vδ2 T cells[20,30]. As we studied foetal and postnatal thymus, we could show that the adult-type blood Vγ9Vδ2 TCR repertoire (e.g. a private repertoire with a higher number of N insertions) was present in the post-natal but not in the foetal thymus. In addition, phosphoantigen selection *in vitro* by microbial-derived HMB-PP further sculptured the postnatal thymic repertoire to a very similar repertoire as found in the adult blood (e.g. further enrichment of TRGJP usage). Therefore, we believe that the adult blood Vγ9Vδ2 repertoire is generated in the postnatal rather than the foetal thymus and is further selected in the periphery by microbial phosphoantigen exposure. The HMB-PP-induced selection of the adult Vγ9Vδ2 TCR repertoire is consistent with the higher response of adult Vγ9Vδ2 T cells compared to their foetal counterparts upon stimulation *in vitro* with HMB-PP[20,193]. The distinct development in the foetal versus post-natal thymus could be mimicked in the *in vitro* T cell development system OP9DL1: the foetal canonical CDR3γ nucleotype (TRGV9-TRGJP 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3', encoding the clonotype CALWEVQELGKKIKF) was generated much more efficiently by foetal-derived HSPC compared to post-natal-derived HSPC, thus indicating that stem/precursor cell autonomous properties underpin this distinct development. While we found that the canonical CDR3γ nucleotype and associated clonotype is highly prevalent in early life and less in the adult, it appears that the situation for the canonical CDR3α (defined by selected usage of TRAV, TRAJ and restricted CDR3 length) of human MAIT cells, another main innate T cell subset, is the opposite: the canonical

MAIT TCR is more frequent in adult than in cord blood MAIT cells[194]. This difference between cord and adult MAIT cells appears to be due to antigen-driven expansion[194], rather than by a distinct development as found here for foetal/cord versus adult Vγ9Vδ2 T cells.

In both foetal and adult blood Vγ9Vδ2 T cells the TRGV9-TRGJP pairing and CDR3γ length restriction was conserved, consistent with the demonstration that these features are essential for phosphoantigen-reactivity[22,23]. This is also compatible with the notion that the Vγ9Vδ2 TCR can be regarded as a pattern recognition receptor and that Vγ9Vδ2 T cells are the main innate γδ T cell subset in human (Ravens 2017, Davey 2018, Liuzzi 2015). However, our data highlighted a series of differences in the CDR3γ repertoire of foetal and adult Vγ9Vδ2 T cells. While the germline-encoded and phosphoantigen-reactive canonical TRGV9-TRGJP nucleotype was present both in early and later life, it was much more prevalent in the foetal blood Vγ9Vδ2 repertoire. This nucleotype has been described to occupy more than 45% of all CDR3γ sequences in the adult blood circulation, but a more recent study[37] reported a much lower percentage (4% of all CDR3γ) consistent with our findings in the adult blood. We propose that the absence of N insertions in the foetal Vγ9Vδ2 thymocyte repertoire allows the usage of a short-homology repeat (GCA, germline-encoded in the TRGJP and generated via the addition of P nucleotides in TRGV9) driving the recombination of the TRGV9-TRGJP nucleotype 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT-3' , resulting in its high prevalence in the foetus. In the adult, N additions prevent this short-homology repeat-mediated recombination[189] and rather generate public N-containing phosphoantigen-reactive CDR3γ sequences via convergent recombination[35], consistent with a different origin of the foetal and adult Vγ9Vδ2 T cells. We found that the TRGV9-TRGJP GCA short-homology repeat is highly conserved among primates, similar to the conservation of amino acid residues important for phosphoantigen reactivity[33,40,195]. Based on the high evolutionary conservation of the short-homology-generated germline-encoded TRGV9-TRGJP nucleotype and its high prevalence in the foetus, we propose that protection against congenital infections with HMB-PP-producing pathogens such as *Plasmodium falciparum*, *Toxoplasma gondii*, *Treponema pallidum* or *Brucella abortus*[54,196,197], has provided a selective pressure for a germline-encoded phosphoantigen-reactive TCR early during foetal development[46]

and that the short-homology repeat identified here contributes to the efficient generation of such a TCR.

The TRDV2-associated CDR3 length was much more variable compared to the CDR3γ. Despite this variation, the amino acid at position 5 was highly enriched for a hydrophobic residue, both in foetal and adult blood Vγ9Vδ2 T cells, consistent with its importance for phosphoantigen-reactivity[22,23]. But the coding of this particular hydrophobic acid was germline based in the foetus, while in the adult a large fraction was formed by N-containing codons, thus again consistent with a distinct development of foetal and adult Vγ9Vδ2 T cells. In addition, the CDR3δ of foetal blood Vγ9Vδ2 T cells were strikingly enriched for TRDJ3 usage while adult blood Vγ9Vδ2 T cells mainly used TRDJ1. A recent HTS study based on sorted Vδ2+ T cells (thus containing both Vγ9+Vδ2+ and Vγ9-Vδ2+ cells) found that the enriched cord blood TRDV2-TRDJ3 CDR3 sequences contained only a low percentage of a hydrophobic residue at position 5[30]. It has been suggested that this contributes to the low phosphoantigen-reactivity of cord blood Vγ9Vδ2 T cells and thus to the high enrichment of TRDJ1 in adult Vγ9Vδ2 T cells after birth upon phosphoantigen exposure[30]. However, we found that both the TRDV2-TRDJ1 and TRDV3-TRDJ3 CDR3 sequences of sorted Vγ9Vδ2 T cells were enriched for a hydrophobic amino acid at this position. Furthermore, *in vitro* expansion with the microbe-derived phosphoantigen HMB-PP did not result in a bias towards TRDJ1 usage, which is consistent with the enrichment of the hydrophobic amino acid in both TRDJ3- and TRDJ1-containing CDR3δ sequences.

Our data highlight the importance of several Vγ9Vδ2 TCR features for phosphoantigen-reactivity as they are conserved in foetal and adult life regardless of the distinct way these features are encoded. Despite the major advancement of the discovery of BTN3A1 as a crucial protein in the activation of Vγ9Vδ2 T cells with phosphoantigens, the exact mechanism of interaction with the Vγ9Vδ2 TCR is yet to be revealed[174]. Thus it is also unclear what the exact role of the TRGJP sequence is, in particular the conserved amino acids important for phosphoantigen reactivity[22,23,42], why a restricted CDR3γ length of 14 +/- 1 aa is needed and what the role is of the hydrophobic amino acid at position 5 of the CDR3δ. Indeed, it remains to be established what the direct ligand is of the Vγ9Vδ2 TCR and thus the potential interacting domains with these CDR3γ and CDR3δ features[174].

Our data may have implications for immunotherapeutic approaches that target Vγ9Vδ2 T cells. Although the *in vivo* expansion of Vγ9Vδ2 T cells by phosphoantigens or nitrogen-containing bisphosphonates such as zoledronate has been translated to early-phase clinical trials, problems such as activation-induced Vγ9Vδ2 T cell anergy and a decrease in the number of peripheral blood Vγ9Vδ2 T cells after infusion of these stimulants have not yet been solved[175–177]. In addition, it is difficult to expand *ex vivo* Vγ9Vδ2 T cells from advanced cancer patients with decreased initial numbers of peripheral blood Vγ9Vδ2 T cells[175]. This is important as favourable clinical outcomes are related to higher frequency of peripheral blood Vγ9Vδ2 T cells[175,177]. Thus, novel approaches are needed to stably expand and maintain the responsiveness and functions of Vγ9Vδ2 T cells. Since our data strongly indicate that Vγ9Vδ2 T cells within the blood circulation of adults are derived postnatally, strategies could be developed to enhance *de novo* generation of Vγ9Vδ2 T cells in cancer patients[198,199].

IV. Foetal public Vγ9Vδ2 T cells expand and gain potent cytotoxic functions early after birth

Abstract

Vγ9Vδ2 T cells are a major human blood γδ T cell population that respond in a T cell receptor (TCR)-dependent manner to phosphoantigens which are generated by a variety of microorganisms. It is not clear how Vγ9Vδ2 T cells react towards the sudden microbial exposure early after birth. We found that Vγ9Vδ2 T cells with a public/shared foetal-derived TCR repertoire expanded within 10 weeks postpartum. Such an expansion was not observed in the non-Vγ9Vδ2 γδ T cells, containing a private TCR repertoire. Furthermore, only the Vγ9Vδ2 T cells differentiated that early into potent cytotoxic effector cells, despite their foetal origin. Both the expansion of public foetal Vγ9Vδ2 T cells and their functional differentiation were surprisingly not affected by vaccination at birth with the phosphoantigen-containing vaccine BCG. These findings suggest a strong and early priming of the public foetal-derived Vγ9Vδ2 T cells promptly after birth, most likely upon environmental phosphoantigen exposure.

1. Introduction

γδ T cells have been conserved, together with αβ T cell and B cells, since the emergence of jawed vertebrates more than 450 million years ago and can play an important role in anti-microbial and anti-tumour immunity[27,170,171]. γδ T cells are the first T cells made in virtually all species examined and are thought to play an important role especially in conditions when αβ T cell responses are impaired such as in early life[29,32,44,157,170,200–202].

γδ T cells, like αβ T cells and B cells, use V(D)J gene rearrangement with the potential to generate a set of highly diverse receptors to recognize antigens. This diversity is generated mainly in the complementary-determining region 3 (CDR3) of the T cell receptor (TCR) via combinatorial and junctional diversity[172,173]. Vγ9Vδ2 T cells express a TCR containing the γ-chain variable region 9 (Vγ9, TRGV9) and the δ-chain variable region 2 (Vδ2, TRDV2) and are the dominant population of γδ T cells in the blood circulation of human adults. They are activated and expanded in a TCR-dependent manner by microbe- and host-derived phosphorylated prenyl metabolites (phosphorylated antigens or 'phosphoantigens'), derived from the isoprenoid metabolic pathway[34,54,174]. This recognition of phosphoantigens allows Vγ9Vδ2 T cells to develop potent antimicrobial and anticancer responses[54,171,203–206]. While Vγ9Vδ2 T cells are also abundant in the blood of mid-gestation foetuses, they represent only a small percentage of γδ T cells at birth[20,50]. Foetal and adult blood Vγ9Vδ2 T cells have a different developmental origin (as revealed by TCR sequencing), show different phosphoantigen activation thresholds and adult Vγ9Vδ2 T cells possess cytotoxic effector functions that are absent from their foetal counterparts[20,157,207]. It is not clear, however, how Vγ9Vδ2 T cells respond upon the sudden environmental change at the transition from the (almost) sterile in utero environment to the microbial phosphoantigen exposure at birth. Furthermore, it is not known whether early after birth the Vγ9Vδ2 T cells in the blood circulation are still 'foetal-like' or whether a switch towards 'adult-like' Vγ9Vδ2 T cells is already initiated.

Since the tuberculosis (TB) vaccine Bacille Calmette-Guérin (BCG) is used to vaccinate infants at birth, this vaccination allows investigation of T cell responses early after birth[208]. BCG contains phosphoantigens[59,61] and using adult non-human primate

models (Vγ9Vδ2 T cells do not exist in rodents[42]), it has been shown that Vγ9Vδ2 T cells expand upon BCG vaccination, which correlated with protection against TB[143]. Consistent with these observations is the description of γδ T cell responses in human BCG-vaccinated adults and infants[145–147,209,210]. Taken together, BCG can be regarded as a potent Vγ9Vδ2 T cell activator to study Vγ9Vδ2 T cell responses *in vivo*, including in early life.

Here we found that Vγ9Vδ2 T cells expanded early after birth (within 10 weeks), possessing a public TCR repertoire which was related to their foetal origin. Furthermore, despite this foetal origin and in contrast to private γδ T cell subsets and conventional αβ T cells, they showed a pronounced differentiation towards adult-like cytotoxic effector cells. Finally, this early and strong Vγ9Vδ2 T cell response was not altered by BCG vaccination at birth, highlighting an important role of the environmental exposure in the expansion and functional differentiation of foetal-derived Vγ9Vδ2 T cells promptly after birth.

2. Materials and Methods

2.1. Study Population - Human cell material

We compared host responses of 10-week-old infants (10w) with those in cord and adult blood. The 10-week-old infants consisted of two groups, one vaccinated with BCG (Danish 1331 strain, Statens Serum Institute, Denmark) at birth as is routine in South Africa (BCG+, median age 64 days, min 56 – max 74), and another group not vaccinated with BCG at birth (BCG-, median age 68 days, min 61 – max 86). In those not vaccinated at birth, BCG vaccine was administered at 10 weeks of age, immediately after blood collection. Control samples were collected from new-borns (cord blood) and adults from the same community (all independent donors).

New-borns, infants and adults were enrolled at the South African Tuberculosis Vaccine Initiative (SATVI) field site, near Cape Town, and at private and public clinics in Worcester, South Africa. The protocol was approved by the University of Cape Town Human Research Ethics Committee (ref 177/2011). Written, informed consent was obtained from legal guardians of all infants and from adult donors.

Exclusion criteria for mothers included delivery through Caesarean section (except for cord blood, which was collected from women undergoing elective Caesarean section), significant complications during pregnancy, possible relocation to a different region, HIV+ or unknown/undisclosed HIV status, known chronic infections or any acute infection during the last trimester of pregnancy, suspicion of TB or known household contact with TB patients.

Exclusion criteria for infants included BCG vaccination before planned blood collection at 10 weeks of age (for the delayed group) or BCG vaccination not received at birth (for the group receiving routine BCG), current suspicion of TB or known household contact with TB patients in the first 10 weeks of life, isoniazid (INH) therapy during the first 10 weeks of life, any chronic disease in the first 10 weeks of life, any acute disease during the 2 weeks before blood collection, infants born before 37 weeks of gestation (preterm) and those with low birth weight (<2500g), congenital malformations or perinatal complications such as birth asphyxia, respiratory distress and severe jaundice, or chronic or current use of immunosuppressant treatments such as steroids.

Exclusion criteria for adults included chronic use of immune-modifying drugs in the last 6 months, any acute or chronic illness, history of TB disease, pregnant or lactating females.

All 10-week-old infants received standard EPI vaccinations before blood draw: oral polio and BCG at birth (for the BCG+ group); oral polio, diphtheria, tetanus, acellular pertussis, inactivated polio, haemophilus influenzae B, hepatitis B, rotavirus, pneumococcal conjugate (PCV7) at 6 weeks of age.

Whole blood was collected in CPT tubes or heparinised polypropylene tubes. PBMC were isolated from blood, cryopreserved, and shipped to Belgium for further analysis.

For the CDR3 repertoire analysis, 10-week-old Vγ9Vδ2 T cells were compared to foetal blood. Since the foetal blood samples originated from Belgium (no access to South-African foetal blood during this study), cord and adult blood from Belgium were included in parallel to the South-African cohort (cord, 10-week-old, adult blood). The Belgian samples (foetal, cord and adult blood) analysed here were previously described(22). Briefly, samples included foetal blood because of interruption of pregnancy (22-30 weeks of gestation), approved by the Hôpital Erasme ethics committee; umbilical cord blood after delivery (39-41 weeks term delivery) with the approval of the University Hospital Center Saint-Pierre; adult peripheral blood, approved by the Ethics committee of the CHU Tivoli, La Louvière. PBMC were isolated from blood and cryopreserved for subsequent experiments.

2.2. Flow cytometry, sorting and cell cultures

For flow cytometry and associated cell sorting, cells were thawed in complete medium, washed twice and either directly labelled with appropriate antibodies/dyes or first cultured for cytokine detection.

Antibodies/dyes used: Zombie dye (Biolegend), CD3 (clone UCHT1, BD), TCR-γδ (11F2, BD or Miltenyi), TCR-Vγ9 (IMMU360, Beckman Coulter), TCR-Vδ2 (IMMU389, Beckman Coulter), HLA-DR (G46-6, BD), CD27 (M-T271, BD), CD28 (CD28.2, Beckman Coulter), CD158a (HP-3E4, BD), CD158b (DX27, Biolegend), CD161 (HP-3G10, Biolegend), NKG2D (1D11, Biolegend), NKG2A (Z199, Beckman Coulter), NKG2C (134591, R&D Systems), KLRG1 (SA231A2, Biolegend), perforin (dG9 (delta G9), eBioscience), granzyme A, granzyme B (GB11, BD), granulysin (eBioDH2 (DH2), eBioscience), Ki-67 (B56, BD), T-bet (4B10, Biolegend), Eomes (WD1928, eBioscience), IFNγ (B27, BD), TNFα (MAb11, BD), IL-2 (MQ1-17H12, BD), IL-8 (E8N1,

Biolegend). For intracellular staining (granzyme A, granzyme B, granulysin, perforin, IFNγ, TNFα) the Cytofix/Cytoperm kit (BD) was used, and for intranuclear staining (T-bet, Eomes) the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) was used.

Samples were acquired for flow cytometry with the CyAn ADP LX9 or BD LSR Fortessa cytometer and analysis was performed with FlowJo v9. PCA analysis of the flow cytometric data was done with the ggfortify package on RStudio based on manual gating of positive cells with FlowJo.

CD3+ γδTCR+ Vγ9+ Vδ2+ were sorted for "Vγ9Vδ2" (mean purity 99.5% of CD3+ γδ+) and CD3+ γδTCR+ Vγ9+Vδ2-, Vγ9-Vδ2+ and Vγ9-Vδ2- γδ T cells were sorted for "non-Vγ9Vδ2" (mean purity 99.4% of CD3+ γδ+) with the BD FACS Aria III. Gating strategy: FS singlets → alive cells (zombie negative) → SSC-FSC lymphocyte/thymocyte gate → CD3+γδTCR+ → Vγ9+Vδ2+ or non-Vγ9Vδ2 T cells. Data were analysed using FlowJo software (BD).

