Effector Vγ9Vδ2 T cell response to congenital *Toxoplasma gondii* infection

Ling Ma, …, Valeria Meroni, David Vermijlen

*JCI Insight.* 2021. [https://doi.org/10.1172/jci.insight.138066](https://doi.org/10.1172/jci.insight.138066).

**Graphical abstract**

Find the latest version:

[https://jci.me/138066/pdf](https://jci.me/138066/pdf)
Effector Vγ9Vδ2 T cell response to congenital *Toxoplasma gondii* infection

Ling Ma¹23, Maria Papadopoulou¹23, Martin Taton²3, Francesca Genco⁴, Arnaud Marchant²3, Valeria Meroni⁴5, David Vermijlen¹23

1 Department of Pharmacotherapy and Pharmaceutics, Université Libre de Bruxelles (ULB), Belgium
2 Institute for Medical Immunology, Université Libre de Bruxelles (ULB), Belgium
3 ULB Center for Research in Immunology (U-CRI), Université Libre de Bruxelles (ULB), Belgium
4 IRCCS San Matteo Polyclinic, Pavia, Italy
5 Molecular Medicine Department, University of Pavia, Italy

Short title: ‘Anti-parasite γδ T cells in utero’

Conflict of interest statement: The authors have declared that no conflict of interest exists

Address correspondence to: David Vermijlen, Department of Pharmacotherapy and Pharmaceutics, Faculty of Pharmacy, Université Libre de Bruxelles (ULB), Campus Plaine, Boulevard du Triomphe, Accès 2, 1050 Brussels, Belgium. E-mail address: David.Vermijlen@ulb.be
Abstract

A major γδ T cell population in human adult blood are the Vγ9Vδ2 T cells that are activated and expanded in a T cell receptor (TCR)-dependent manner by microbe- and endogenous-derived phosphorylated prenyl metabolites (phosphoantigens). Vγ9Vδ2 T cells are also abundant in human fetal peripheral blood, but compared to their adult counterparts they have a distinct developmental origin, are hyporesponsive towards in vitro phosphoantigen exposure and they do not possess a cytotoxic effector phenotype. In order to obtain insight into the role of Vγ9Vδ2 T cells in the human fetus, we investigated their response to in utero infection with the phosphoantigen-producing parasite Toxoplasma gondii (T. gondii). Vγ9Vδ2 T cells expanded strongly in face with congenital T. gondii infection which was associated with differentiation towards potent cytotoxic effector cells. The Vγ9Vδ2 T cell expansion in utero resulted in a fetal footprint with public germline-encoded clonotypes in the Vγ9Vδ2 TCR repertoire 2 months after birth. Overall, our data indicate that the human fetus, from early gestation onwards, possesses public Vγ9Vδ2 T cells that acquire effector functions following parasite infections.

Key words: gammadelta; human; parasite; fetus; newborn; infant; early life; TCR repertoire; Toxoplasma gondii
Introduction

γδ T cells are T lymphocytes which express a T cell receptor (TCR) containing γ and δ chains, instead of α and β chains as in the conventional CD4 and CD8 αβ T cells. As αβ T cells, γδ T cells have been conserved for more than 450 million years of evolution and play an important role against infection and cancer (1–3). A major γδ T cell population in human adult blood are the Vy9Vδ2 T cells that are defined by the expression of a TCR containing the γ-chain variable region 9 (Vγ9, TRGV9) and the δ-chain V region 2 (Vδ2, TRDV2). They express a potent cytotoxic effector phenotype and are activated and expanded in a TCR-dependent manner by microbe- and host-derived phosphorylated prenyl metabolites (phosphorylated Ags, or “phosphoantigens”), derived from the isoprenoid metabolic pathway (4–6). The prototypical example of a microbial phosphoantigen is (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP) produced by the 2-Cmethyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid synthesis (also known as the nonmevalonate pathway) that is present in bacteria and in protozoan parasites of the phylum Apicomplexa (4). The recognition of phosphoantigens allows adult Vy9Vδ2 T cells to develop potent antimicrobial and anti-cancer immune responses (2, 4, 7–9). While Vy9Vδ2 T cells are also abundant in fetal peripheral blood, they are hyporesponsive towards phosphoantigen stimulation in vitro, they are highly regulated by PD-1 and they do not show a cytotoxic effector phenotype (10–15). These features are likely related to (tolerance) requirements of the fetal immune system, which involves a distinct thymic development (16–18).

During development of T cells in the thymus, TCR gene rearrangements take place where single V (variable), D (diversity; only for TRD), and J (joining) gene segments join to form a final chain (α, β, γ or δ). The variability created during the V(D)J recombination is significantly enhanced by the junctional diversity which comprises: 1) incorporation of palindromic sequences (“P nucleotides”); 2) the introduction of additional random nucleotides (“N additions”) in the junction by the terminal deoxynucleotidyl transferase (TdT enzyme); and 3) deletion of nucleotides (by exonuclease) (19). The pairing of a single γ (TRG) with a δ (TRD) chain will give rise to the final TCR expressed on the surface of the γδ T cell. The most variable domain, usually responsible for antigen recognition, is found in the complementarity determining region 3 (CDR3) and is the region most often analyzed. In human, γδ T cells are the most abundant lymphoid population in the embryonic thymus in early gestation, with a shift around gestation week 11 when they decrease significantly and the αβ T cells take the lead (20). The very first γδ T cell population to arise in human is the Vy9Vδ2 subset, detected in the embryonic (prethymic) liver from as early as 5- to 6-week gestation (21) and in fetal thymus after 8 weeks of gestation (17, 22), which is then likely to exit the thymus towards the fetal blood (10, 19). We have
recently shown that fetal and adult Vγ9Vδ2 T cells are generated by the thymus at different timepoints in life, and we have identified key differences between the TCR repertoire of fetal and adult blood Vγ9Vδ2 T cells, including the very low number of N additions in the fetal Vγ9Vδ2 TCR repertoire (17).

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan that belongs to the phylum Apicomplexa and, thus, produces the potent phosphoantigen HMBPP. It infects up to one third of the world’s population and infection acquired during pregnancy (congenital infection) may cause severe damage to the fetus (23, 24). Previous studies indicate that γδ T cells play a role in the immune response against T. gondii. Indeed, mice depleted of γδ T cells are more susceptible to T. gondii infection (25), human adult Vγ9Vδ2 T cells are expanded upon acute toxoplasmosis and an in vitro study showed that Vγ9Vδ2 T cells are activated by incubation with T. gondii-infected cells resulting in the killing of the infected cells (26). While some changes have been observed in cord blood Vγ9Vδ2 T cells in placental malaria (27), it remains unclear whether fetal Vγ9Vδ2 T cells can develop immune responses against pathogens that cross through the placenta, such as in congenital toxoplasmosis (13, 15, 28, 29). We found that fetal Vγ9Vδ2 T cells expanded strongly and differentiated towards potent killer effector cells in infants with congenital T. gondii infection.
Results

Study population

Pregnant mothers with primary \textit{T. gondii} infection were enrolled in this study. In order to address the fetal/newborn immune response towards \textit{T. gondii} infection, blood samples from their infants were collected. Fifteen out of 74 infants (20\%) were diagnosed with congenital toxoplasmosis (‘Toxo+’). The Toxo+ infants were age-matched with their non-infected (‘Toxo-’) counterparts (Fig. 1A; age of Toxo- versus Toxo+ subjects: \(p=0.0688\)). For most infants (66 out of 74) one blood sample was collected, for 7 infants two blood samples and for 1 infant three blood samples were collected between birth and 2 years of age (Fig. 1A). Clinical characteristics, such as age at the moment of diagnosis and treatment schedules of the Toxo+ infants, can be found in Table 1. All deliveries (of Toxo+ and Toxo- infants) were term deliveries (> 37 weeks gestation). No clinical problems were observed in the Toxo- infants.

\textbf{Vγ9Vδ2 T cells expand strongly upon congenital \textit{T. gondii} infection}

First, we determined the percentage of γδ T cells (out of total T cells) and found that they were significantly increased in Toxo+ infants (Fig. 1A). As can be seen from the plotting of the percentage of γδ T cells versus the age of the infants, this higher percentage of γδ T cells was especially evident early after birth (0-2 month old newborns, Fig. 1A). At older ages (> 2 months), the difference between Toxo+ and Toxo- infants diminished, mainly because the percentage of γδ T cells started to increase in the Toxo- group (Fig. 1A). The increase in γδ T cells percentages early after birth was not associated with an increased expression of the proliferation marker Ki-67 (Fig. 1B). To ascertain that this high percentage of γδ T cells was not due to changes in αβ T cells, we quantified absolute numbers and confirmed that the γδ T cells were increased, while no change could be detected in the αβ T cell compartment (supplemental Fig. 1 A-B).

Next, we investigated the Vγ9Vδ2 subset more specifically and compared it to other γδ subsets. Using antibodies specifically against the Vy9 and Vδ2 chain (combined with CD3 and pan-γδ antibodies), we could delineate four different populations: Vy9+Vδ2+, Vy9+Vδ2-, Vy9-Vδ2+ and Vy9-Vδ2- γδ T cells (Fig. 2; supplemental Fig. 1 C; supplemental Fig. 2). The increase in newborn γδ T cells upon congenital \textit{T. gondii} infection was highly restricted to the Vy9+Vδ2+ subset (Fig. 2, supplemental Fig. 1C, supplemental Fig. 2); the other subsets did now show different percentages nor numbers compared to Toxo- newborns, including the more abundant Vy9-Vδ2- γδ T cell subset (Fig. 2B; supplemental Fig. 1C, supplemental Fig. 2). Gating on newborn Vy9Vδ2 T cells, again no increase in the Ki-67 proliferation marker could be observed between Toxo+ and Toxo- subjects (supplemental Fig. 3), indicating that the proliferation of Vy9Vδ2 T cells has already occurred in utero, accounting for the higher percentage and
number of newborn Vγ9Vδ2 T cells in the Toxo+ compared to the Toxo- group (Fig. 2, supplemental Fig. 1C; supplemental Fig. 2).