PBMC were cultured at 37 °C, 5% CO2 in 14-mL polypropylene, round-bottom tubes (Falcon; BD) at a final concentration of 1 $\times 10^6$ cells/mL. Culture medium consisted of 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 FBS (Sigma). PMA and ionomycin were from Sigma; IL-2 (Proleukin) was from Chiron/Novartis; HMB-PP was from Echelon Bioscience, zoledronate was from Novartis. For the detection of cytokines after polyclonal stimulation, PBMC were stimulated for 4 hours with 10 ng/mL PMA (phorbol 12-myristate 13-acetate, specific activator of Protein Kinase C) and 2 μM ionomycin (stimulates Ca^{2+} release from the endoplasmic reticulum, activates Ca^{2+}-sensitive enzymes and synergises with PMA[211]) in the presence of protein transport inhibitors: 2 μM monensin and 5μg/ml Brefeldin A (Sigma). (Monensin prevents protein secretion by interacting with the Golgi transmembrane Na^{++}/H^{+} transport, while brefeldin A redistributes intracellularly produced proteins from the Golgi complex to the endoplasmic reticulum[212]). For detection of cytokines after phosphoantigen stimulation, PBMC were stimulated for 3 days or overnight with HMB-PP (10 nM) or zoledronate (10 μM) in the presence of IL-2; 4 hours before the cells were harvested for flow cytometry staining, monensin (2 μM) and Brefeldin A (5μg/ml) were added to the cultures.

2.3. TCRγ and TCRδ high-throughput sequencing

RNA was isolated from sorted cells (10000 Vγ9Vδ2 T cells per sample) with the RNeasy Micro Kit (Qiagen) or from OP9DL1 co-cultures with the RNeasy Mini Kit (Qiagen). cDNA was generated performing a template switch anchored RT-PCR. RNA was reverse transcribed via a template-switch cDNA reaction using TRCG (5'-CAAGAAGACAAAGGTATGTTCCAG) and TRDC (5'-GTAGAATTCCTTCACCAGACAAG) specific primers in the same reaction tube, a template-switch adaptor (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG) and the Superscript II RT enzyme (Invitrogen). The TRCG primer binds both TRCG1 and TRCG2. 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 TRCG1 and TRCG2 5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*AATAGTGGGCTTGGGGGAA ACATCTGCAT, adapter in italic) and a specific TRDC primer (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*ACGGATGGTTTGGTATGAG GCTGACTTCT, adapter in italic) and a primer complementary to the template-switch adapter (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*AAGCAGTGGTATCAACGCAG, adapter in italic) 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 centre, University of Liège, Belgium].

Raw sequencing reads from fastq files (read 1 and read 2) 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 2.1.12[184]. 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 analysed using VDJtools software version 1.2.1 using default settings[185]. Sequences out of frame and containing stop codons were excluded from the analysis. The CDR3 repertoire data shown are filtered for TRGV9 and TRDV2 sequences. Note that the

nucleotype lengths generated by VDJtools include the C and V ends of the CDR3 clonotypes. Tree maps were created using the Treemap Package on RStudio (https://CRAN.R-project.org/package=treemap).

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6. Parametric tests were used after verifying the normality of the data using the Kolmogorov-Smirnov normality test. Differences between groups were analysed using Kruskal–Wallis ANOVA and Dunn’s post-tests for non-parametric data.

3. Results

3.1. Vγ9Vδ2 T cells expand early after birth

First, we assessed the prevalence of Vγ9Vδ2 T cells and other γδ subsets (including Vγ9-Vδ2+ cells) within the peripheral blood of 10-week-old infants and compared it to cord and adult blood. The percentage of Vγ9Vδ2 T cells was higher in 10-week-old infants compared to cord (**Fig. IV.1a**), which was specific for this γδ subset (**Fig. IV.1a, right panel**). While the percentage of adult Vγ9Vδ2 T cells was also higher compared to cord, the expression of the proliferation marker Ki-67 was clearly the highest in the 10-week old group, compared to both cord and adult blood, highlighting an active phase of proliferation early after birth (**Fig. IV.1b**).

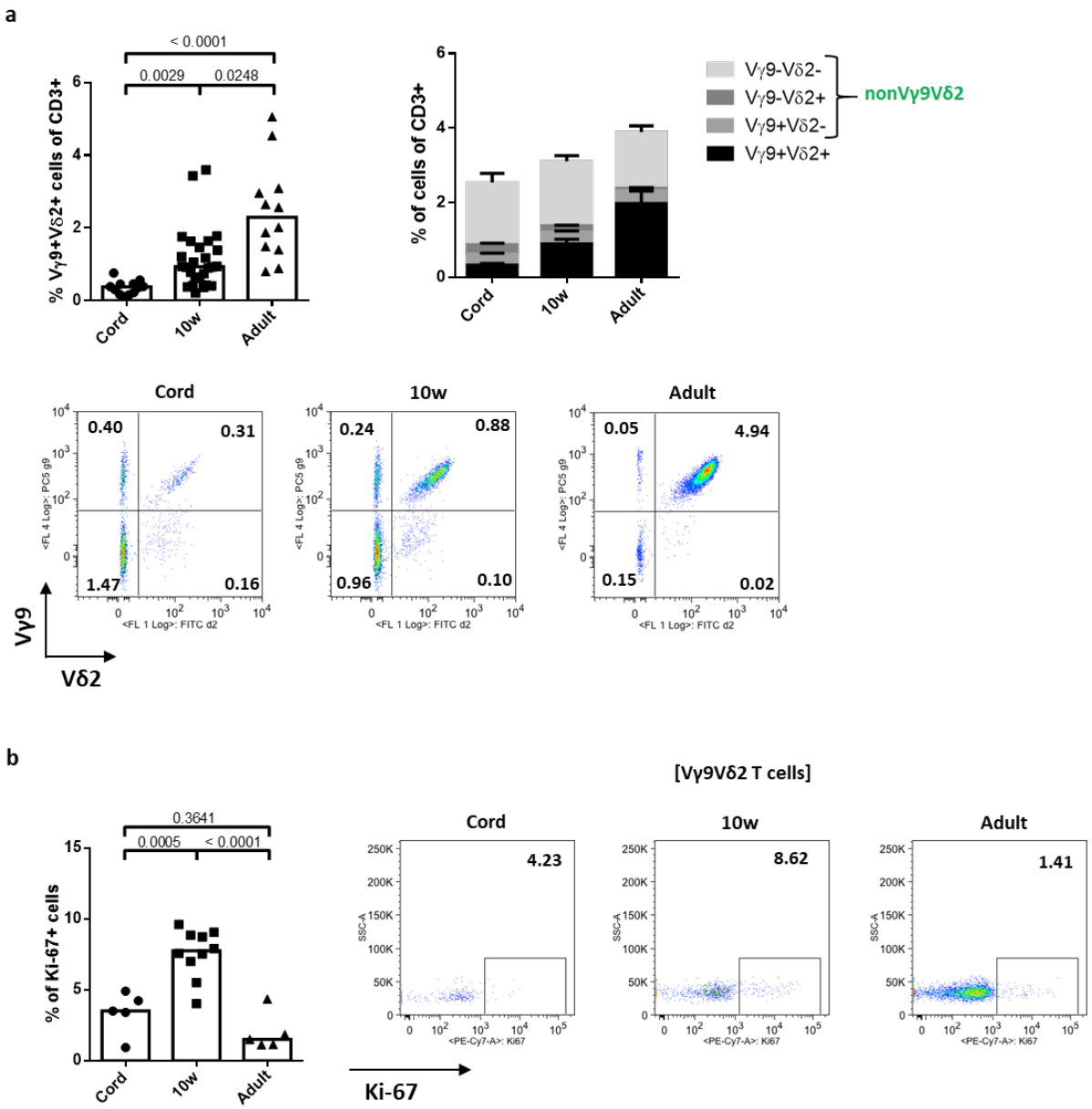


Fig. IV.1. Vγ9Vδ2 T cells expand early after birth. **a** Prevalence of the Vγ9+Vδ2+ subset in CD3+ cells (left panel, bars indicate medians) and accumulative prevalence of Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells among CD3+ cells (right panel, error bars indicate means ± SEM) in cord (n=12), 10-week-old (10w, n=26) and adult (n=12). Representative flow plots (bottom panel) gated on γδ+CD3+ cells, percentages out of CD3+. **b** Percentage of Ki-67+ cells among Vγ9Vδ2 T cells (cord, adult n=5, 10w n=10). Bars indicate medians. Representative flow plots (right panel). Data shown from independent subjects (South-Africa). p values are reported on graphs.

3.2. Only the 10-week-old Vγ9Vδ2 TRD repertoire is public and foetal-derived

Compared to adult Vγ9Vδ2 T cells, foetal and cord blood Vγ9Vδ2 T cells respond only poorly to microbial-derived phosphoantigens[20,157,193,213]. Thus, we wondered whether the expanded Vγ9Vδ2 T cells at 10 weeks after birth can be derived from foetal Vγ9Vδ2 T cells, or whether already an 'adult-like' Vγ9Vδ2 developmental program was initiated immediately after birth. In order to address this question, the TCR repertoire of sorted Vγ9Vδ2 T cells from 10-week-old infants were compared to their counterparts with a foetal and adult origin. For the foetal origin we compared it to the 'foetal' group, i.e. < 30 weeks of gestation, and to the 'cord' group, i.e. term delivery (>37w gestation)[207]. In contrast to the adult Vγ9Vδ2 T cells, the 10-week-old Vγ9Vδ2 TRD repertoire showed a high level of publicity, meaning that the TRD clonotypes of different subjects were shared (**Fig. IV.2a-b, left panels**). It was even more public than the cord blood TRD repertoire and reached a similar level of publicity as observed for foetal Vγ9Vδ2 T cells (**Fig. IV.2a-b, left panels**). A range of different clonotypes contributed to this publicity (**Fig. IV.2b, left panel**); the most public clonotypes at 10 weeks are shown in **Table IV.1**. Of note, the two most abundant clonotypes, CACDVLGDTDKLIF and CACDILGDTDKLIF, have been described to be highly prevalent in pre-thymic foetal liver[46]. Importantly, this observation was highly specific for the Vγ9Vδ2 T cell subset. Indeed, the TRD repertoire of 10-week-old non-Vγ9Vδ2 T cells was completely private like in adult non-Vγ9Vδ2 T cells, despite showing a significant level of publicity at the foetal stage (**Fig. IV.2a-b, right panels; supplementary Fig. IV.1**). An important feature in the detection of the developmental origin is the number of N additions used during the formation of the CDR3 by V(D)J recombination[207]. The 10-week-old Vγ9Vδ2 CDR3δ repertoire possessed a low foetal-like level of N additions (**Fig. IV.2c, left panel**), which was again specific for the Vγ9Vδ2 T cells as the non-Vγ9Vδ2 T cells from the same infants showed a high adult-like level of N additions in their CDR3δ sequences (**Fig. IV.2c, right panel**). In line with an important contribution of foetal-derived Vγ9Vδ2 T cells to the 10-week Vγ9Vδ2 TCR repertoire, was the relative high usage of the foetal-like TRDJ2-3 segment at the expense of the adult-like TRDJ1 (**Fig. IV.2d, left panel**). TRDJ2-3 are longer than TRDJ1 and therefore probably contribute to the maintenance of the CDR3δ length at 10 weeks compared to the cord Vγ9Vδ2 T cells (**Fig. IV.2e, left panel**), despite a reduction in the number of N additions. In contrast, the TRDJ usage of the 10-week-old non-Vγ9Vδ2 T cells was already similar to their adult counterparts (**Fig. IV.2d, right panel**). Of note, the high sharing of the

10-week-old Vγ9Vδ2 TRD repertoire was not directly associated with the preferential usage of TRDJ3. Indeed, TRDJ1-containing CDR3δ sequences of 10-week-old Vγ9Vδ2 T cells showed even a higher degree of publicity than TRDJ3-containing CDR3δ sequences (**Fig. IV.2f).** To investigate the foetal origin of the 10-week-old Vγ9Vδ2 T cells more directly, we examined the level of overlap between the 10-week-old CDR3δ repertoire and the other groups and observed that around 30% is similar to the foetal, while the overlap with the cord and adult repertoire was significantly lower (**Fig. IV.2g-h, left panels**). Once more, this was highly specific for the Vγ9Vδ2 subset, as such sharing was not observed between 10-week-old and foetal non-Vγ9Vδ2 T cells (**Fig. IV.2g-h, right panels**). The high sharing between foetal and 10-week-old Vγ9Vδ2 T cells was due to a relatively high number of clonotypes and not just a few abundant foetal ones (**Fig. IV.2h, left panel** overlap plot Nb figures; **Fig. IV.2i** overlappair plot for the Vγ9Vδ2 subset). The lower overlap found in cord (**Fig. IV.2a**) was in line with a higher diversity estimation (D25) which was later dampened at 10-weeks with the expansion of (foetal-derived) Vγ9Vδ2 T cells (**Fig. IV.2j**). The analysis of CDR3γ repertoire (**supplementary Fig. IV.2**) showed similar tendencies as the CDR3δ repertoire (**Fig. IV.2**), with the main exception that the adult TRGV9 is still largely public (**supplementary Fig. IV.2**), in line with previous studies[30,37,207].

In summary, TCR sequencing indicates that a large fraction of the early post-natal expanded infant Vγ9Vδ2 T cells is derived from <30week gestation foetal public Vγ9Vδ2 T cell clones.