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

Next, we examined whether the expanded Vγ9Vδ2 T cells upon congenital toxoplasmosis were differentiated, as assessed by the downregulation of CD28 and CD27 (30–33). The Vγ9Vδ2 T cells of Toxo+ newborns were highly differentiated (CD27-CD28-) compared to Toxo- newborns, while this was not the case for nonVγ9Vδ2 γδ T cells (Fig. 3A). The few Toxo- newborns showing a CD28-27-phenotype (Fig. 3A) could be due to an early response to the post-natal exposure to the microbiome (34, 35), but an influence of exposure to *T. gondii*-derived metabolites in utero cannot be excluded (36). At later ages, the difference between the Toxo+ and Toxo- group became less pronounced due to an increase in differentiated Vγ9Vδ2 T cells in Toxo- infants (Fig. 3A). The level of HLA-DR expression, reflecting recent activation (26, 37), on Vγ9Vδ2 T cells, but not on nonVγ9Vδ2 T cells, was also higher in the Toxo+ group (Fig. 3C-D), although this was more subtle compared to the differences observed regarding the differentiation status (compare Fig. 3A with Fig. 3C). Thus, it appears that the Vγ9Vδ2 T cells were activated and differentiated in utero along the strong expansion, after which proliferation and activation declined while the differentiation status remained stable and high until early after birth.

**Vγ9Vδ2 T cells express high levels of cytotoxic effector molecules upon congenital *T. gondii* infection**

We investigated whether the differentiation of the Vγ9Vδ2 T cells in utero was associated with the expression of cytotoxic effector molecules, which can be important for fighting infections (38). While fetal Vγ9Vδ2 T cells express granzyme A (GzmA) in the absence of infection, they do not express granzyme B (GzmB) nor perforin (10), the main cytotoxic effector molecules that can efficiently kill infected cells (38). Indeed, newborn Vγ9Vδ2 T cells of the Toxo- group did not express GzmB nor perforin (Fig. 4A-C). However, upon congenital *T. gondii* infection, the expression of these two cytotoxic mediators strikingly increased: a vast majority of (newborn) Vγ9Vδ2 T cells expressed GzmB, while perforin was co-expressed in the GzmB$^{\text{high}}$ Vγ9Vδ2 T cells (Fig. 4 A-C). The co-expression of GzmB and perforin is in line with the need of their combined actions to mediate their cytotoxic activity (38). At older ages, the Vγ9Vδ2 T cells of Toxo- infants started to also express GzmB and perforin (Fig. 4A-B). A relatively small difference in GzmB$^{\text{+}}$ and perforin$^{\text{+}}$ nonVγ9Vδ2 T cells could be observed between Toxo+ and Toxo- subjects, possibly due to a bystander effect caused by cytokine production in the local environment (Supplemental Fig. 4A-B). Since the T-box family transcription factors T-bet and eomesodermin (eomes) can be important for the expression of GzmB and/or perforin (38), we investigated the expression of these transcription factors in Vγ9Vδ2 T cells. While the expression of T-bet followed the same expression pattern as GzmB and perforin (Fig. 4D), this was not observed for
cymes (Fig. 4E). Granulysin is a cytotoxic granule pore-forming peptide that can permeabilize bacteria and parasites directly, and deliver death-inducing GzmB into these pathogens (39). Furthermore, adult Vγ9Vδ2 T cells, which are expanded in the blood stage of malaria-infected patients (Plasmodium falciparum), are able to reduce parasite reinvasion in a granulysin-dependent manner (40, 41). However, in contrast to GzmB and perforin, granulysin remained low in Toxo+ infants, even at older ages (Fig. 4F), indicating that this cytotoxic mediator does not play an important role in the defense of fetal Vγ9Vδ2 T cells against congenital T. gondii infection. Finally, we confirmed the programmed expression of GzmA (10) in Toxo- newborns, which was further increased by congenital T. gondii infection (Fig. 4G).

In order to have a global overview of all the flow cytometry data of Vγ9Vδ2 T cells in Toxo+ and Toxo-infants and how this compares to the data obtained in nonVγ9Vδ2 T cells and conventional αβ T cells, we performed tSNE and PCA analysis. This analysis revealed that early after birth Vγ9Vδ2 T cells from Toxo+ infants are clearly forming a distinct cytotoxic effector-related cluster, while this is not the case for nonVγ9Vδ2 T cells and αβ T cells (Fig. 4H and supplemental Fig. 5). Later in life, the Vγ9Vδ2 T cells from Toxo- and Toxo+ infants grouped together into one cluster (Fig. 4H and supplemental Fig. 5). Thus, this global analysis highlights the early and potent response of Vγ9Vδ2 T cells towards congenital T. gondii infection, including the acquisition of a high co-expression of the cytotoxic effector molecules GzmB and perforin.

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

In order to address whether the observed expansion of Vγ9Vδ2 T cells upon congenital toxoplasmosis (Fig. 1-2, supplemental Fig. 1-2) shaped their TCR repertoire, we analyzed the CDR3 of the γ and δ chain of sorted blood γδ T cells of Toxo+ and Toxo-infants at 2 months after birth (the earliest age at which we had a sufficient amount of sample material at the right conditions for TCR repertoire analysis; Toxo+ n=5, Toxo- n=10) and at 1 year (Toxo+ n=3, Toxo- n=4). At both age points, one of the Toxo+ infants had symptoms (retinitis).