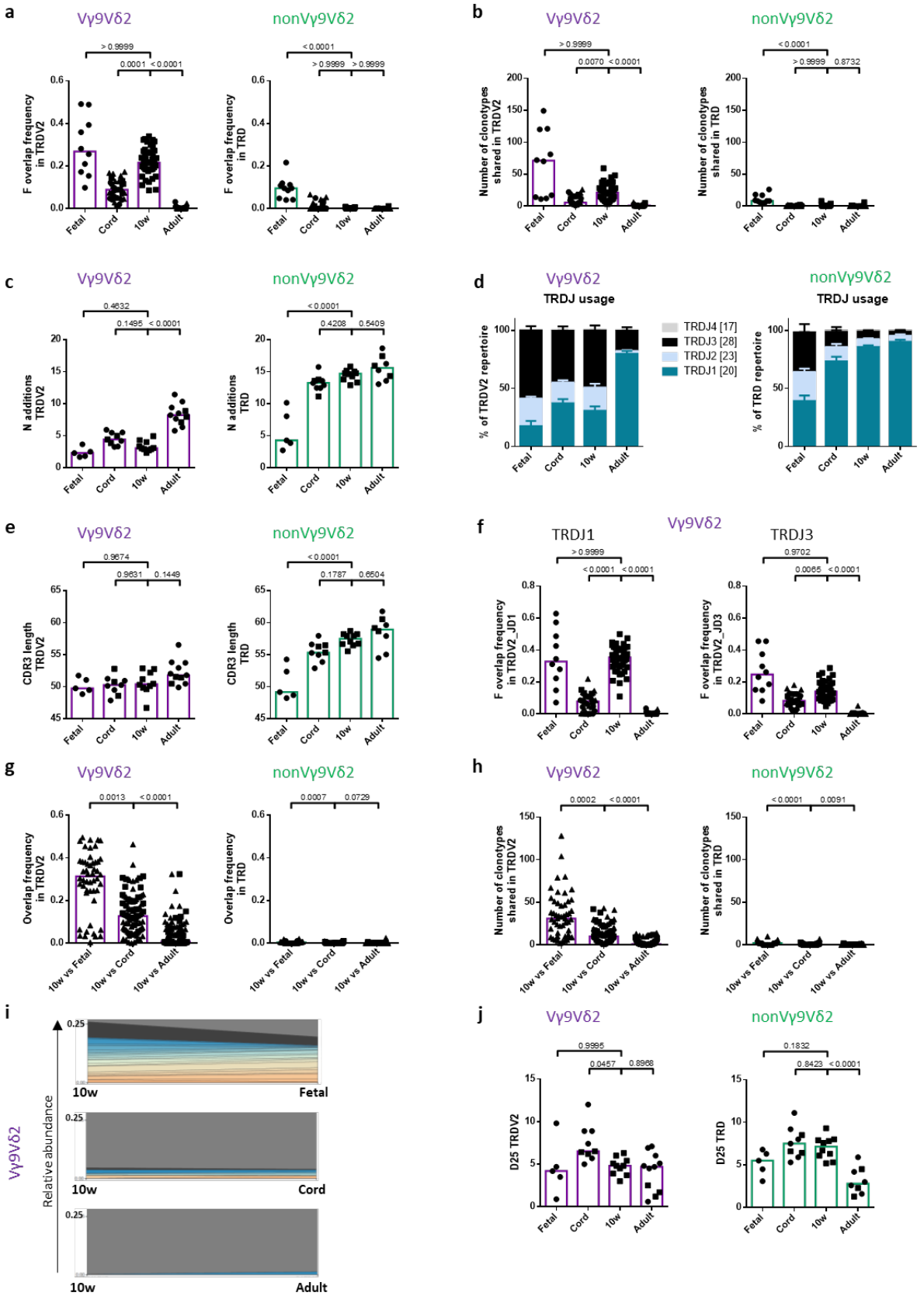
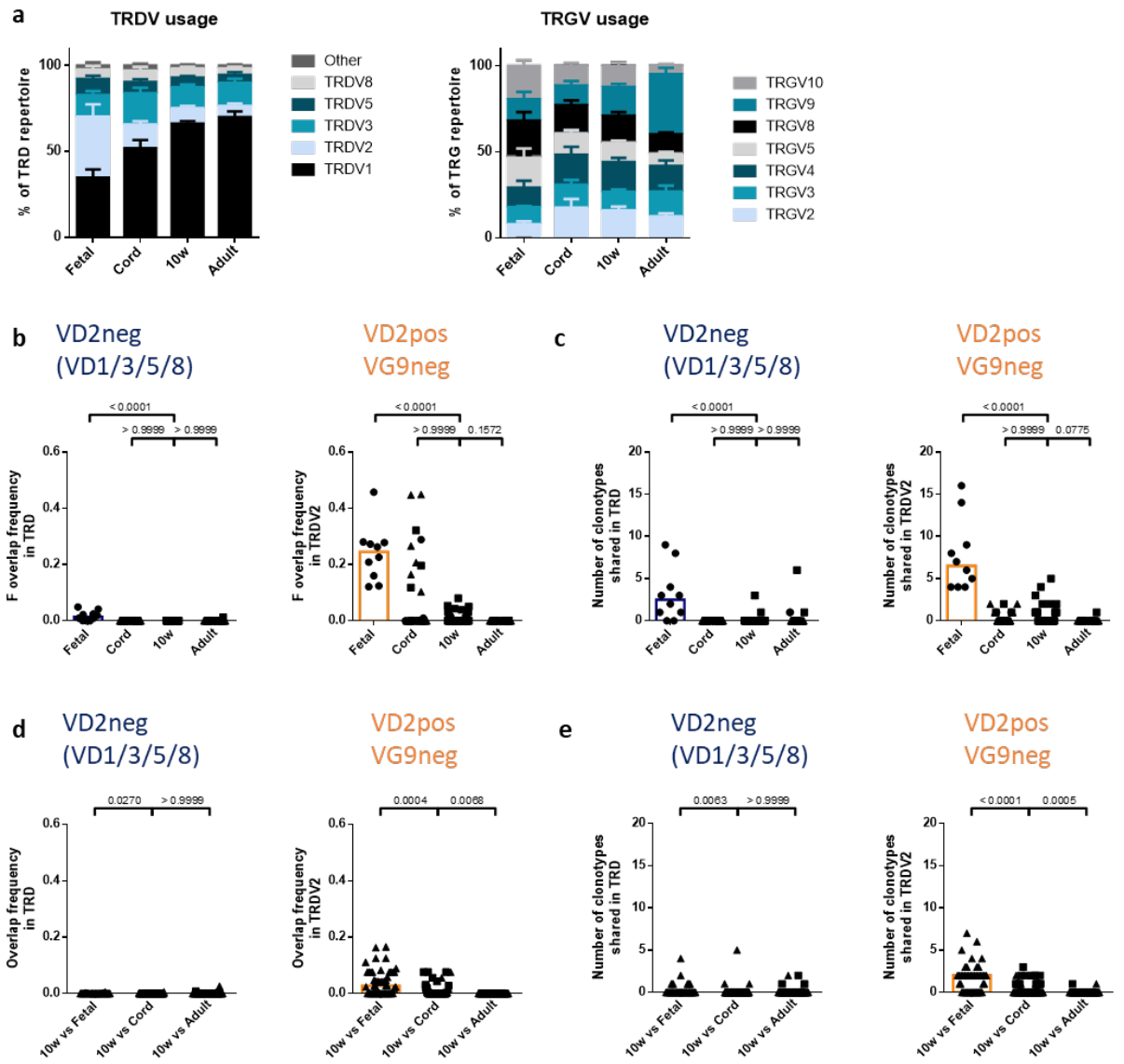
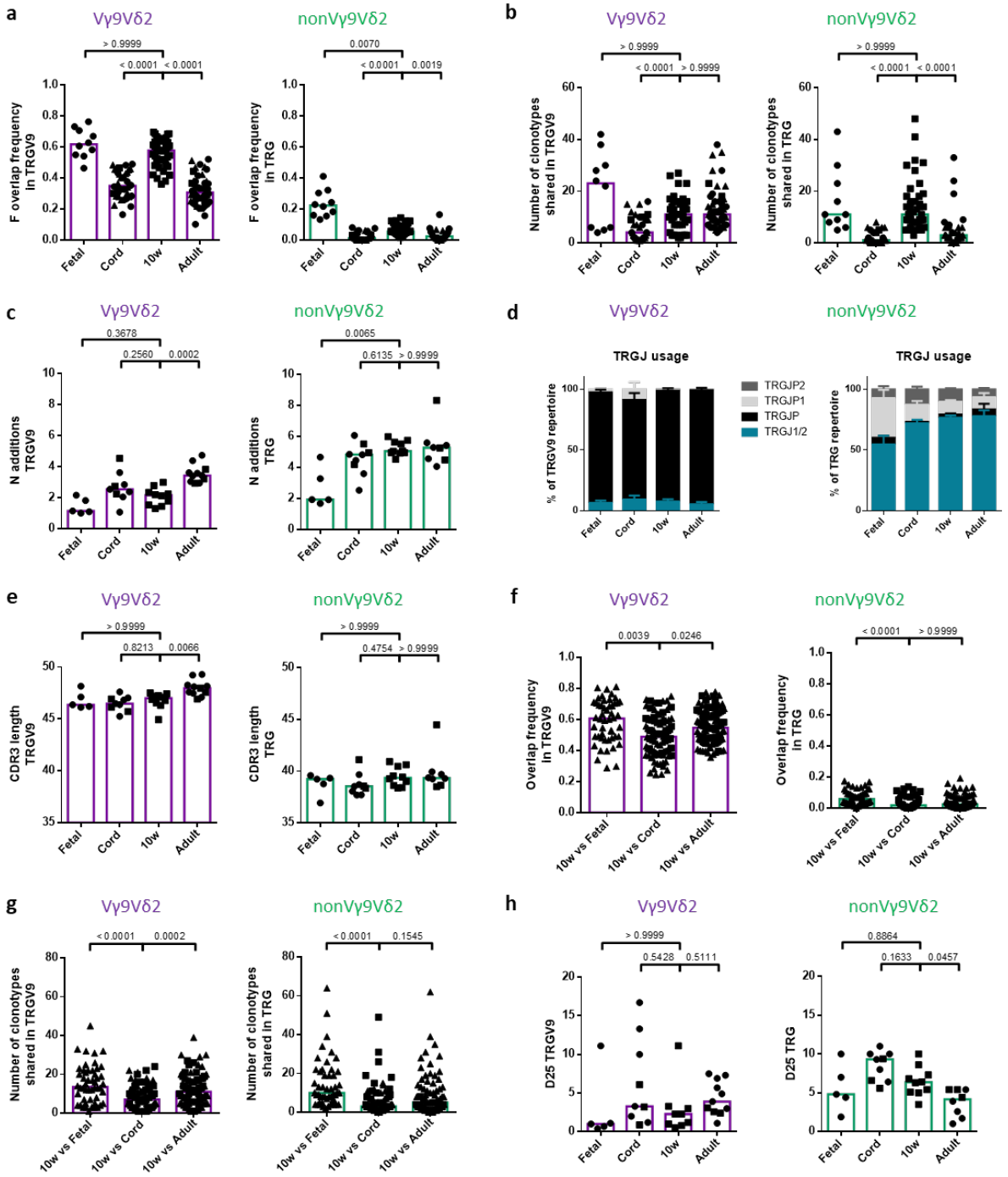

a
Vγ9Vδ2
nonVγ9Vδ2
F overlap frequency in TRDV2
F overlap frequency in TRD
b
Number of clonotypes shared in TRDV2
Number of clonotypes shared in TRD
c
N additions TRDV2
N additions TRD
d
TRDJ usage
% of TRDV2 repertoire
% of TRD repertoire
TRDJ4 [17]
TRDJ3 [28]
TRDJ2 [23]
TRDJ1 [20]
e
CDR3 length TRDV2
CDR3 length TRD
f
TRDJ1
TRDJ3
F overlap frequency in TRDV2_JD1
F overlap frequency in TRDV2_JD3
g
Overlap frequency in TRDV2
Overlap frequency in TRD
10w vs Fetal
10w vs Cord
10w vs Adult
h
Number of clonotypes shared in TRDV2
Number of clonotypes shared in TRD
i
Vγ9Vδ2
Relative abundance
10w
Fetal
Cord
Adult
j
D25 TRDV2
D25 TRD
Fetal
Cord
10w
Adult

Fig. IV.2. Only the 10-week-old Vγ9Vδ2 TCR repertoire is public and foetal-derived. a-e Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (left panels) and non-Vγ9Vδ2 γδ T cells (right panels), derived from foetal (n=5), cord (n=9), 10w (n=10) and adult (n=11 for Vγ9Vδ2 and n=8 for non-Vγ9Vδ2) blood. **a** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents the F value of a pair of samples. **b** Number of clonotypes shared within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents a pair comparison. **c** Number of N additions, each dot represents the weighted mean of an individual sample. **d** J gene segment usage distribution. (Error bars indicate means ± SEM; numbers in brackets refer to the J gene segment length). **e** CDR3 length (nucleotide count including the C-start and F-end residues), each dot represents the weighted mean of an individual sample. **f** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of foetal, of cord, of 10w and of adult blood subjects, in TRDJ1 repertoire (left panel) and TRDJ3 repertoire (right panel) of sorted Vγ9Vδ2 T cells derived from foetal (n=5), cord (n=6), 10w and adult (n=8) blood. Each dot represents the F value of a pair of samples. **g-h, j**. Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (left panels) and non-Vγ9Vδ2 γδ T cells (right panels), derived from foetal (n=5), cord (n=9), 10w (n=10) and adult (n=11 for Vγ9Vδ2 and n=8 for non-Vγ9Vδ2) blood. **g** Relative abundance of the 10w repertoire overlapping with foetal, cord or adult repertoire. Each dot represents a pair comparison. **h** Number of clonotypes shared between the 10w repertoire and foetal, cord or adult repertoire. Each dot represents a pair comparison. **i** Representative shared clonotype abundance plots for one 10w Vγ9Vδ2 TRD repertoire versus one foetal (top), versus cord (middle) and versus adult (bottom) comparison. Top 20 clonotypes shared are in distinct colours, collapsed in dark grey and non-overlapping in light grey. **j** Comparison of D25 values (percentage of unique clonotypes required to account for 25% of total repertoire). Data shown from independent subjects (from Belgium in round, from South-Africa in square, mixed pair comparisons in triangle). Bars indicate medians (a-c, e-h, j). p values are reported on graphs.



Supplementary Fig. IV.1. CDR3 repertoire analysis of non-Vγ9Vδ2 γδ T cells. a-e Description of the CDR3 repertoire of sorted non-Vγ9Vδ2 γδ T cells derived from foetal (n=5), cord (n=9), 10w (n=10) and adult (n=8) blood. **a** TRDV and TRGV usage distribution. B-E. TRD repertoire of Vδ2- γδ T cells (left panels) and Vδ2+Vγ9- γδ T cells (right panels). **b** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents the F value of a pair of samples. **c** Number of clonotypes shared within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents a pair comparison. **d** Relative abundance of the 10w repertoire overlapping with foetal, cord or adult repertoire. Each dot represents a pair comparison. **e** Number of clonotypes shared between the 10w repertoire and foetal, cord or adult repertoire. Each dot represents a pair comparison. Data shown from independent subjects (from Belgium in round, from South-Africa in square, mixed pair comparisons in triangle). p values are reported on graphs.



Supplementary Fig. IV.2. CDR3 TRG repertoire analysis. **a-h**. Description of the CDR3 TRG repertoire of sorted Vγ9Vδ2 T cells (left panels) and non-Vγ9Vδ2 γδ T cells (right panels), derived from foetal (n=5), cord (n=9), 10w (n=10) and adult (n=11 for Vγ9Vδ2 and n=8 for non-Vγ9Vδ2) blood. **a** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents the F value of a pair of samples. **b** Number of clonotypes shared within pairs of foetal, of cord, of 10w and of adult blood subjects, each dot represents a pair comparison. **c** Number of N additions, each dot represents the weighted mean of an individual sample. **d** J gene segment usage distribution. (Error bars indicate means ± SEM). **e** CDR3 length (nucleotide count including the C-start and F-end residues), each dot represents the weighted mean of an individual sample.

f Relative abundance of the 10w repertoire overlapping with foetal, cord or adult repertoire. Each dot represents a pair comparison. **g** Number of clonotypes shared between the 10w repertoire and foetal, cord or adult repertoire. Each dot represents a pair comparison. **h** Comparison of D25 values (percentage of unique clonotypes required to account for 25% of total repertoire). Data shown from independent subjects (from Belgium in round, from South-Africa in square, mixed pair comparisons in triangle). p values are reported on graphs.

CDR3 nucleotype (nt)	CDR3 clonotype (aa)	Number of N additions	Occurrences
TGTGCCTGTGACACCGTACTGGGGGATACCTGGGACACCCGACAGATGTTTTTC	CACDTVLGDTWDTRQMFF	0	10/10
TGTGCCTGTGACGTACTGGGGGACACCGATAAACTCATCTTT	CACDVLGDTDKLIF*	0	9/10
TGTGCCTGTGACATACTGGGGGACACCGATAAACTCATCTTT	CACDILGDTDKLIF**	0	9/10
TGTGCCTGTGACACCGTACTGGGGGATAGCTCCTGGGACACCCGACAGATGTTTTTC	CACDTVLGDSSWDTRQMFF	0	9/10
TGTGCCTGTGACATACTGGGGGATACCGATAAACTCATCTTT	CACDILGDTDKLIF**	0	8/10
TGTGCCTGTGACATACTGGGGGATACAGCACAACTCTTCTTT	CACDILGDTAQLFF	0	8/10
TGTGCCTGTGACGTACTGGGGGATACCGATAAACTCATCTTT	CACDVLGDTDKLIF*	0	8/10
TGTGCCTGTGACACCGTGGGGGACACCGATAAACTCATCTTT	CACDTVGDTDKLIF***	1	7/10
TGTGCCTGTGACGTACTGGGGGATTTGACAGCACAACTCTTCTTT	CACDVLGDLTAQLFF	0	7/10
TGTGCCTGTGACACCTGGGGGACCGATAAACTCATCTTT	CACDTWGTDKLIF	0	7/10
TGTGCCTGTGACATACTGGGGGATACTTTGACAGCACAACTCTTCTTT	CACDILGDTLTAQLFF	0	7/10
TGTGCCTGTGACACCTGGGGATACACCGATAAACTCATCTTT	CACDTWGYTDKLIF	1	6/10
TGTGCCTGTGACACCGTGGGGGATACCGATAAACTCATCTTT	CACDTVGDTDKLIF***	1	6/10
TGTGCCTGTGACAATACTGGGGGATACTCCTGGGACACCCGACAGATGTTTTTC	CACDNTGGYSWDTRQMFF	1	6/10
TGTGCCTGTGACATACTGGGGGATTTGACAGCACAACTCTTCTTT	CACDILGDLTAQLFF	0	6/10
TGTGCCTGTGACACCTGGGACACCCGACAGATGTTTTTC	CACDTWDTRQMFF	0	6/10
TGTGCCTGTGACACCGCTGGGGGATACTCCTGGGACACCCGACAGATGTTTTTC	CACDTAGGYSWDTRQMFF	1	6/10
TGTGCCTGTGACAGTACTGGGGGATACTCCTGGGACACCCGACAGATGTTTTTC	CACDSTGGYSWDTRQMFF	0	6/10

Table IV.1. The most shared CDR3δ clonotypes among 10-week-old (10w) Vγ9Vδ2 T cells. Sequences detected in at least 50% of the 10w subjects are shown. Occurrences: number of subjects where the clonotype was detected (out of 10). Median abundance: median percentage of repertoire in the ten 10-week-old subjects. */**/*** same amino-acid sequence. (nt: nucleotide; aa: amino acid).

3.3. Foetal-derived Vγ9Vδ2 T cells get activated and become highly cytotoxic rapidly after birth

Next, we investigated whether the foetal-derived expanded Vγ9Vδ2 T cells early after birth have the ability to functionally mature. The 10-week-old Vγ9Vδ2 T cells were highly activated compared to cord Vγ9Vδ2 T cells, and, more surprisingly, also compared to adult Vγ9Vδ2 T cells (**Fig. IV.3a, supplementary Fig. IV.3a**). In addition, at 10 weeks they started gradually to differentiate by losing the expression of the CD27 and CD28 markers (**Fig. IV.3b**). However, this did not lead to an increase of fully differentiated cells, as observed for adult Vγ9Vδ2 T cells (**supplementary Fig. IV.3b**). Aside their TCR, Vγ9Vδ2 T cells can also use NK receptors (NKR) in order to recognize target cells[85]. We verified the expression of a series of NKR and found that 10-week old Vγ9Vδ2 T cells specifically showed increased (compared to cord blood) expression of the inhibitory NKR NKG2A: other T cell subsets and other NKR did not show such expression pattern (**Fig. IV.3c,d; supplementary Fig. IV.3**). NKG2D, an important activating NKR for γδ T cells including the Vγ9Vδ2 subset[85], was already highly expressed by cord blood Vγ9Vδ2 T cells and was not further increased after birth (**Fig. IV.3d, supplementary Fig. IV.3d**).