The random insertion of nucleotides (denoted by N) by the enzyme TdT into the junctions of the joining V(D)J gene segments can significantly increase the junctional diversity of the CDR3 region (42). The level of TdT expression is low in fetal life, which is associated with the absence or a low number of N additions in the CDR3 repertoire of fetal γδ T cells (17, 43). Compared to Toxo- infants, the TRGV9- and TRDV2-containing CDR3 repertoire of Toxo+ infants at 2 months contained a lower number of N additions (Fig. 5A), especially in the TRDV2-containing CDR3 sequences using TRDJ1 as a joining gene segment (Fig. 5B). At 1 year, differences were less clear (supplemental Fig. 6A-B). Note that the Toxo+ infant showing symptoms had the highest number of N additions, especially in the TRDV2-containing
CDR3 (Fig. 5A-B). The difference in N additions between Toxo+ and Toxo- infants was specific for the
TRDV2- and TRGV9-containing sequences (supplemental Fig. 6C), which is in line with the distinct
expansion of Vγ9Vδ2 T cells upon congenital T. gondii infection (Fig. 2; supplementary Fig. 1-2).
The lower number of N additions in the TRGV9- and TRDV2-containing TCR repertoire of 2 month old
Toxo+ infants (with the notable exception of the infant with symptoms), indicates a fetal origin since
these features are known to be enriched in fetal blood Vγ9Vδ2 T cells (17). In order to address this
more directly we investigated the overlap of the TRGV9 and TRDV2 CDR3 infant repertoires with the
repertoires derived from fetal blood (22w-30w gestation), cord blood (39w-41w gestations) and adult
blood (26-64y) (17) (supplemental Fig. 7). Compared to Toxo- infants, the TRDV2 CDR3 repertoire of
Toxo+ infants (when excluding the ‘outlier’ infant with symptoms, Fig. 5A N additions TRDV2) was
shared more with the fetal blood repertoire (supplemental Fig. 7B, right panel). A tendency for such
increased sharing was also observed when compared with term delivery cord blood, but was
completely absent when compared to adult blood (supplemental Fig. 7B, right panel). The TRGV9
repertoire did not show such differences in sharing between Toxo+ and Toxo- infants (supplemental
Fig. 7B, left panel), which is probably due to the high prevalence of the public clonotype
CALWEVQELGKKIKVF (10, 27, 34) in both the Toxo- and Toxo+ group (supplemental Fig. 6D). These data
indicate that the Toxo+ TRDV2 repertoire at 2 months has an origin early in fetal life. Therefore, we
verified the presence of early fetal germline-encoded (i.e. without N additions) TRDV2 CDR3 sequences
(21, 34, 43) in the top 20 clonotypes of the Toxo+ infants (Fig. 5C) and found that their accumulated
frequency was more prevalent in 2 month Toxo+ compared to 2 month Toxo- infants (Fig. 5D). A
possible explanation for the high prevalence of the CACDVLGDTDKLIF and CACDILGDTDKLIF TRDV2
CDR3 sequences in early fetal life (21, 34) is that the P nucleotide(s) needed to form these sequences
can be derived from both the TRDV2 and the TRDD3 gene segment (supplemental Fig. 8). This can
occur in an efficient way in the absence of N additions because of the low expression of the TdT enzyme
in fetal life (43). In addition, there is a short-homology repeat present between the TRDD3 and TRDJ1
gene segments (supplemental Fig. 8). The prevalence of the third fetal liver sequence,
CACDTGGYTDKLIF, can be explained by short-homology recombination (supplemental Fig. 8). Note that
the short-homology repeat between TRDV2 and TRDD3 (the nucleotides ac) to form CACDTGGYTDKLIF
has been described previously (43).
In summary, it appears that Vγ9Vδ2 clonotypes with a fetal origin expand extensively in utero when
face with congenital T. gondii infection, resulting in a TCR repertoire footprint that is still present at 2
months after birth.
Discussion

Despite their high activation threshold in vitro, we show here that fetal Vy9Vδ2 T cells can respond vigorously to a parasite infection in utero. A main finding of our study was the presence among congenital Toxo+ infants of a fetal footprint in their γδ TCR repertoire (including germline-encoded TCR sequences), most likely because of the high expansion of fetal Vy9Vδ2 T cells in utero. In line with these in vivo observations, Vy9Vδ2 T cell clones expressing fetal germline-encoded TCR sequences are responsive in vitro towards phosphoantigen-containing mycobacterial extracts (21). Moreover, cord blood Vy9Vδ2 T cells proliferate upon incubation with T. gondii-infected PBMC in vitro (26). We propose that protection against phosphoantigen-generating pathogens, such as T. gondii, may have provided a selective pressure during evolution for the maintenance of the germline-encoded genetic elements needed for the generation of phosphoantigen-reactive TCRs early during fetal development (17, 19). This is in agreement with the high level of heritability of Vy9Vδ2 T cells compared to other innate-like T cells (44). Vy9Vδ2 T cells from the older infants of the Toxo- group showed post-natal expansions, likely due to a more general phosphoantigen exposure (e.g. microbiome) (34, 35), thus reaching similar Vy9Vδ2 T cell percentages as their Toxo+ counterparts. It is worth noting that the newborn who showed sequelae due to congenital T. gondii-infection (retinitis) did not show a fetal footprint like the Toxo+ infants without symptoms. Further studies are needed to investigate whether the lack of a fetal Vy9Vδ2 T cell footprint can be linked to the development of symptoms upon congenital T. gondii infection.