A major function of Vγ9Vδ2 T cells in adults is killing of infected and cancer cells[171,203,205]. Thus we evaluated in detail the cytotoxic potential of the Vγ9Vδ2 T cells by analysing a series of cytotoxic mediators that play each a different role in the killing machinery[96]. At birth, Vγ9Vδ2 T cells lack the expression of granzyme B and perforin, of which the combined action is known to kill efficiently infected cells via apoptosis[96]. Strikingly, at 10-weeks, despite their relative limited differentiation status (**supplementary Fig. IV.3b**), the Vγ9Vδ2 T cells expressed these cytotoxic mediators at adult-like levels (**Fig. IV.3e,f**). Interestingly, while all the T cell subsets in the adult expressed granzyme B and perforin, in early life their expression was restricted to the Vγ9Vδ2 T cell subset (**Fig. IV.3e,f**). Perforin and granzyme B were co-expressed which is in line with the need of their combined action in order to mediate their cytotoxic activity[96] (**Fig. IV.3g**). Granulysin is a protein specific for killing intracellular and extracellular microbes[97,140]. In contrast to perforin and granzyme B, granulysin was almost absent in early life and reached only high levels of expression in the adult (**Fig. IV.3h**). Granzyme A uses different mechanisms to kill target cells compared to granzyme B, and it is known to have alternative roles besides cytotoxic activity[95,96,214]. Of note, granzyme A was already expressed in cord specifically by Vγ9Vδ2 T cells

(**Fig. IV.3i**), as observed previously in foetal (<30 weeks gestation) Vγ9Vδ2 T cells[20]. This expression further increased in 10-week Vγ9Vδ2 T cells and remained highly restricted to the Vγ9Vδ2 T cell subset (**Fig. IV.3i**). In addition, the expression per cell was even higher in infant compared to adult Vγ9Vδ2 T cells (**Fig. IV.3g, supplementary Fig. IV.4a**).

In summary, 10-week-old Vγ9Vδ2 T cells are highly activated and differentiate towards a particular expression pattern of cytotoxic mediators that is clearly different from Vγ9Vδ2 T cells at birth (high perforin and granzyme B), but also different from Vγ9Vδ2 T cells that are found in the adult blood circulation (absence of granulysin, higher granzyme A).

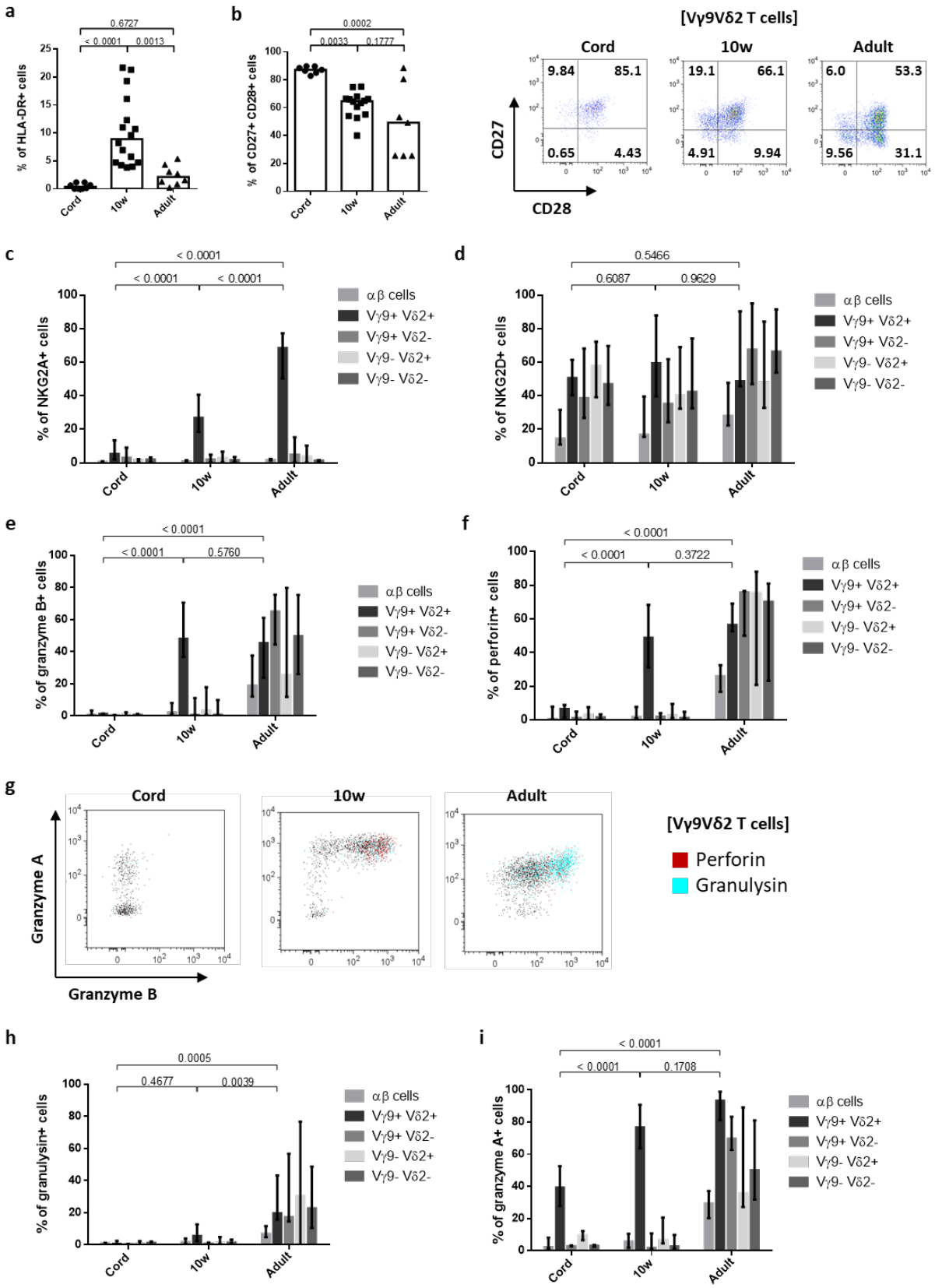
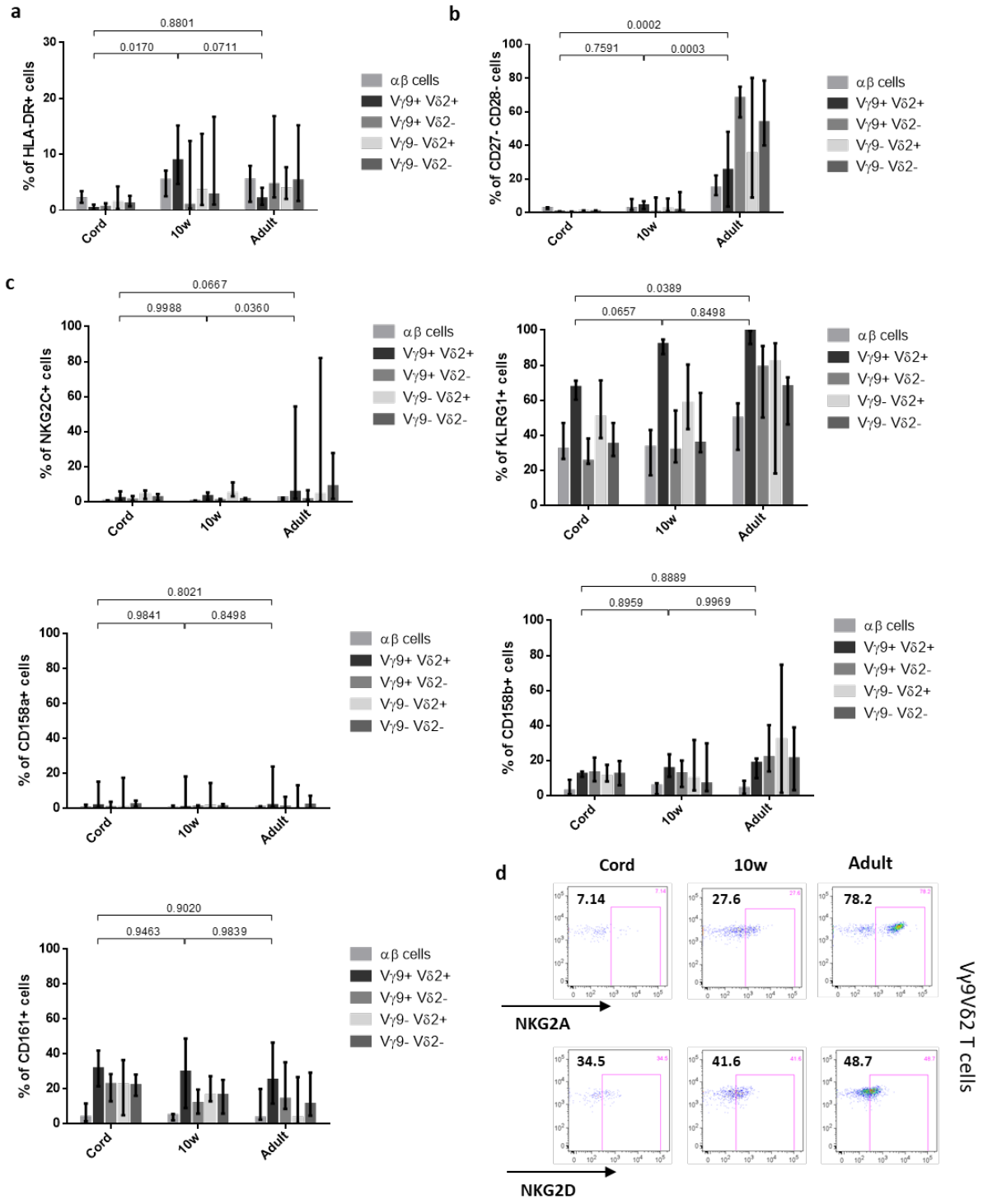
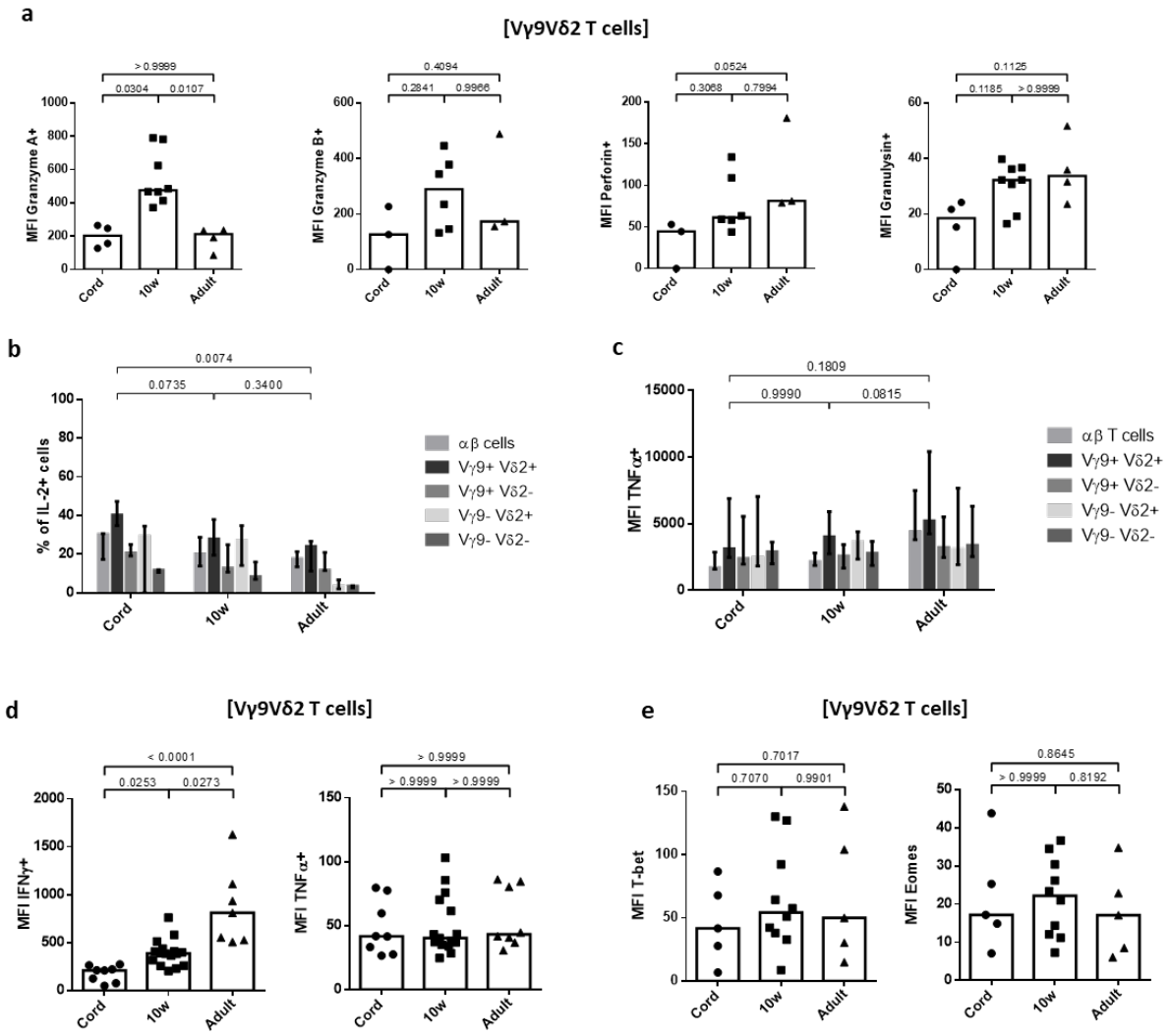


Fig. IV.3. Vγ9Vδ2 T cells get activated and become cytotoxic rapidly after birth. **a** Percentage of activated (HLA-DR+) cells among Vγ9Vδ2 T cells (cord, adult n=8, 10w n=16). **b** Prevalence of naive (CD27+CD28+) Vγ9Vδ2 T cells (left panel) and representative flow plots (right panel). **c-f, h-i** Percentage of positive cells in αβ T cells,

Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells. **c** Prevalence of NKG2A in cord (n=4), 10w (n=8-10) and adult (n=3-4). **d** Prevalence of NKG2D in cord (n=4-5), 10w (n=8-10) and adult (n=4-5). **e** Prevalence of granzyme B in cord (n=7), 10w (n=14) and adult (n=7). **f** Prevalence of perforin in cord (n=5), 10w (n=10) and adult (n=5). **g** Representative co-expression flow plots of granzyme A, granzyme B, perforin and granulysin in cord, 10w and adult Vγ9Vδ2 T cells. **h** Prevalence of granulysin in cord (n=7), 10w (n=14) and adult (n=7). **i** Prevalence of granzyme A in cord (n=7), 10w (n=14) and adult (n=7). Data shown from independent subjects (South-Africa). Bars indicate medians (a,b). Error bars indicate medians ± IQR (e-f, h-i). p values are reported on graphs.



Supplementary Fig. IV.3. Activation and differentiation status of T cells. **a-c** Percentage of positive cells in αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells. **a** Percentage of activated (HLA-DR+) cells (cord, adult n=8, 10w n=16). **b** Prevalence of differentiated (CD27-CD28-) cells. **c** Prevalence of NKR: NKG2C (cord, adult n=4,5, 10w=8,10); KLRG1, CD158a, CD158b, CD161 (cord, adult n=4, 10w n=7). **d** Representative flow plots of NKG2A+ (top) and NKG2D+ (bottom) Vγ9Vδ2 T cells. Data shown from independent subjects (South Africa). Error bars indicate medians ± IQR. p values are reported on graphs.



Supplementary Fig. IV.4. Expression of cytotoxic mediators and cytokines in T cells. **a** Median fluorescent intensity of granzyme B, perforin, granulysin and granzyme A positive Vγ9Vδ2 T cells (cord, adult n=3,4, 10w n=6,8). **b-d** Expression of cytokines after 4-hour PMA-Ionomycin stimulation in αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells. **b** Percentage of IL-2 positive cells (cord, adult n=3, 10w=6). **c** Median fluorescent intensity of TNFα positive cells (BD Fortessa, cord n=3, 10w n=8, adult n=4). **d** Median fluorescent intensity of IFNγ positive cells (left panel) and TNFα positive cells (right panel) among Vγ9+Vδ2+ T cells (Dako Cyan, cord, adult n=8, 10w n=16). **e** Median fluorescent intensity of T-bet (left panel) and Eomes (right panel) positive cells *ex vivo* (cord, adult n=5, 10w n=10). Data shown from independent subjects (South Africa). Bars indicate medians (a, d-e). Error bars indicate medians ± IQR (b-c). p values are reported on graphs.

3.4. Cytokine expression capacity by Vγ9Vδ2 T cells is mainly determined before birth

As γδ T cells can be rapidly activated to produce effector cytokines such as IFNγ and TNFα[27,170,171], we explored this effector capacity with strong short-term stimulation by PMA and ionomycin. We observed high expression of the two cytokines by cord, 10-week-old and adult Vγ9Vδ2 T cells (**Fig. IV.4a-b**). The Vγ9Vδ2 T cells were the main producers of IFNγ in early life, while in the adult other T cells expressed IFNγ as well (**Fig. IV.4a**). In contrast, IL-2 was expressed by all T cells in early life and showed lower expression in adult (**supplementary Fig. IV.4b**). Of note, while the percentage of Vγ9Vδ2 expressing IFNγ and TNFα remained stable at 10 weeks (compared to cord blood), the 10-week-old Vγ9Vδ2 T cells expressed much more IFNγ (but not TNFα) per cell (**Fig. IV.4a, right panel, Fig 4b right panel, supplementary Fig. IV.4c,d**). The high percentage of Vγ9Vδ2 T cells expressing IFNγ within cord and infant Vγ9Vδ2 T cells was paralleled by expression of the transcription factors T-bet and Eomes (**Fig. IV.4c, supplementary Fig. IV.4d**) known to be important for IFNγ production in γδ T cells[215].

Thus, the cytokine expression capacity (IFNγ, TNFα and associated transcription factors) of the Vγ9Vδ2 T cells is mainly programmed before birth while the IFNγ levels per Vγ9Vδ2 T cell is highly increased early after birth.

3.5. Phosphoantigen-reactivity remains stable early after birth

It is known that foetal and cord blood-derived Vγ9Vδ2 T cells, compared to adult-derived Vγ9Vδ2 T cells, show a significantly reduced response towards phosphoantigens such as the microbial-derived HMB-PP[20,54,193,213], but it is not clear whether this response would change early after birth[157]. HMB-PP induced comparable low levels of IFNγ in infant Vγ9Vδ2 T cells as in cord blood (**Fig. IV.4d**), while the higher response of adult Vγ9Vδ2 was confirmed (**Fig. IV.4d**). Also, intracellular isopentenyl pyrophosphate (IPP) accumulation induced by zoledronate treatment[193] did not lead to a higher IFNγ production in 10-week-old Vγ9Vδ2 T cells compared to cord (**Fig. IV.4e**). Thus, upon functional differentiation early after birth, the foetal-derived Vγ9Vδ2 T cells do not show an increase of their phosphoantigen-reactivity towards adult-like levels.

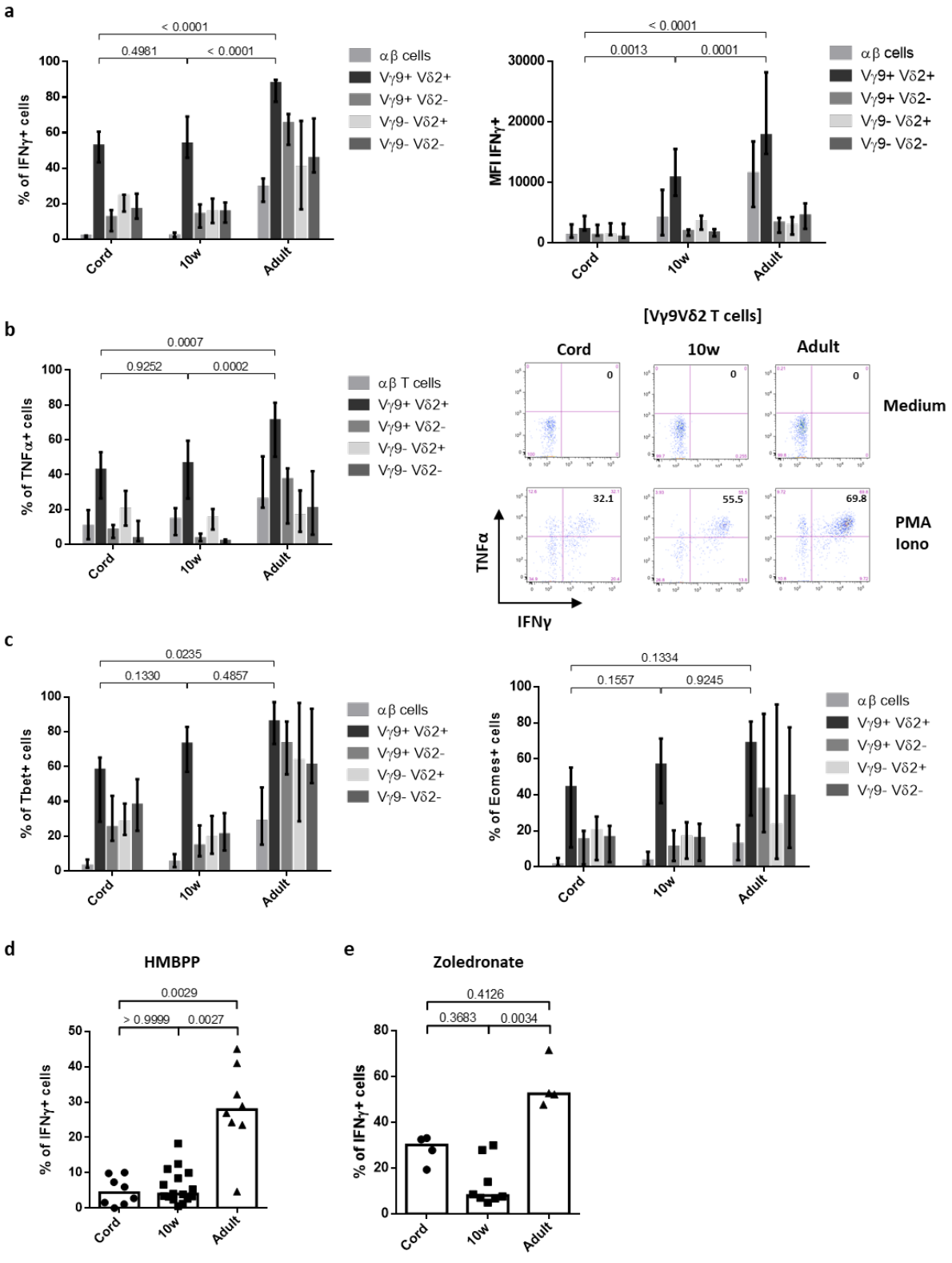


Fig. IV.4. The cytokine expression capacity of Vγ9Vδ2 T cells is mainly determined before birth. a-c Flow cytometry data on αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells. **a** IFNγ expression after 4-hour PMA-Ionomycin stimulation: percentage of positive cells (left panel; cord, adult n=12, 10w=24) and median fluorescent intensity (BD Fortessa; right panel; cord, adult n=4, 10w=8). **b** TNFα expression after 4-hour PMA-Ionomycin stimulation:

percentage of positive cells (left panel; cord n=8, 10w=17, adult n=9) and representative co-expression flow plots of IFNγ and TNFα in cord, 10w and adult Vγ9Vδ2 T cells (right panel). **c** Ex-vivo expression of T-bet (left panel) and Eomes (right panel) (cord, adult n=5-6, 10w=10-12). **d** Percentage of IFNγ+ cells among Vγ9Vδ2 T cells after stimulation with the phosphoantigen HMB-PP (3 days or overnight, in presence of IL-2; cord, adult n=8, 10w=16). Values derived from 'medium+IL-2' condition are subtracted. **e** Percentage of IFNγ+ cells among Vγ9Vδ2 T cells after stimulation with zoledronate (3 days, in presence of IL-2; cord, adult n=4, 10w=8). Values derived from 'medium+IL-2' condition are subtracted. Data shown from independent subjects (South-Africa). Error bars indicate medians ± IQR (a-c). Bars indicate medians (d-e). p values are reported on graphs.

3.6. BCG vaccination at birth does not alter the TCR repertoire nor functional differentiation

Next, we investigated whether the expansion and associated activation and functional maturation of the 10-week-old Vγ9Vδ2 T cells could be influenced by vaccination at birth with BCG, a known Vγ9Vδ2 T cell activator[59–61,143]. Thus, we studied the function and repertoire of Vγ9Vδ2 T cells from 10-week-old infants who received BCG vaccination at birth (BCG+) and compared them to 10-week old Vγ9Vδ2 T cells from infants who did not receive vaccination at birth (BCG-).

BCG is known to expand cord blood Vγ9Vδ2 T cells *in vitro*[213]. To our surprise, Vγ9Vδ2 T cells were not increased in the BCG+ compared to the BCG- group (**Fig. IV.5a, top panel**). This was confirmed by a very similar proliferation rate ex-vivo (**Fig. IV.5a, bottom panel**). We further investigated in detail the shaping of the repertoire early after birth (**Fig. IV.1**), which might shed light on the different way the TCR repertoire reacted to a specific stimulus (BCG)[204]. However, the level of diversity did not change significantly between the BCG- and BCG+ groups (**Fig. IV.5 b-c**), neither did a series of TCR/CDR3 characteristics such as number of N additions, usage of J segment, CDR3 length, and level of overlap of the repertoire (**Fig. IV.5 d-h**).

A detailed analysis of the phenotype and effector functions of the Vγ9Vδ2 T cells coming from the two 10-week old groups indicated that the BCG vaccination could not influence the rapid and striking differentiation of the neonatal Vγ9Vδ2 T cells early after birth (**Fig. IV.6, Supplementary Fig. IV.5, Supplementary Fig. IV.6, Supplementary Fig. IV.7**). Note that at 10 weeks the conventional αβ T cells showed the same phenotype as in cord blood (**supplementary Fig. IV.8**).

Overall, it appears that the expansion of public foetal-derived Vγ9Vδ2 T cells and their functional differentiation early after birth, most likely upon environmental (phosphoantigen) exposure, is so powerful that it cannot be altered by the administration of the phosphoantigen-containing vaccine BCG at birth.

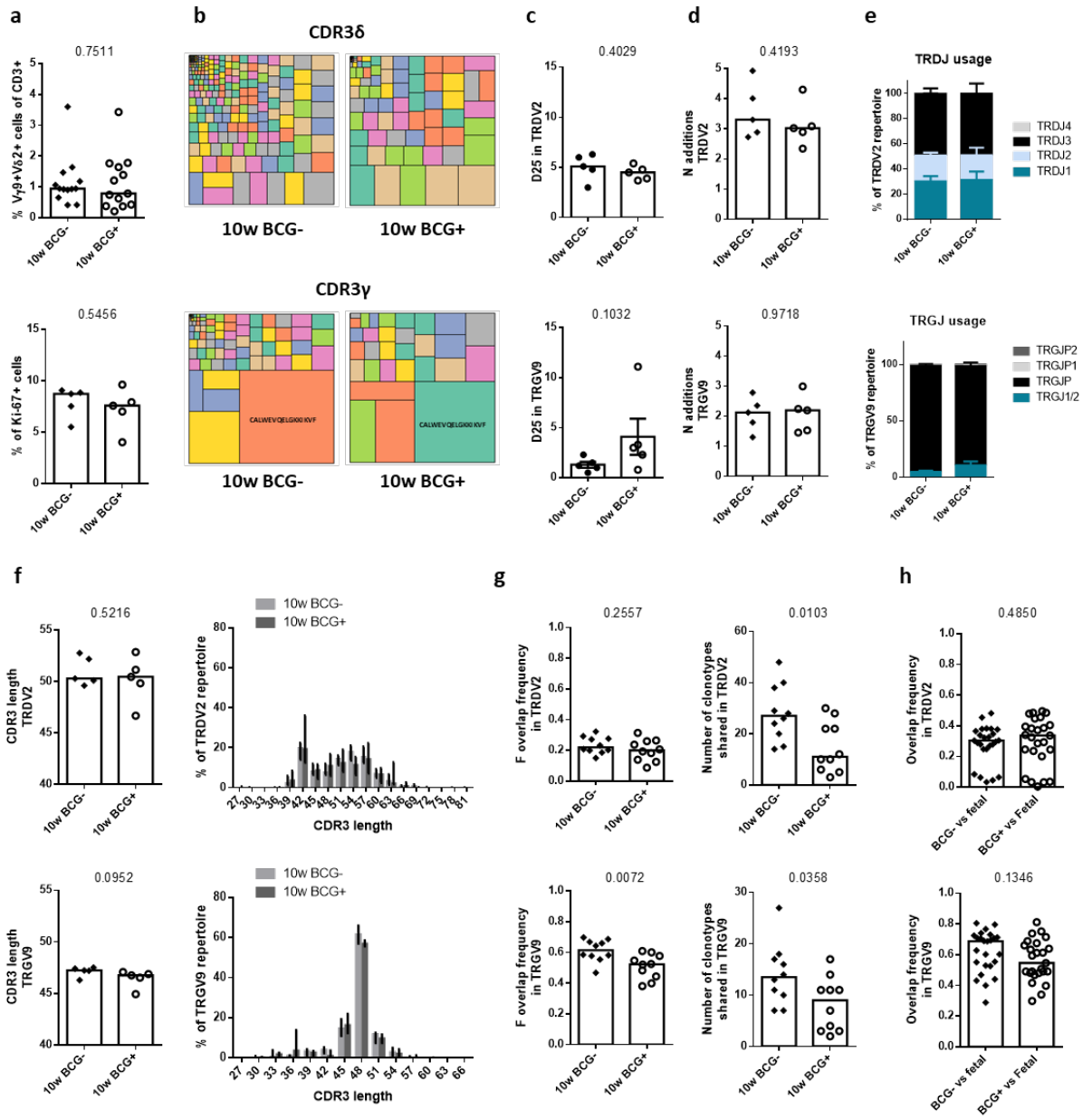


Fig. IV.5. Neonatal BCG vaccination does not influence the shaping of the 10-week-old Vγ9Vδ2 TCR repertoire. a Prevalence of the Vγ9+Vδ2+ subset in 10-week-old unvaccinated infants (10w BCG-) and 10-week-old vaccinated infants (10w BCG+) CD3+ cells (top panel; n=14) and percentage of Ki-67+ cells among Vγ9Vδ2 T cells (bottom panel; n=5)). **b-h** Comparison of the CDR3 TRDV2 (top row) and TRGV9 (bottom row) repertoire of sorted Vγ9Vδ2 T cells derived from 10w BCG- (n=5) and 10w BCG+ (n=5) blood. **b** Representative tree-maps showing CDR3 clonotype usage for BCG- (left) and BCG+ (right) Vγ9Vδ2 T cells; each rectangle represents one CDR3 clonotype and its size corresponds to its relative frequency in the repertoire (rectangle colours are chosen randomly and do not match between plots). **c** Comparison of D25 values (percentage of unique clonotypes required to account for 25% of total

repertoire). **d** Number of N additions, each dot represents the weighted mean of an individual sample. **e** J gene segment usage distribution (Error bars indicate mean ± SEM). **f** CDR3 length (nucleotide count including the C-start and F-end residues), each dot represents the weighted mean of an individual sample (left panel); frequency of repertoire per CDR3 length (right panel). **g** Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) (left panel) and number of clonotypes shared (right panel) within pairs of BCG- or pairs of BCG+ subjects; each dot represents the F value or the number of shared clonotypes of a pair of samples. **h** Relative abundance of the BCG- repertoire overlapping with foetal, or the BCG+ overlapping with foetal repertoire. Each dot represents a pair comparison. Data shown from independent subjects (South-Africa). Bars indicate medians (a, c-d, f left panel, g-h). Error bars indicate medians ± IQR (e, f right panel). p values are reported on graphs.