In contrast to our data, a previous study, mainly based on vitro restimulation data, indicated that congenital T. gondii infection induces an anergic state in infant Vy9Vδ2 T cells (45). However, other Vy9Vδ2 T cell functions were not assessed and age-matched controls were lacking (10, 45–47). Our data indicate that a major wave of proliferation of fetal Vy9Vδ2 T cells occurs in utero upon T. gondii encounter and is accompanied by the acquisition of the potent expression of cytotoxic mediators (GzmB+perforin+). These effector functions can be used to kill T. gondii-infected cells, as illustrated by the in vitro study of Subauste et al with Vy9Vδ2 T cell lines and clones (26). Such a response that combines innate (germline-encoded TCR acting as a pathogen recognition receptor) and adaptive (high proliferation upon pathogen encounter) features has been referred to as ‘adaptate’ biology (48). Hara et al suggested that Vy9Vδ2 T cells are susceptible to anergy induction because of their extra-thymic development (45). However, we have recently shown that the human thymus clearly contains Vy9+Vδ2+ cells (17). Based on our data from the current study, we conclude that congenital T. gondii infection does not induce an anergic state of fetal Vy9Vδ2 T cells but rather transforms them to
lymphocytes with a potent cytotoxic phenotype that contributes to protection against infection by killing T. gondii-infected cells.

We have previously shown that fetal nonVγ9Vδ2 γδ T cells, such as the public Vγ8Vδ1 T cells, play a major role in the response towards congenital human cytomegalovirus (HCMV) infection (30). Together with the data of our current study it appears that the human fetus is equipped with γδ T cell subsets that show a division of labor in their response to congenital infections: (i) the Vγ9Vδ2 T cells respond to T. gondii and possibly other phosphoantigen-generating pathogens and (ii) the nonVγ9Vδ2 T cells that target HCMV-infected cells. Data from human in vitro studies (26, 30) and in vivo studies in mice (25, 49, 50) indicate that γδ T cells play a protective role against infections with T. gondii and HCMV, but it cannot be excluded that the potent effector γδ T cells contribute to the development of pathologies observed upon congenital infections (51, 52).

A main correlate of protection of the malaria vaccine PfSPZ (attenuated Plasmodium falciparum sporozoite) are Vγ9Vδ2 T cells (53, 54). Our data, showing the importance of Vγ9Vδ2 T cells in the response towards congenital T. gondii infection, indicate that vaccines, or other strategies, could be developed targeting these cells to protect infants against (congenital T. gondii) infections. Tools to manipulate Vγ9Vδ2 T cells in vivo are becoming increasingly available and include modified phosphoantigens with improved pharmacological characteristics and monoclonal antibodies targeting BTN3A1 (55, 56). Both Plasmodium falciparum and T. gondii contain an organelle, the apicoplast, which has specific metabolic functions including the MEP pathway of isoprenoid synthesis. In this pathway, the metabolite HMBPP is generated, the most potent natural phosphoantigen (4, 57). This indicates that T. gondii-derived HMBPP is a major driving force for the expansion of fetal Vγ9Vδ2 T cells in utero. However, in contrast to our observations in congenital toxoplasmosis, Cairo et al observed a depletion of phosphoantigen-reactive Vγ9Vδ2 T cells in placental malaria (27). A main difference between congenital T. gondii infection and placental malaria is that the malaria parasite very rarely crosses the placenta into the fetal circulation to establish an infection (58). Furthermore, the type of placental malaria infection can have opposing effects on the immune system in early life, thus possibly contributing to the differential effect on the fetal Vγ9Vδ2 T cells (27, 58).

In immunocompromised (adult) patients (AIDS and transplant patients), toxoplasmosis is a major cause of morbidity and mortality (23). HIV infection leads to a decrease of Vγ9Vδ2 T cells (59), but it is not clear to what extent the depletion of these potential T. gondii-responsive cells contributes to T. gondii-induced morbidities. HCMV infection is a major driving force of nonVγ9Vδ2 γδ T cell expansion in organ transplant and hematopoietic stem cell transplant patients (60–62) and these expansions are
associated with reduced cancer development (63, 64). In contrast, the role of *T. gondii* infection in driving Vγ9Vδ2 T cell expansion/differentiation in these transplant settings and their potential anti-cancer role is not known. Therefore, the role of Vγ9Vδ2 T cells in toxoplasmosis in transplant and AIDS patients deserves further investigation.