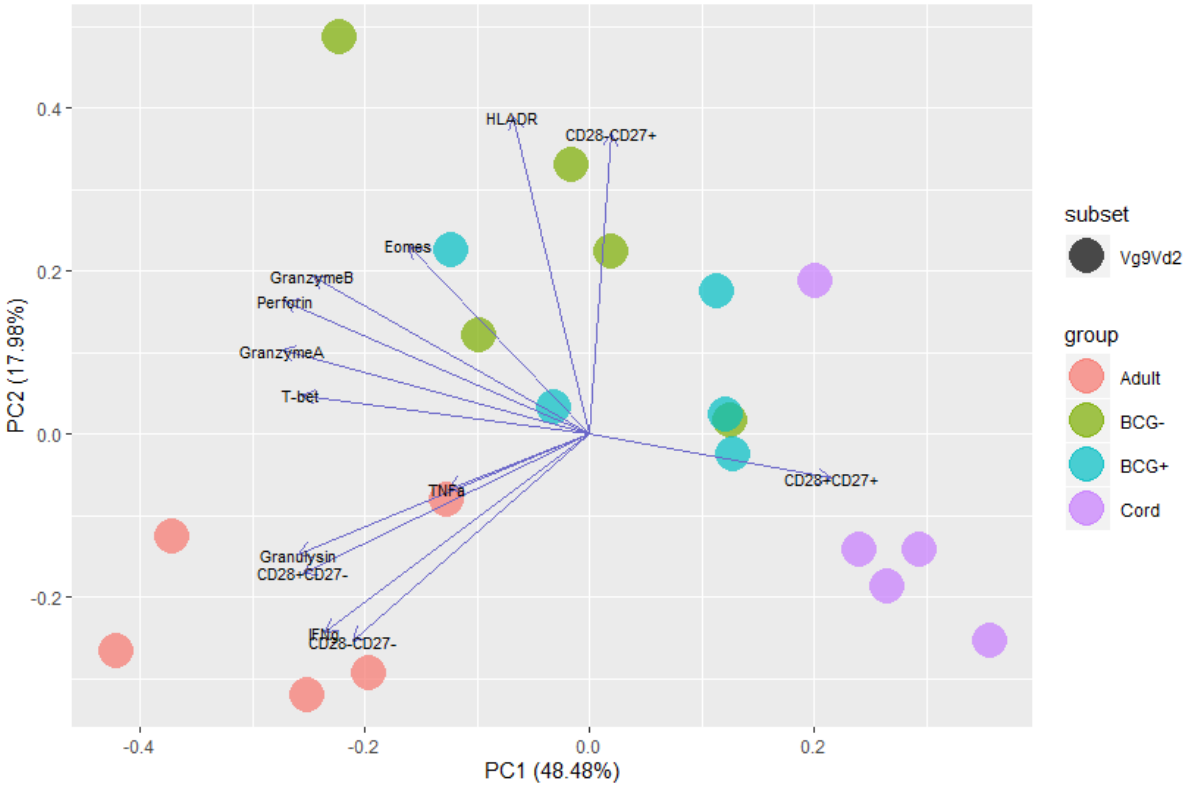
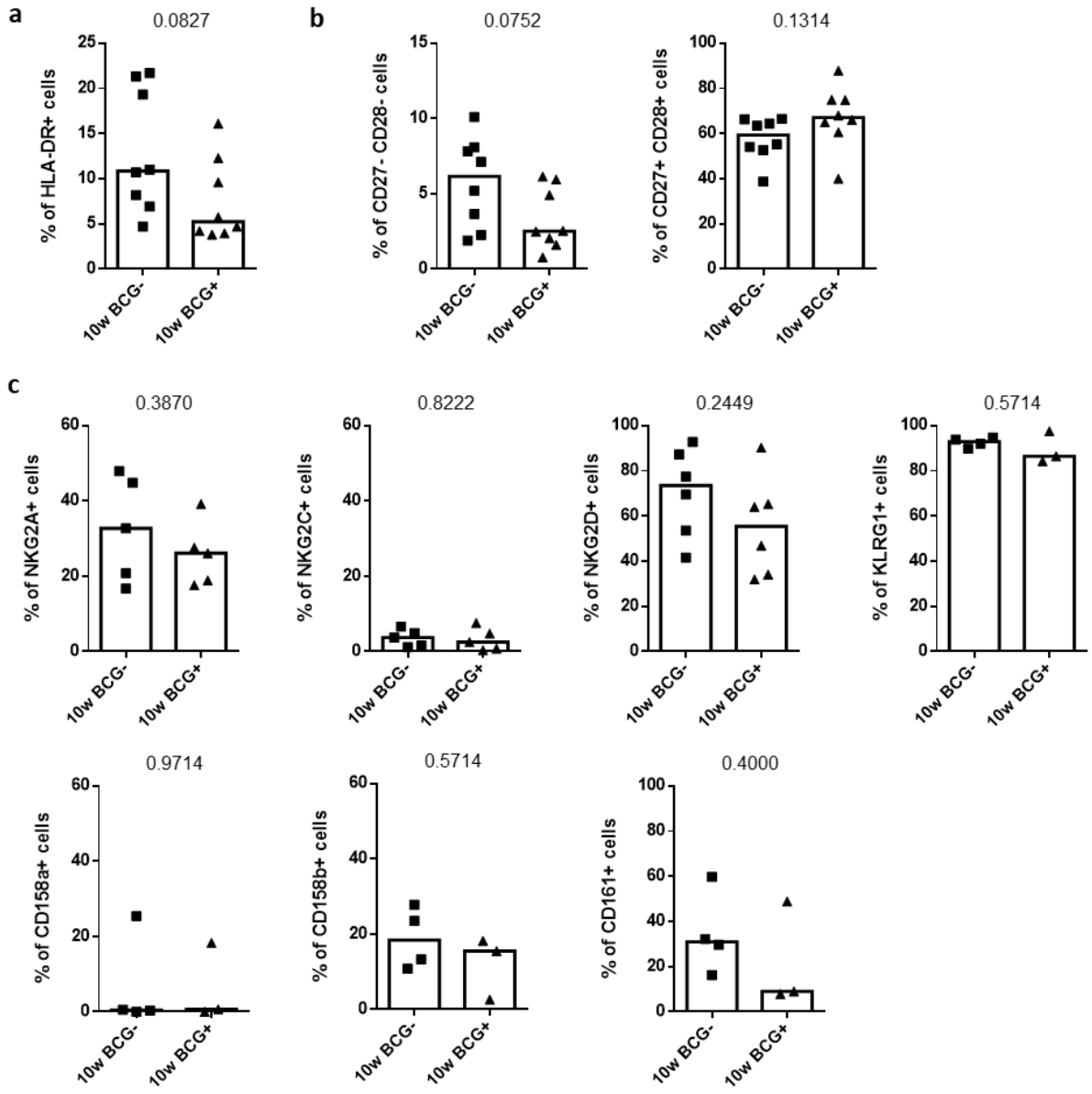
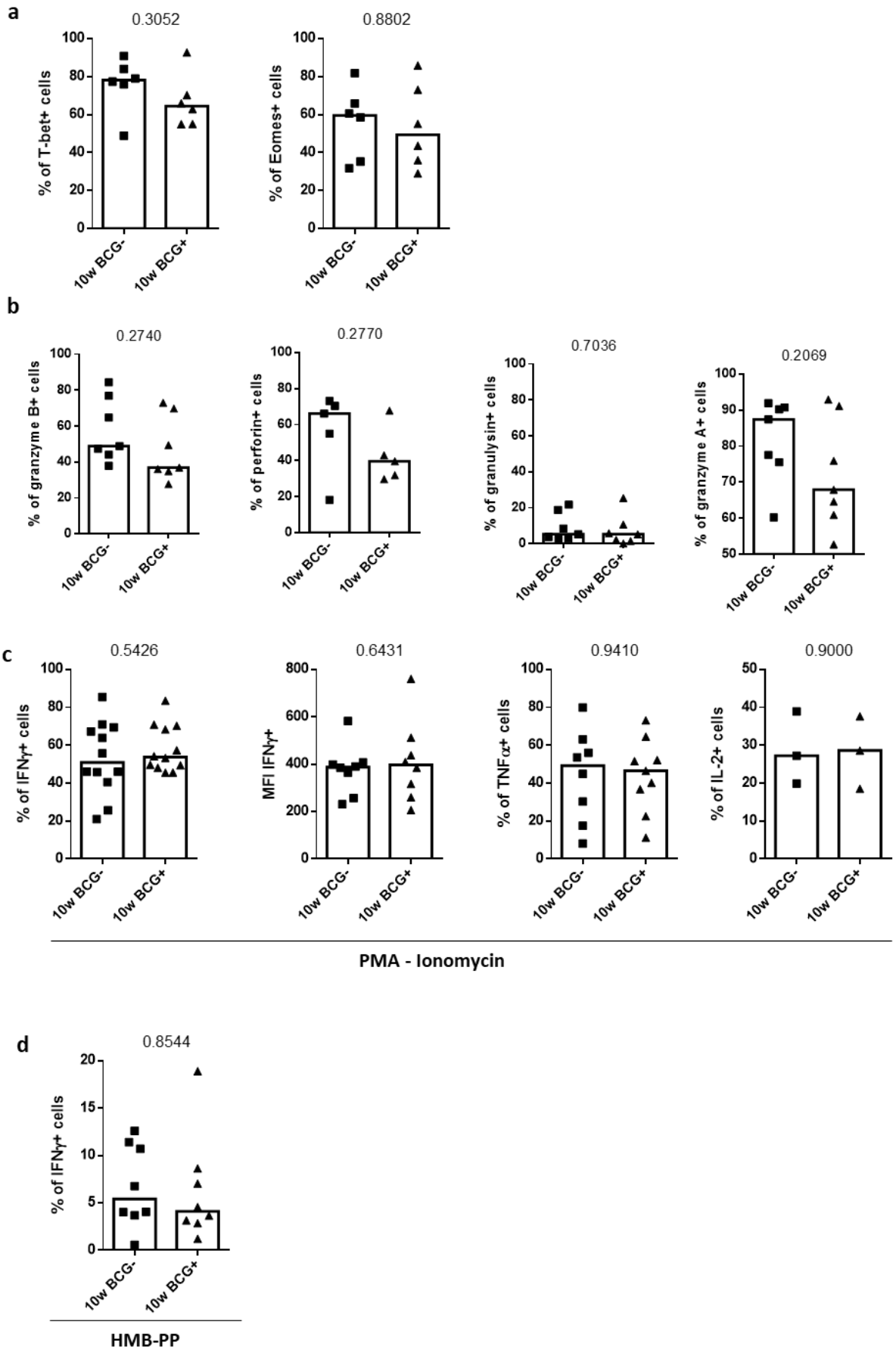


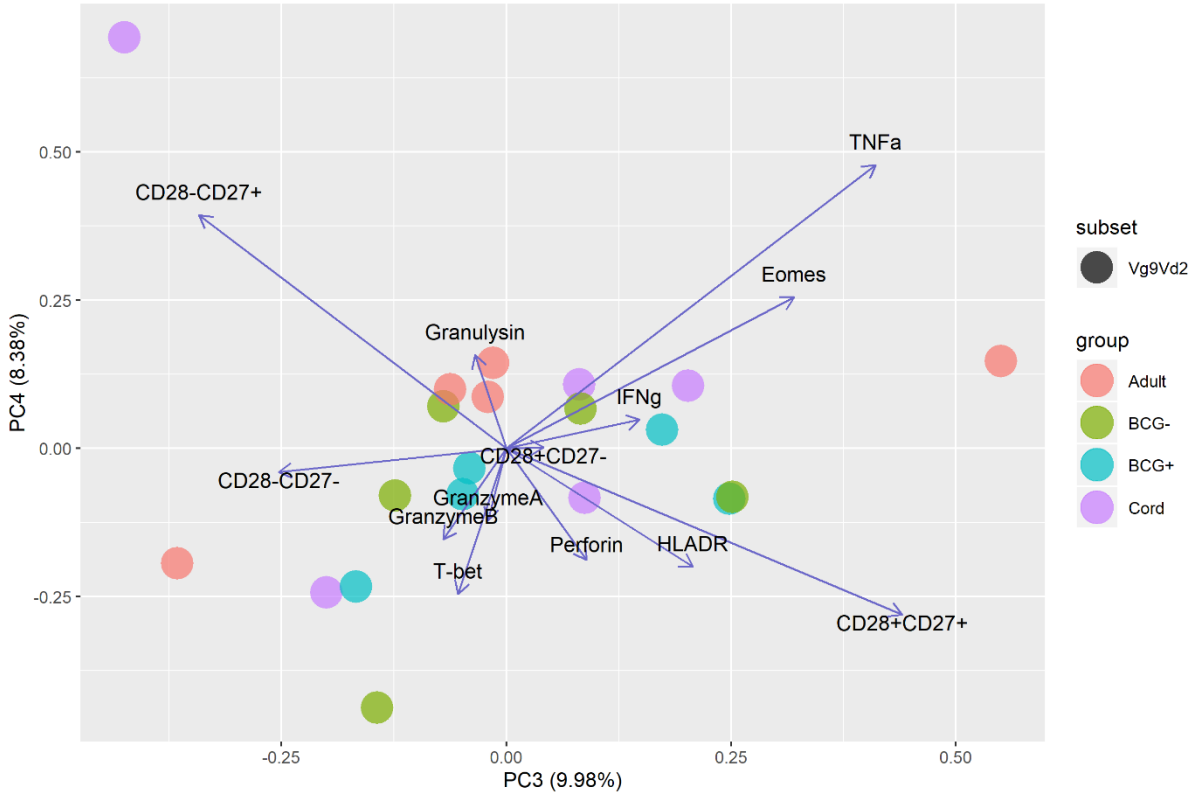
Fig. IV.6. Neonatal BCG vaccination does not influence the functional differentiation of 10-week-old Vγ9Vδ2 T cells. PCA analysis profiling of Vγ9Vδ2 T cells derived from cord, 10-week-old BCG-, 10-week-old BCG+ and adult blood (n=5), based on the percentage of: HLA-DR+, CD27-CD28-, CD27-CD28+, CD27+CD28-, CD27-CD28-, T-bet+, Eomes+, granzyme A+, granzyme B+, perforin+, granulysin+, IFNγ+ and TNFα+ cells. Data shown from independent subjects (South-Africa).



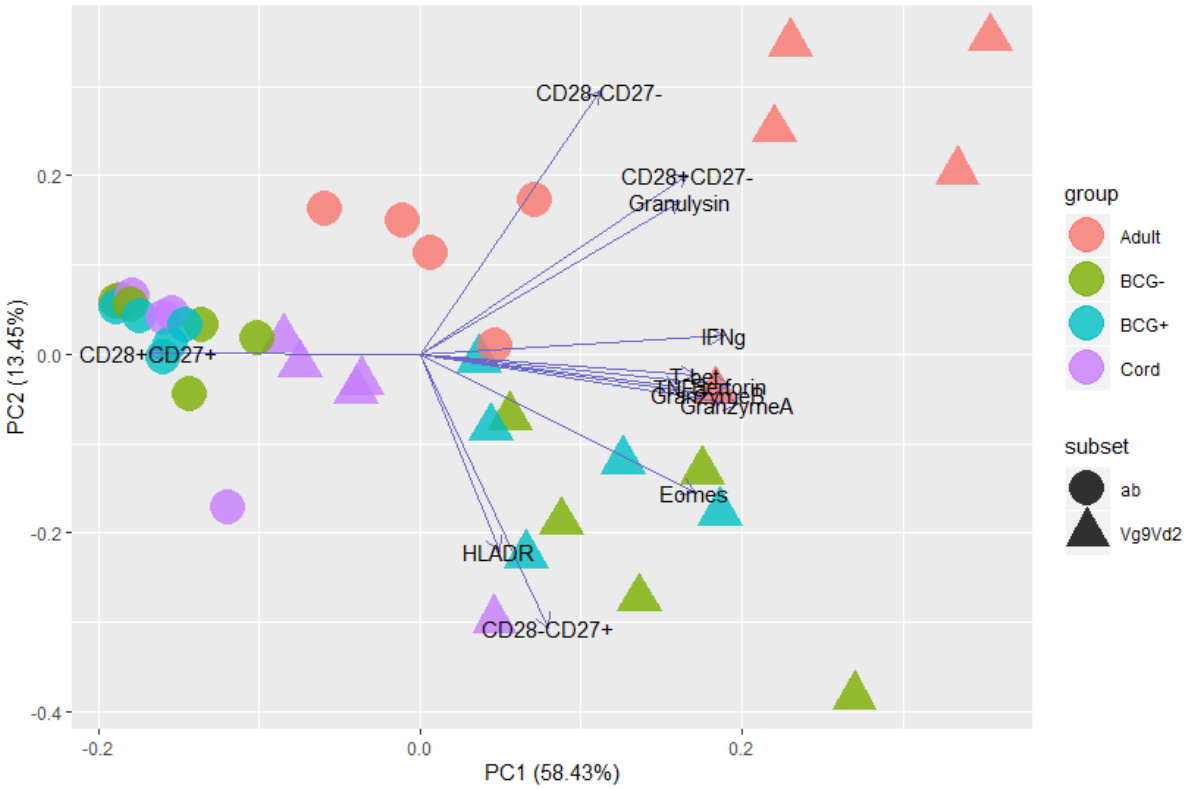
Supplementary Fig. IV.5. Activation and differentiation status and NKR expression in BCG+ and BCG- 10-week-old Vγ9Vδ2 T cells. **a** Percentage of activated (HLA-DR+) cells. **b** Prevalence of differentiated (CD27-CD28-) (left panel) and naive (CD27+CD28+) (right panel) cells. **c** Prevalence of NKR: NKG2A, NKG2C, NKG2D, KLRG1, CD161, CD158a, CD158b). Data shown from independent subjects (South Africa). Bars indicate medians. p values are reported on graphs.


a
0.3052
0.8802
% of T-bet+ cells
% of Eomes+ cells
10w BCG-
10w BCG+
b
0.2740
0.2770
0.7036
0.2069
% of granzyme B+ cells
% of perforin+ cells
% of granulysin+ cells
% of granzyme A+ cells
c
0.5426
0.6431
0.9410
0.9000
% of IFNγ+ cells
MFI IFNγ+
% of TNFα+ cells
% of IL-2+ cells
PMA - Ionomycin
d
0.8544
% of IFNγ+ cells
HMB-PP

Supplementary Fig. IV.6. Expression and regulation of cytotoxic mediators and cytokines in BCG+ and BCG- 10-week old Vγ9Vδ2 T cells. **a** *Ex-vivo* expression of the transcription factors T-bet (left panel) and Eomes (right panel). **b** *Ex vivo* expression of granzyme B, perforin, granulysin and granzyme A. **c** Expression of cytokines after 4-hour PMA-ionomycin stimulation: IFN-γ, median fluorescent intensity of IFNγ+, TNF-α and IL-2. **d** Percentage of IFN-γ positive cells after HMB-PP stimulation (for 3 days or overnight in presence of IL-2). Data shown from independent subjects (South Africa). Bars indicate medians. p values are reported on graphs.



Supplementary Fig. IV.7. BCG+ and BCG- cluster close together based on PC3-4. PCA analysis profiling of Vγ9Vδ2 T cells derived from cord, 10-week-old BCG-, 10-week-old BCG+ and adult blood (n=5), based on the percentage of: HLA-DR+, CD27-CD28-, CD27-CD28+, CD27+CD28-, CD27-CD28-, T-bet+, Eomes+, granzyme A+, granzyme B+, perforin+, granulysin+, IFNγ+ and TNFα+ cells. Data shown from independent subjects (South-Africa).



Supplementary Fig. IV.8. Functional differentiation of Vγ9Vδ2 T cells compared to αβ T cells. PCA analysis profiling of Vγ9Vδ2 and αβ T cells derived from cord, 10-week-old BCG-, 10-week-old BCG+ and adult blood (n=5) based on the percentage of: HLA-DR+, CD27-CD28-, CD27-CD28+, CD27+CD28-, CD27-CD28-, T-bet+, Eomes+, granzyme A+, granzyme B+, perforin+, granulysin+, IFNγ+ and TNFα+ cells. Data shown from independent subjects (South Africa).

4. Discussion

Compared to adult blood Vγ9Vδ2 T cells, cord blood Vγ9Vδ2 T cells show only a limited expansion upon *in vitro* phosphoantigen stimulation[20,157,193,213]. Despite this, we show here that within ten weeks after birth the Vγ9Vδ2 T cells of healthy infants were already expanding. Furthermore, TCR repertoire analysis indicated a preferential expansion of early foetal-derived (<30 weeks of gestation) clonotypes showing a high level of sharing (publicity). Consistent with this is the identification of CACDVLGDTDKLIF and CACDILGDTDKLIF among the top shared TRD sequences of 10-week-old Vγ9Vδ2 T cells, previously described to be highly abundant and shared in pre-thymic livers of 6/7-week gestation fetuses[46]. Thus, it appears that public Vγ9Vδ2 clonotypes, derived from foetal thymus[207] and/or foetal liver[46] are maintained till birth and show a preferential expansion upon birth. In sharp contrast, the other blood γδ T cell subsets (grouped as non-Vγ9Vδ2 T cells, not responding to phosphoantigens), while also showing TCR repertoire publicity at the foetal stage, became already private at term delivery and did not expand early after birth. In adults, the TRD repertoire of both Vγ9Vδ2 and non-Vγ9Vδ2 T cells were private, consistent with other studies[30,36,37]. A major potential source of phosphoantigens in the first weeks after birth is the developing microbiota[55]. Indeed, HMB-PP, the most potent natural phosphoantigen, is produced by multiple bacterial species that are present in the gut microbiome and can induce a polyclonal expansion of foetal/cord Vγ9Vδ2 T cells in vitro[54,55,207,216,217]. These phosphoantigens may act together with other factors such as innate cytokines (IL-18, IL-23) that are highly expressed early after birth[159]. In line with this possibility is the increased in vitro phosphoantigen responsiveness of foetal/cord blood Vγ9Vδ2 T cells when co-incubated with IL-18 or IL-23, the high expression of the receptor for IL-18 on foetal Vγ9Vδ2 T cells and the induction of the receptor for IL-23 on cord blood Vγ9Vδ2 T cells upon phosphoantigen exposure[20,193,218]. A previous study has shown increasing percentages of total Vδ2+ T cells (thus not making the distinction between Vγ9+Vδ2+ and Vγ9-Vδ2+ T cells) in children between 3-10 years, possibly reflecting expansion of 'adult-like' Vγ9Vδ2 T cells[50]. Compared to samples collected >1 year after birth, the number of samples collected <1 year were more limited in this study, possibly explaining the lack of observing a clear increase in Vδ2+ percentages early after birth. In addition, the decrease of Vγ9-Vδ2+ cells after birth[29–31] could have masked a clear increase of Vγ9+Vδ2+ cells when gating on total Vδ2+ T cells at this age.