Overall, our data indicate that the human fetus, from early gestation onwards, possesses Vγ9Vδ2 T cells that can expand and transform into killer effector cells upon congenital *T. gondii* infection. Thus, these fetal innate T cells could provide protection against parasite infections in utero.
Materials and methods

Blood sample collection and processing
Peripheral blood was collected in the Microbiology and Virology outpatient center of IRCCS San Matteo Hospital Foundation, Pavia, Italy. The diagnosis of congenital toxoplasmosis was performed with Liaison®XL Toxo IgG II / IgM CLIA, Novalisa IgA (DiaSorin, Saluggia, Italy), VIDAS Toxo IgG II, ISAGA IgM ((bioMérieux, Marcy l’Etoile, France), IgG-IgM western blot (LDBio, Lyon, France ) and homemade Interferon gamma release assay (65). IGRA test was performed in the same way as the test developed by Chapey and colleagues (65) with some modification, i.e.: the antigen employed (the same antigen utilized for Liaison commercial tests) with a final concentration of 3µg/ml, which was kindly provided by DiaSorin®. This concentration yielded the best results according to previous studies. The ELISA test to evaluate interferon gamma production is a commercially available kit (Qiagen, GMBH Germany).

All the mothers from both infected and non-infected groups were diagnosed with *T. gondii* infection during pregnancy and were treated in the same way (with pyramicin and /or pyrimethamine+sulfodiazine + folinic acid) (66). Depending on the volume of collected blood, samples were either directly lysed (~0.5ml) with FACS-lysing solution (BD FACS™ Lysing Solution) or processed (~1ml) to isolate peripheral blood mononuclear cells (PBMC) with Lymphoprep gradient centrifugation (Lymphoprep™, Stemcell Technology) and stored in liquid nitrogen. Frozen PBMC samples and FACS lysed blood samples were then sent to the Institute for Medical Immunology (IMI) of the Université Libre de Bruxelles (ULB) in Belgium.

Flow cytometry and Antibody reagents
FACS-lysed samples were thawed from liquid nitrogen at 37 °C and washed with PBS containing 0.1% bovine serum albumin (BSA) (Sigma). For surface staining, cells were incubated with antibody mix at 4 °C for 15-20min, then washed and resuspended with 0.1%BSA/PBS. For intracellular staining, after surface staining, the Perm 2 kit (BD) was used to permeabilize cell membrane. All samples were acquired either on CyAn ADP cytometer (Dako Cytomation) or LSRFortessa (BD); analysis was done using FlowJo software and R.

The following antibodies were used in this study: CD3-PB (clone SP34-2, BD), CD3-BV510 (UCHT1, BD), TCR γδ-APC (11F2, Miltenyi Biotec), TCR Vγ9-PC5 (IMMU 360, Beckman Coulter), TCR Vδ2-FITC (IMMU 389, Beckman Coulter), CD27-PE (M-T271, BD Bioscience), CD28-ECD (CD28.2, Beckman Coulter), CD45RA-PC7 (L48, BD Bioscience), HLA-DR-V450 (G46-6, BD), Ki-67-PC7 (B56, BD), T-bet-BV421 (4B10,
Biolegend), eomes-PE (WD1928, eBioscience), granzyme A-PB (CB9, BioLegend), granzyme B-PE-CF594 (GB11, BD), granulysin-PE (eBioDH2 (DH2), Invitrogen), perforin-PC7 (dG9 (delta G9), eBioscience).

Cell sorting, RNA isolation and CDR3 analysis

For PBMC samples, cells were thawed at 37 °C in complete medium [(RPMI 1640 (Gibco, Invitrogen), supplemented with L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 U/mL), and 1% nonessential amino acids (Lona) and 10% (vol/vol) heat-inactivated FCS (PPA Laboratories]), then labelled with Zombie NIR™ dye (Biolegend) at room temperature for 10 min, and stained with CD3/TCR γδ/T CR Vγ9/TCR Vδ2 antibodies at 4 °C for 15min. CD3+TCR γδ+ T cells were sorted on FACS Aria III (BD) with a mean purity of 98.0%. Cells were snap frozen in liquid nitrogen and preserved in -80 °C.

RNA was isolated from sorted γδ T cells (~10000 cells) with the RNeasy Micro Kit (Qiagen). cDNA was generated performing a template switch anchored RT-PCR. RNA was reverse transcribed via a template-switch cDNA reaction using TRGC (5’-CAAGAAGACAAAGGTATGTTCCAG) and TRDC (5’-GTAGAATTCTTACCCAGACAAAG) specific primers in the same reaction tube, a template-switch adaptor (5’-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG) and the Superscript II RT enzyme (Invitrogen). The TRGC primer binds both TRGC1 and TRGC2. The cDNA was then purified using AMPure XP Beads (Agencourt). Amplification of the TRG and TRD region was achieved using a specific TRGC primer (binding both TRGC1 and TRGC2 5’-GTCTCGTGAGCGAGATCTTGAAGAGACAGAATAGTGGGCTTTGGGGAACATCTCAT-3’, adapter underlined) and a specific TRDC primer (5’-GTCTCGTGAGCGAGATCTTGAAGAGACAGAATAGTGGGCTTTGGGGAACATCTCAT-3’, adapter underlined) and a primer complementary to the template-switch adapter (5’-TCGTCGGCGAGTGATGATGTATAAGAGACAGAATAGTGGGCTTTGGGGAACATCTCAT-3’, adapter underlined) with the KAPA Real-Time Library Amplification Kit (Kapa Biosystems).. Adapters were required for subsequent sequencing reactions. After purification with AMPure XP beads, an index PCR with Illumina sequencing adapters was performed using the Nextera XT Index Kit. This second PCR product was again purified with AMPure XP beads. High-throughput sequencing of the generated amplicon products containing the TRG and TRD sequences was performed on an Illumina MiSeq platform using the V2 300 kit, with 150 base pairs (bp) at the 3’end (read 2) and 150 bp at the 5’end (read 1) [at the GIGA center, University of Liège, Belgium].

After passing the quality check using fastqc (version 0.11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), 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 3.0.3) (67). Default parameters were used except to assemble the TRDD gene segments where 3 instead of 5 consecutive nucleotides were applied as assemble parameter. CDR3 sequences were then exported and analyzed using VDJtools software (version 1.2.1) using default settings in order to calculate the number of N additions, the CDR3 length, the length of J gene segments and the level of clonotype sharing between different samples (68). Sequences out of frame and containing stop codons were excluded from the analysis. Files generated from VDJtools were uploaded into Rstudio (version 1.1.463, R version 3.5.2) and analysis involved the following packages: ggplot2, dplyr, reshape, ggpubr and ggseqlogo (https://CRAN.R-project.org/package=). Fastq files of TRG and TRD sequences are deposited in the Sequence Read Archive under accession no. PRJNA625515.

Dimensionality reduction and clustering
Flow cytometry results generated from Flowjo and CDR3 data generated from VDJtools were uploaded into Rstudio. Package ggfortify (https://CRAN.R-project.org/package=ggfortify) and ggbiplot (http://github.com/vqv/ggbiplot) were used to generate principal-components analysis (PCA analysis). Package Rtsne (https://github.com/jkrijthe/Rtsne) was used to generate t-distributed stochastic neighbor embedding (t-SNE) clustering analysis (69). Parameters were adapted according to sample size. t-SNE analyses were run multiple times using different parameters.

Statistics
Statistical analysis was done by using R. Student's t-test was used for normally distributed data (Shapiro-Wilk test, p>0.05) and with equal variances (Levene's test, p>0.05). Otherwise, Mann Whitney U test was used. When more than one blood sample was obtained from the same subject at different ages (e.g. Figure 1: in 8 out of 74 infants), only the earliest sample was used from this subject in the statistical test for the calculation of the p value between Toxo+ and Toxo- infants. For analysis of the data according to age, the linear model was used for prediction; the pointwise 95% confidence interval was indicated around the mean.

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

LM, MP, MT conducted experiments; FG and VM acquired crucial blood samples; AM, VM and DV designed the study; LM and DV analyzed data; DV wrote the manuscript.
Acknowledgments

We are grateful to all the mothers and infants for participating in this study. We thank the GIGA Genomics platform (Latifa Karim and Wouter Coppieters) for their outstanding technical support. This work was supported by the Fonds de la Recherche Scientifique - FNRS (CDR 0244.17) and the Fund John W. Mouton Pro Retina. Ling Ma is supported by the Chinese Scholarship Council (CSC), Fonds Van Buuren-Jaumotte-Demoulin and Fonds Hoguet. Maria Papadopoulou is supported by the FNRS (FRIA and short-term post-doctoral fellowship), Fonds Van Buuren-Jaumotte-Demoulin and Fonds Hoguet.


44. Mangino M, et al. Innate and adaptive immune traits are differentially affected by genetic and environmental factors [Internet]. Nat Commun. 2017;8(1). doi:10.1038/ncomms13850


Figure 1. Congenital *T. gondii* infection induces expansion of γδ T cells in utero.

(A) Percentage of γδ T cells (of total CD3+ T cells) versus age (sample number: Toxo-: n=66, Toxo+: n=17; subject number: n=74, Toxo+ n=15).

(B) Expression of the proliferation marker Ki-67 in γδ T cells versus age (sample number: Toxo-: n=55, Toxo+: n=10; subject number: n=56, Toxo+ n=8).

Toxo+ samples are indicated in orange triangles, Toxo- samples are indicated in black dots. Lines connect samples of the same subject. Samples from subjects with symptoms (retinitis) are indicated. P values of Toxo+ (with or without the subjects with symptoms) samples versus Toxo+ samples are calculated by Mann Whitney U test. The 95% confidence interval (linear model) of >0 area is indicated for each group (orange for Toxo+, grey for Toxo-).
Figure 2. The expansion of γδ T cells upon congenital *T. gondii* infection is highly restricted to Vγ9^+^Vδ2^+^ T cells.

(A) Representative flow cytometry plots of a Toxo- (2 days old) and a Toxo+ (1 day old) newborn. Gate is on CD3^+^ T lymphocytes; percentage of Vγ9^+^Vδ2^+^ cells are indicated.

(B) γδ subsets percentage (of CD3^+^ T cells) (sample number: Toxo-: n=66, Toxo+: n=17; subject number: n=74, Toxo+ n=15).

(C) Percentage of Vγ9^+^Vδ2^+^ cells (of total CD3^+^ T cells) versus age (sample number : Toxo-: n=66, Toxo+: n=17; subject number: n=74, Toxo+ n=15).