A recent study investigated the association of 62 leukocyte subsets from birth till 6 years with a series of nongenetic determinants (prenatal maternal lifestyle-related or immune-mediated determinants, birth characteristics and bacterial/viral exposure–related determinants) levels[219]. Interestingly, among the 26 different determinants investigated, only the determinant 'premature rupture of membranes' was found to be associated with Vγ9Vδ2 T cell levels[219]. At premature gestation times Vγ9Vδ2 T cells are the main subset, while at term-delivery Vδ1+ γδ T cells are predominant[20,220]. Since we show here that upon delivery the foetal-derived Vγ9Vδ2 T cells expand immediately, the higher initial Vγ9Vδ2 T cell levels upon premature birth can explain the association of Vγ9Vδ2 T cells in infants and young children that were born prematurely.

In contrast to the private γδ T cell subsets (non-Vγ9Vδ2), at 10 weeks after birth the public Vγ9Vδ2 T cells were activated and differentiated towards high expressers of cytotoxic mediators (perforin, granzyme B, granzyme A). While variable perforin expression has been described in paediatric Vδ2+ cells[157] (containing both the Vγ9+Vδ2+ and Vγ9-Vδ2+ subset[20,30,31,157]), we define here that its expression is limited to the Vγ9+Vδ2+ subset early after birth. Granzyme B, together with perforin, can efficiently kill infected target cells[96]. Thus, their co-expression in 10-week-old Vγ9Vδ2 T cells suggests a role as potent cytotoxic effector cells against (phosphoantigen-generating) infections early after birth. Granzyme A, highly expressed by 10-week-old Vγ9Vδ2 T cells, even higher than in adult, can induce a different cell death pathway than granzyme B[96]. Furthermore, granzyme A produced by Vγ9Vδ2 T cells promotes macrophages to inhibit the intracellular growth of mycobacteria[95]. Thus, the very high granzyme A expression observed in 10-week-old Vγ9Vδ2 T cells can play an important role in the killing of infected cells and/or the inhibition of intracellular growth of pathogens. In contrast to perforin and granzyme B expression, the cytokine expression capacity (IFNγ, TNFα and associated transcription factors) of the Vγ9Vδ2 T cells was mainly programmed before birth. Among NKR, NKG2A was highly upregulated early after birth on the cell surface Vγ9Vδ2 T cells. Sudden phosphoantigen exposure can be a triggering factor for this specific NKG2A expression pattern, since cell surface expression of NKG2A can be induced by phosphoantigen exposure[91]. The similar expression patterns of the cytotoxic mediators perforin/granzyme B and the inhibitory

NKR NKG2A in 10-week old infant Vγ9Vδ2 T cells suggests that NKG2A signalling could regulate the potent cytotoxic activity of infant Vγ9Vδ2 T cells. Granulysin is a cytotoxic mediator that, like Vγ9Vδ2 T cells, is not present in rodents. It can target pathogens directly rather than the infected cells[97]. In contrast to cord-and 10-week Vγ9Vδ2 T cells, granulysin was highly expressed by adult Vγ9Vδ2 T cells. Furthermore, adult Vγ9Vδ2 T cells showed clearly a higher response towards HMB-PP compared to 10-week and cord blood Vγ9Vδ2 T cells. These specific features of Vγ9Vδ2 T cells in the adult blood circulation may be due to their distinct development compared to foetal-derived Vγ9Vδ2 T cells[207].

No influence of vaccination with BCG (a known Vγ9Vδ2 T cell activator) at birth could be observed in 10-week-old Vγ9Vδ2 T cells, with regards to their expansion, TCR repertoire and function. It has been previously suggested that Vγ9Vδ2 T cells can be activated in early life upon BCG vaccination[145,146,210], but these studies did not consider age-matched unvaccinated controls. A possible explanation for the absence of BCG-induced effects on 10-week-old Vγ9Vδ2 T cells is that the expansion due to the sudden microbial phosphoantigen exposure at birth (including the developing microbiome) overrides the possible effect of the BCG administration. This could explain why clear expansions of Vγ9Vδ2 T cells can be seen in non-human primates (in clean facilities) upon vaccination with BCG[143], while species-related effects cannot be excluded either. Also the distinct development of foetal and adult Vγ9Vδ2 T cells may contribute to different responses towards vaccination with BCG, depending on the age of the vaccinated subjects[147,207,209]. Of note, γδ T cells have been increasingly recognized as important players in vaccine-mediated protection from infection[221]. As our study shows that foetal-derived Vγ9Vδ2 T cells are expanded and functionally differentiated early after birth independently from BCG vaccination, it highlights the need for correct (age-matched) control groups when investigating γδ T cells in vaccination studies. While vaccination with BCG has been shown to lead to heterologous or non-specific effects, including via the induction of trained immunity in innate immune cells such as monocytes (also known as innate memory)[164,165], our study indicates that innate Vγ9Vδ2 T cells are rather ‘trained’ by the overt phosphoantigen exposure they encounter after birth.

Collectively, our study shows that in the first two months after birth, public foetal-derived Vγ9Vδ2 T cells expand and differentiate to a cytotoxic subset with functions closer to those seen in adults than the foetal counterparts. This differentiation is not affected by BCG vaccination at birth, a strong γδ stimulus, which is likely due to the prominent environmental exposure. This post-natal polyclonal burst of Vγ9Vδ2 T cells combined with strong functional maturation thus shapes an innate T cell subset in new-borns that may be important to fight infections at a time the conventional (memory) αβ T cell response is not fully active yet[155].

V. General discussion

The contribution of Vγ9Vδ2 T cells in anti-microbial and anti-cancer immunity and their potential important role in early life infections are of increasing interest in the γδ T cell field. The uniqueness of Vγ9Vδ2 T cells in transitional immunity stems from two main properties, their “pattern-recognition” receptors activated by the nearly ubiquitous phosphoantigens, which still follow VDJ recombination, and the rapid onset of cytotoxic and immunomodulatory functions. In this work, different aspects of the biology and development of γδ T cells were elucidated which can potentially aid in the cancer immunotherapy and early-life infection management.

1. Insight by CDR3 repertoire analysis

In depth analysis of the TCR CDR3 by HTS sequencing shed light into the character and intricacies of Vγ9Vδ2 T cells and their TCRs. Detailed analysis of their repertoire within a range of different sources, including blood and tissue, varying age and country of origin, *ex vivo* and after *in vitro* treatment, led to valuable interpretations and conclusions on the Vγ9Vδ2 T cell development and the immune surveillance they confer.

1.1. Gamma versus delta chain repertoire

Vγ9Vδ2 T cells are considered the main innate human γδ subset with public repertoire principally based on the gamma chain[30,33]. Based on previous data and our own, it is clear that the delta chain has a much more private repertoire compared to gamma and this is partly due to the presence of D (diversity) domains, sometimes multiple, adding

on top further available sites for non-template nucleotides (N additions) that are incorporated randomly, creating a diverse repertoire easily distinguished from individual to individual. This difference between gamma and delta chain in the Vγ9Vδ2 TCR is maybe designed for distinct roles. Each chain may be involved in a different kind of recognition with the phosphoantigen-BTN complex. The publicity of Vγ9 chain may ensure a first wave of recognition and function, stable, like a pattern-recognition receptor, while the Vδ2 chain, with increasing diversity and length with age, may grant a higher avidity. Remarkably, very recently, Rigau and colleagues discovered that BTN2A1, which is also essential for phosphoantigen reactivity, binds to the Vγ9 chain while BTN3A1 probably binds to a separate domain incorporating Vδ2[68]. This difference in the binding mechanism could explain the difference in CDR3 characteristics important for phosphoantigen recognition. On the one hand, the hydrophobicity of the 5th amino acid in the Vδ2 CDR3 chain is crucial for phosphoantigen reactivity while the length shift does not affect it[22,23,25]. On the other hand, the length of Vγ9-JγP CDR3 is critical for the phosphoantigen reactivity[23] and as very recently pointed out certain residues of the Vγ9 chain are important in the BTN2A1 mediated recognition of phosphoantigens[68].

1.2. Early life versus adult repertoire

In contrast to certain similarities between the foetal and adult Vγ9Vδ2 T cell repertoire, there were two distinct waves of generation in the thymus. Microbial exposure still shapes the post-natal repertoire, but this process alone could not fully explain the differences observed.

The distinct features between foetal and adult Vγ9Vδ2 CDR3 repertoire may also contribute to shifts in roles as in the gamma and delta comparison. In adult Vδ2 sequences, the relatively low sharing decreases even more and the length increases, as seen with the mean length of Vγ9 that is shorter than Vδ2. As all events in human biology, this variation could not be by chance. The dual wave of Vγ9Vδ2 thymocytes indicates two distinct immunobiology tasks of these cells. With regard to adult-like TCRs, they seem to be linked to a better *in vitro* activation by HMB-PP producing higher IFNγ (section IV.3.5) and more effective expansion with lower amounts of phosphoantigen[20]. Thus, the threshold required to initiate immune responses is set higher in early life compared to adult life. Vγ9Vδ2 T cells seem to be tailored depending on the context. In early life, especially in the foetus, potential congenital infections could be controlled by Vγ9Vδ2 effectors. However, these cells should not escalate the

immune response as most times what is required is to maintain a regulatory landscape with a balance in the maternal-foetal interface and absence of immunopathology.

1.3. Driving the generation of Vγ9Vδ2 T cells

What drives the generation of distinct Vγ9Vδ2 T cells populations has been intriguing. The nature of the precursor cell seems to define the type of TCR of the thymocyte (section III.3.6) rather than the intrathymic exposure to phosphoantigens. However, a potential role of the BTN3A1 and BTN2A1 molecules is not excluded, as they are conserved in vertebrates, but not rodents, concomitant to the Vγ9 and Vδ2 genes[42]. An example of evolutionary conservation is the human BTNL3 and BTNL8, which are largely restricted to intestinal epithelial cells and provoked TCR downregulation specifically by human Vγ4 T cells, the major colonic γδ intraepithelial lymphocyte subtype[201,222,223].

While the specific enrichment of the public Vγ9-JγP clone (CALWEVQELGKKIKVF) in early life could point to a positive selection through ligand interaction in the thymic development, another mechanism leading to the enrichment of this specific recombination might explain its generation. The absence of N additions provides a favourable setting for short homology repeats that drive the VDJ recombination at specific sites[188,189]. As proposed in the results shown above (section III. 3.4), the repetition of the nucleotides GCA at the V-end of the TRGV9 and the J-start of the TRGJP following some uneven cutting (P additions) could explain the skewed selection for the TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAAAAATCAAGGTATTT nucleotype enriched in the foetus. This involves an example of conservation among primate species that makes this TCR so unique. The abundance of this public Vγ9 clonotype in early life could be “cost-effective” providing sufficient protection without maximal deployment which could be associated to potential immunopathology.

In contrast, in post-natal Vγ9Vδ2 T cell generation, higher CDR3 diversity is obtained with the incorporation of increased number of N additions which is in big part responsible for the increased diversity and CDR3 length. This is due to the enhanced activity of the TdT enzyme which is upregulated postnatally due to downregulation of the Lin28b RNA binding protein and concomitant upregulation of let7[44]. Interestingly, the Vγ9 public clonotype is still rearranged postnatally but now thanks to the convergent recombination, N additions are involved[35].

1.4. Country of origin impact on repertoire

CDR3 repertoire analysis was performed in Vγ9Vδ2 T cells from different countries. So, cord blood and adult blood were analysed both from Belgium and South Africa. Even though race has been described to influence the cellularity and cytotoxicity of Vδ2 γδ T cells, the Vγ9 repertoire was substantially very similar between Caucasian American and African American donors[49]. In the cohorts studied here, Belgian and South-African donors shared great similarities in all the readouts examined, in both the gamma and delta repertoire, with only slight tendency for a more public repertoire in the South-African samples (section IV. 3.2). Altogether, the CDR3 repertoire data were comparable in terms of key features, such as N additions and J usage, and allowed direct comparisons between different ages (foetal versus 10 weeks).

2. Expansion of foetal-derived Vγ9Vδ2 at 10 weeks after birth

The repertoire and function of blood Vγ9Vδ2 T cells was studied in the first couple months post-partum and was compared to foetal, cord and adult blood Vγ9Vδ2 T cells. Apart from the public germline Vγ9-JγP clonotype which was still present in the neonatal period, other foetal-derived TCRs expanded early after birth and at 10-weeks they possessed recently acquired cytotoxic functions. So, the increase of the Vγ9Vδ2 population at 10 weeks compared to cord was not due to recent thymic immigrants but to foetal-derived cells that have now encountered various microbial cues.

2.1. Vγ9Vδ2 innateness versus nonVγ9Vδ2 γδ T cells

The expansion of Vγ9Vδ2 T cells early after birth was associated with a polyclonal response of Vγ9Vδ2 T cells to phosphoantigens. We observed that in early life there were particular clonotypes expanding and were shared among young individuals while in adults they were almost absent. The sharing of repertoire was associated to foetal-derived sequences. This goes in line with the innate character attributed to Vγ9Vδ2 T cells and intrathymic programming. However, these cells still evolved in the periphery adapting on environmental signals they encountered (10-week-old Vγ9Vδ2 T cells).

The expansion of foetal-derived Vγ9Vδ2 T cells was specific for this γδ subset, as nonVγ9Vδ2 γδ T cells did not expand while they showed gradual increase of N additions (adult-like repertoire). Notably, there was no indication of Vδ1 specific expansion in the BCG+ group compared to BCG-, even though there has been

evidence that Vδ1 T cells can recognise mycobacterial lipids via CD1c presentation[224]. In general, nonVγ9Vδ2 T cells are considered as the adaptive leg of human γδ T cells, with concomitant differentiation and functional maturation[36,225]. However, this seems not to happen in early life as they remained naïve without any important cytokine or granule expression (section IV. 3). In contrast, Vγ9Vδ2 T cells acquired potent cytotoxic functions already at 10 weeks post-partum following polyclonal expansion.

2.2. Function at 10 weeks after birth

At 10 weeks after birth, Vγ9Vδ2 T cells have started to produce granzyme B and perforin which work very efficiently in tandem[94]. Their capacity to produce IFNγ has improved compared to cord blood and their differentiation and activation status has shifted. This represents the earliest immunological maturation of human lymphocytes. Although, similar abrupt shift in differentiation was observed in postnatal blood samples[157], the distinction between Vγ9+Vδ2+ and Vγ9-Vδ2+ was not done. As the latter population is quite important in early life in contrast to adults[20,30], it is important to attribute this early potent activation to the phosphoantigen-reactive ones (Vγ9+Vδ2+ γδ T cells).

The sudden exposure after birth instigated immune responses by foetal-derived Vγ9Vδ2 T cells. This transition could be achieved by interaction with commensal microbiota, especially in the very first weeks of life[226] (see also section I.6.2). Diverse bacterial species consisting of the neonatal and infant microbiome produce phosphoantigens, such as Bifidobacterium, Clostridium and Bacteroides[54,55], which could activate Vγ9Vδ2 T cells. This could be considered as preparation of the host to rapidly mount immune responses upon pathogen encounter. For example, germ-free or antibiotic-treated mice demonstrate impaired clearance of systemic bacterial infection supporting the general crosstalk between microbiota and immune cells[155].

3. 10 weeks versus adulthood

Even though 10-week-old Vγ9Vδ2 T cells acquired multiple cytotoxic and adult-like immune functions, there was not a complete parallel between 10-week-old and adult profiles. Indeed, Vγ9Vδ2 T cells were more prevalent in adult donors compared to early-life donors and were much more differentiated as previously described[50,157]. The infant Vγ9Vδ2 T cells acquired most of the adult effector functions early after birth with exceptions such as the expression of granulysin which was considerably lower in infant

T cells and almost absent in cord blood, while cytokine expression was higher in adult compared to early-life Vγ9Vδ2 T cells, especially after phosphoantigen stimulation. It seems that there are two different formats of effector functions of Vγ9Vδ2 T cells, making the infant ones selectively cytotoxic.

Differences in the early life and adult repertoire go in line with differences in the effector function but they are not always enhanced in the adult (section IV.3.3). The first foetal wave may be important in development and growth of the foetus and infant. For example, the high expression of granzyme A already in the foetus but in the absence of the highly cytotoxic granzyme B and perforin indicates a special role designated to granzyme A. As discussed earlier (section I.3.1), granzyme A performs alternative functions such as degrading extracellular matrix proteins, including collagen type IV, and fibronectin, as well as mediating in the release of growth factors (reviewed in [96]) which can be important in the development and growth of the new-born. Thus, non-immune functions of Vγ9Vδ2 T cells may cover a big part of their role in early life and development for which different mediators are required.

4. Effect of BCG immunisation on Vγ9Vδ2 T cells

4.1. BCG influence on repertoire and function

In order to monitor a specific microbial encounter potentially affecting the Vγ9Vδ2 functional maturation, BCG was administered at birth and Vγ9Vδ2 T cells at 10 weeks postvaccination were compared to age-matched unvaccinated infant Vγ9Vδ2 T cells. As BCG is able to expand neonatal Vγ9Vδ2 T cells in vitro[213], we expected a similar proliferation state after *in vivo* vaccination. Furthermore, as γδ T cells have been described to be activated by neonatal BCG[145,146], the parallel study of age-matched vaccinated and unvaccinated infants was crucial. Indeed, surprisingly, the abundance, repertoire and phenotype of blood Vγ9Vδ2 T cells was quite similar between the BCG+ and BCG- groups in 10-week-old infants.

The expansion of particular Vγ9Vδ2 TCRs observed at 10 weeks after birth was not affected by BCG vaccination *in vivo*, in contrast to *in vitro* studies with adult Vγ9Vδ2 T cells[204] or cases of tuberculosis infection[227,228]. Interestingly, several of the most enriched sequences at 10-week-old Vγ9Vδ2 T cells were public, previously detected in foetal liver and found to be reactive to *Mycobacterium tuberculosis*[46]. However, their prevalence was comparable in the groups with or without BCG vaccination, indicating that their expansion was due to other environmental stimuli besides BCG.

The absence of difference between the BCG+ and BCG- groups was also observed in the phenotype and functions investigated with sometimes even a tendency towards a weaker activation and cytotoxic potential in the BCG+ group (e.g. HLA-DR, perforin). This observation together with the similar expansion state could support the hypothesis that BCG-activated Vγ9Vδ2 T cells might have migrated to the tissues as it has been previously described in the context of infection[229,230]. However, as blood was the only sampling site in the studied cohort, conclusions cannot be drawn with certainty regarding possible Vγ9Vδ2 migration to the tissues.

4.2. Speculations on absence of BCG effect on neonatal Vγ9Vδ2 T cells

The route of vaccine administration and the timeline can be important for the outcome of studies focusing on the immune effect of vaccination. Here, BCG was administered intradermally as per guidelines and blood was withdrawn 10 weeks postvaccination. In a recent study in non-human primates[231], they compared different routes of BCG administration and intravenous immunisation led to higher levels of antigen-responsive αβ T cells in various tissues, including lung, and 90% of the animals were protected against *Mycobacterium tuberculosis* infection six months after BCG immunisation. However, there was no significant effect of BCG vaccination on γδ T cells (including Vγ9+), either on the tissue homing of γδ T cells or an effect of route of administration. Interestingly, there was a trend towards increased Vγ9 γδ T cells two weeks after BCG vaccination with contraction to prevaccination proportions 8 weeks postvaccination for both intravenous and intradermal administration[231]. So, the hypothesis that an earlier timepoint of blood withdrawal in our human cohort might lead to mildly different results, with blood Vγ9Vδ2 T cells showing a potential expansion and activation due to BCG which would later be curtailed, cannot be excluded.

Studies in non-human primates are important specifically for studies targeting Vγ9Vδ2 T cells, as rodents do not possess them[42]. Although, critical knowledge and detailed mechanism of action has been acquired in the context of BCG vaccination and *Mycobacterium tuberculosis* infection in non-human primates (e.g. macaques), there is one main factor to consider. These studies are executed in clean facilities or specific-pathogen free facilities where encounter with microbes is monitored. This contrasts with real life conditions. When studying immune cells, especially cells with innate properties such as Vγ9Vδ2 T cells, it is possible that non-controlled variables such as environmental microbial exposure influences their immune response and relative conclusions. Based on a comparative study between wild mice and laboratory mice,

the immune system of wild mice is in a primed, highly activated state due to high level pathogen exposure[232]. Interestingly, *in vitro* cytokine responses to pathogen-related ligands are generally lower in cells from wild mice, probably indicating the importance of preserving immune homeostasis towards intense antigenic triggers in the wild[232]. This kind of differences might explain in part the discrepancy in the effect of BCG vaccination on γδ T cells observed in previous laboratory macaques' model[143] and this current study.

Furthermore, the absence of significant differences between the vaccinated and unvaccinated group might be characteristic of the young age of the cohort studied. This study focuses on early life and the evolution of neonatal Vγ9Vδ2, while previous study showing a BCG effect on human Vγ9Vδ2 T cells was conducted in adult donors[147]. As discussed in the first part of this work, early life Vγ9Vδ2 T cells differ from their adult counterparts and follow distinct development[207], so the memory mediated immune responses[147] might be distinguishable only in later life Vγ9Vδ2 T cells.

It is surprising that Vγ9Vδ2 T cells proliferate from foetal derived ones and gain cytotoxic potential in such a height that any effect by BCG immunisation seems hidden. Although, the early immunological maturation of Vδ2 T cells has been previously described[157], variable paediatric ages were followed up to one year old with almost no clear indication as early as 10 weeks of age. This early shift in immune phenotype and function of Vγ9Vδ2 T cells indicates their potent role in early life infections, consisting of a ready-to-fight army.

In human infants, a stereotypic immune system development has been described with children of different levels of maturity and postnatal environmental conditions converging on a shared developmental trajectory early in life[159]. In our study, it is possible that early life equilibrium was attained either by vaccination and/or other microbial stimuli with the two 10-week-old groups converging to a similar immune phenotype. Therefore, no significant difference was detectable between BCG+ and BCG- infants regarding γδ T cells. This hypothesis would also be in line with the transient effect on Vγ9 cells discussed earlier in the NHP model[231].

VI. Conclusion

This work contributed in the understanding of human Vγ9Vδ2 T cell function and development in foetal and postnatal life and its shaping towards environmental or artificial encounters. The functional specialisation of Vγ9Vδ2 T cells results from both developmental programming and plasticity in the periphery.

Human foetal blood Vγ9Vδ2 T cells find their origin in the foetal thymus whereas adult blood Vγ9Vδ2 T cells are generated to a large degree postnatally. This comes in contrast with the established model of mouse innate γδ T cell development.

Early after birth, environmental exposure drives the expansion and differentiation of human public foetal-derived Vγ9Vδ2 T cells which acquire potent cytotoxic functions. This finding supports the idea that the immune system in early life is characterised by a dynamic capacity to handle both genetically encoded and environmental driven programming.

These main results are visualised in the overview figure below (**figure VI.1**).

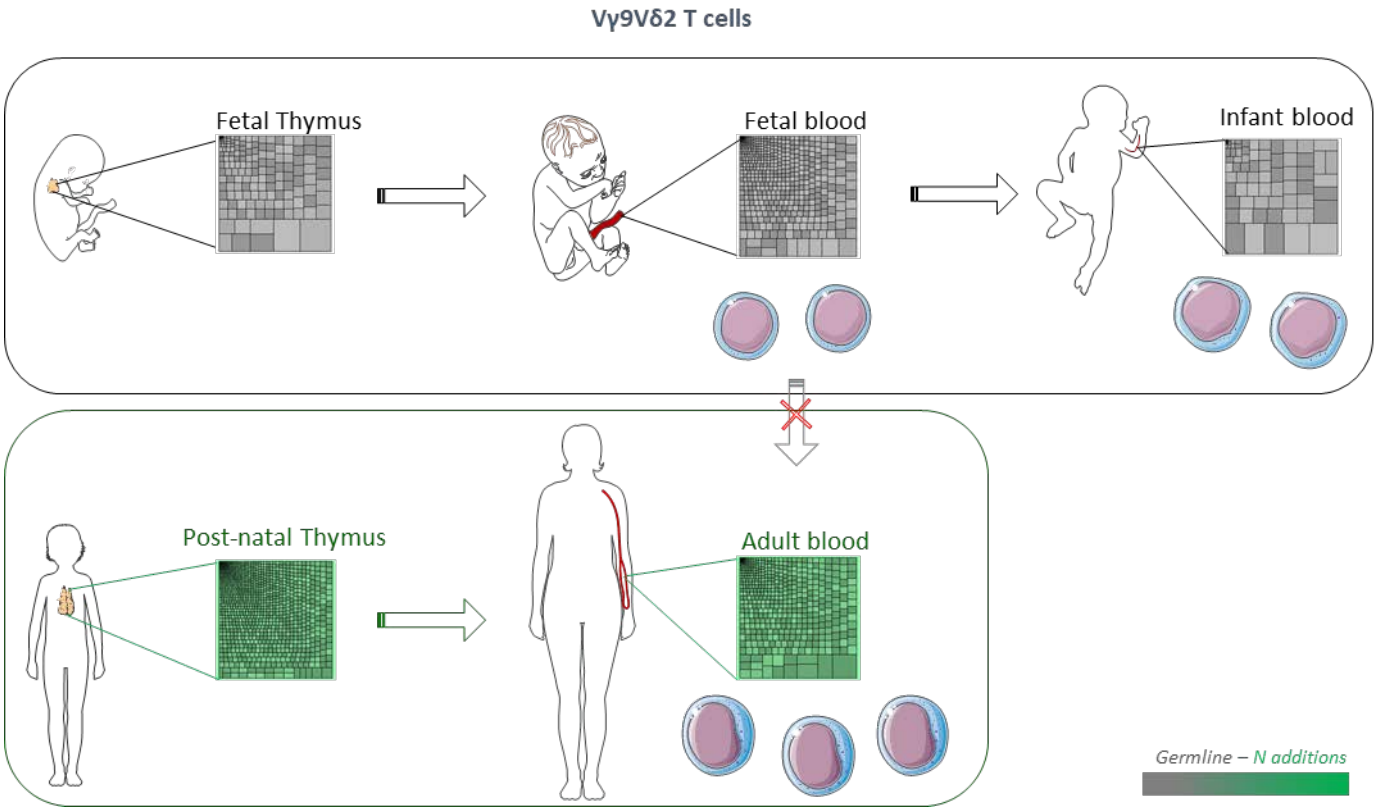


Figure VI.1. Thymic development and functional polarisation of human Vγ9Vδ2 T cells. The foetal thymus generates a TCR repertoire with low N additions (grey) like the repertoire found in foetal blood. Public foetal-derived Vγ9Vδ2 T cells expand early after birth and gain cytotoxic properties (more granules in cells). However, adult blood Vγ9Vδ2 T cells principally originate from postnatal thymocytes and not foetal, with high number of N additions (green) and highest cytotoxic potential. (Human forms and immune cells modified from[5]; CDR3 TCR repertoires depicted in tree maps).

VII. Perspectives

Through TCR sequencing, substantial advance was made on the CDR3 analysis and key features that distinguish early life TCRs from later life. The insight on TCR CDR3 characteristics can be useful in the design of immunological tools, in the recognition of ligands and sensing mechanism of phosphoantigens by Vγ9Vδ2 T cells.

The observation that adult blood Vγ9Vδ2 do not, principally, originate from their foetal counterparts can orientate immunotherapy strategies that use Vγ9Vδ2 T cells. In the context of cancer treatment, different TCRs could be tested for their efficacy and tenacity, and public shorter clones could be compared with longer adult-like ones to assess which are more competent. It has been described that foetal and new-born effector αβ T lymphocytes might be limited by a more rapid onset of functional exhaustion (upon CMV infection)[233], while repeated malaria exposure was associated with increased expression of immunoregulatory genes in Vδ2 γδ T cells in young children (<1 year of age)[234]. If the adult-like Vγ9Vδ2 T cells reveal better fitness for a longer time *in vivo*, new ways to produce more of the post-natal γδ T cells would be beneficial.

Next, the features and membrane markers that are linked to robust and long-lasting anticancer effect could be determined. The tumour-killing efficiency of post-natal thymocytes could be compared to foetal ones, and/or foetal and adult blood Vγ9Vδ2 T cells, in *in vitro* experiments using cancer cell lines. If the activity of adult-like cells is substantially better and enduring, a comparison of their phenotype and gene expression profile would be informing, as well as the evolution during phosphoantigen treatment and during contact with the cancer cell line, for example the expression of exhaustion markers (PD-1, Tim-3). Recently, the role of the transcription factors TOX and TOX2 has been demonstrated in the “exhaustion” of anti-cancer CD8 αβ T cells (cells becoming hyporesponsive)[235,236]. It is not known whether γδ T cells would follow similar programming and it would be interesting to evaluate the expression and influence of the TOX2 transcription factor within foetal- and adult-like Vγ9Vδ2 T cells[237]. For example, if specific (post-natal) Vγ9Vδ2 TCRs are associated with absence of exhaustion programming, differential expression of surface markers could be defined so that a selection on Vγ9Vδ2 T cells derived from cancer patients could be done before *in vitro* phosphoantigen stimulation.

The fact that the generation of foetal-like or adult-like Vγ9Vδ2 T cells (and γδ T cells in general) is dependent on the precursor cell, could trigger the investigation of the detailed mechanism underlying this distinction. The transcriptional and epigenetic programming could be investigated at the level of the precursor cells, before the DNA is rearranged toward a specific TCR. Such insight could be helpful in the *in vitro* generation of specific tumour-reactive γδ T cells.

The initial hypothesis for the infant Vγ9Vδ2 T cells was that upregulating activation and/or functional responses of this subset by BCG vaccination may enhance general protection against the agent targeted by immunisation and others (trained immunity[238]). However, taken into consideration the absence of effect in the conditions tested here, further work is needed to assess potential mechanisms driving heterologous effects of vaccination. Timing of interventions could therefore be optimised to induce maximal γδ T cell recall responses or target different age populations.

It is noteworthy, that the cohort where the effect of neonatal BCG vaccination was studied included 10-week-old infants. The possibility that a more transient temporary effect on blood Vγ9Vδ2 T cells could be brought by BCG immunisation could still be evaluated at an earlier timepoint after BCG administration (as discussed above, section V.4.2). In fact, our collaborators in South-Africa are currently studying other non-conventional T cells in 5-week-old infants vaccinated or not with BCG.

Furthermore, the direct effect of *in vivo* BCG vaccination was studied in infant Vγ9Vδ2 T cells where no significant difference was detected compared to Vγ9Vδ2 T cells coming from same age infants without previous BCG immunisation. Even though there were specific differences compared to cord blood Vγ9Vδ2 T cells, the functional maturation of Vγ9Vδ2 T cells could not be attributed to the BCG administration. This finding highlights the need of inclusion of age-matched controls in the design of such studies.

Infant Vγ9Vδ2 T cells were defined as foetal-derived based on their TCR CDR3 repertoire. It would be interesting to investigate the expression of molecules associated with a foetal CDR3 phenotype such as the RNA-binding protein Lin28b[44,239] and examine associated transcription factors such as the “innate lymphocyte” regulator

PLZF[44,240–242] to follow the transition from foetal to postnatal behaviour. At 10 weeks after birth, a big part of the public foetal-derived Vγ9Vδ2 T cell repertoire has undergone significant functional polarisation subsequent to the environmental exposure. This finding elucidates the evolution of the immune system in the neonatal and infant period, guiding future directions while dealing with infections in early life.

Moreover, the innate nature of Vγ9Vδ2 T cells with high expression of perforin and granzyme B so early after birth with potential anti-microbial activity could be investigated on other unconventional T cells such as NKT and MAIT cells[243].

Finally, the shift in Vγ9Vδ2 T cell function might be due to encounters with commensal microbiota as discussed earlier. This hypothesis encourages the administration of probiotics that enhance the effect of the microbiota[155] and could be a useful consideration in future studies aiming to investigate how to boost the neonatal and infant immune system with minor interventions.

VIII. References

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Annex

Nomenclature of gamma chains in human and mice.

1. Human TRG genes, from Lefranc and Rabbits 1990[26].

Variable genes	
Subgroups	Genes
VγI	*V1*
	V2
	V3
	V4
	V5
	V5P
	V6
	V7
	V8
VγA	*VA*
VγII	V9
VγIII	V10
VγB	*VB*
VγIV	V11

Human variable TRG (TRGV) genes. Pseudogenes are shown in italics.

2. Mouse TRG genes (from imgt.org[17]).

Correspondence between the different nomenclatures

Cassette	IMGT gene name	Previous gene designations							
		Hayday et al. [1]	Heilig and Tonegawa [2]	Traunecker et al. [3]	Garman et al. [4]	Iwamoto et al. [5]	Pelkonen et al. [6]	Huck et al. [7]	Iwasato and Yamagaishi [8]
1	TRGV4		V4 *	V4 *	V2 *	V6	V4.3	V4	V4 *
	TRGV5		V5		V3 *	V4	V4.1	V6	V5 *
	TRGV6				V4 *	V5	V4.2	V5	V6
	TRGV7						V4.4 *	V7	V7 *
	TRGJ1	J13.4 *	J1	J4	J1	J2	J4	J4	
	TRGC1		C1	C4	C1 *	C2	C4	C4	
2	TRGV2	V10.8A *	V2	V1	V1.2	V2	V1	V1	V2
	TRGJ2	J10.5 *	J2	J1	J2	J1	J1	J1	
	TRGC2	C10.5 *	C2	C1	C2	C1	C1	C1	
3	TRGV3	V5.7 *	V3	V3	V1.3	V3	V3	V3	V3
	TRGJ3		J3	J3	J3	J3	J3	J3	
	TRGC3	C7.5 *	C3	C3	C3	C3	C3	C3	
4	TRGV1	V10.8B *	V1	V2	V1.1	V1	V2	V2	V1
	TRGJ4		J4	J2	J4	J4	J2	J2	
	TRGC4		C4	C2	(C4)	C4	C2	C2	

* indicates genes with sequence accession numbers quoted in the House mouse *(Mus musculus)* germline gene tables : TRGV, TRGC and TRGJ.

IMGT references:

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