Toxo+ samples are indicated in orange triangles, Toxo- samples are indicated in black dots. Lines connect samples of the same subject. Samples from subjects with symptoms (retinitis) are indicated. P values of Toxo+ (with or without the subjects with symptoms) samples versus Toxo+ samples are calculated by Mann Whitney U test. The 95% confidence interval (linear model) of >0 area is indicated for each group (orange for Toxo+, grey for Toxo-).
Figure 3. Vγ9Vδ2 T cells are differentiated upon congenital T. gondii infection.

(A) percentage of CD27-CD28- cells of Vγ9Vδ2+ γδ T cells versus age (sample number: Toxo-: n=53, Toxo+: n=13; subject number: n=57, Toxo+ n=11).
(B) percentage of CD27-CD28- cells of nonVγ9Vδ2+ γδ T cells versus age (sample number: Toxo-: n=53, Toxo+: n=13; subject number: n=57, Toxo+ n=11).
(C) percentage of HLA-DR+ cells of Vγ9Vδ2+ γδ T cells versus age (sample number: Toxo-: n=53, Toxo+: n=13; subject number: n=57, Toxo+ n=11).
(D) percentage of HLA-DR+ cells of nonVγ9Vδ2+ γδ T cells versus age (sample number: Toxo-: n=53, Toxo+: n=13; subject number: n=57, Toxo+ n=11).

Toxo+ samples are indicated in orange triangles, Toxo- samples are indicated in black dots. Lines connect samples of the same subject. Samples from subjects with symptoms (retinitis) are indicated. P values of Toxo+ (with or without the subjects with symptoms) samples versus Toxo+ samples are calculated by Mann Whitney U test. The 95% confidence interval (linear model) of >0 area is indicated for each group (orange for Toxo+, grey for Toxo-).
Figure 4. Vγ9Vδ2 T cells express high levels of cytotoxic effector molecules upon congenital T. gondii infection.

(A) Percentage of gzMB+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=53, Toxo+: n=10; subject: n=54, Toxo+ n=8).

(B) Percentage of perforin+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=53, Toxo+: n=10; subject: n=54, Toxo+ n=8).

(C) Flow cytometry plots gated on Vγ9Vδ2 T cells of a representative sample of Toxo- (2 days old) and Toxo+ (1 day old) newborns illustrating the expression of gzMA, gzMB and perforin.

(D) Percentage of T-bet+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=55, Toxo+: n=10; subject: n=56, Toxo+ n=8).

(E) Percentage of eomes+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=55, Toxo+: n=10; subject: n=56, Toxo+ n=8).

(F) Percentage of granulysin+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=53, Toxo+: n=10; subject: n=54, Toxo+ n=8).

(G) Percentage of gzMA+ cells of Vγ9Vδ2+ γδ T cells versus age (sample: Toxo-: n=53, Toxo+: n=10; subject: n=54, Toxo+ n=8).
t-SNE analysis of flow cytometry results (11 markers [HLA-DR, CD27, CD28, CD45RA, Ki-67, T-bet, eomes, gzmA, gzmB, granulysin, perforin], n=48 subjects).

Toxo+ samples are indicated in orange triangles, Toxo- samples are indicated in black dots. Lines connect samples of the same subject. Samples from subjects with symptoms (retinitis) are indicated. P values of Toxo+ (with or without the subjects with symptoms) samples versus Toxo+ samples are calculated by Mann Whitney U test. The 95% confidence interval (linear model) of >0 area is indicated for each group (orange for Toxo+, grey for Toxo-).
Figure 5. The Vy9Vδ2 TCR repertoire of Toxo+ newborns contains a fetal footprint.

(A) Number of N additions of TRGV9- and TRDV2-containing CDR3 of sorted blood γδ T cells from 2-month-old infants. P values (indicated on the bar graphs) are obtained by the student’s t-test for TRGV9 N additions (bar indicates mean) and by Mann Whitney U test for TRDV2 N addition (bar indicates median).

(B) Number of N additions of TRDV2-containing CDR3 using either TRDJ1, TRDJ2 or TRDJ3 of sorted blood γδ T cells from 2-month-old infants. P values (indicated on the bar graphs) are calculated by student’s t-test, bar indicates mean.

(C) Accumulated percentage of the top 20 TRDV2-containing CDR3 sequences of 5 Toxo+ 2-month-old samples (obtained from sorted blood γδ T cells). Six germline-encoded sequences are indicated in different colors.

(D) Accumulated percentage of the six germline-encoded sequences indicated in (C) in Toxo+ and Toxo- 2-month-old infants (sorted blood γδ T cells). P value (indicated on the bar graph) is calculated by student’s t-test, bar indicates mean.

One subject with symptoms (retinitis) is indicated. The P values obtained without this subject with symptom are indicated under each bar figure.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample type</th>
<th>Gender</th>
<th>Gestation time at diagnosis (Mother)</th>
<th>Start of treatment (Mother)</th>
<th>Treatment (Mother)</th>
<th>Age at diagnosis (infant)</th>
<th>Start of treatment (infant)</th>
<th>Treatment (infant)</th>
<th>Symptoms (infant)</th>
<th>Age at sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Toxo-001</td>
<td>FACS-lysed</td>
<td>M</td>
<td>20 gw</td>
<td>28 gw</td>
<td>Pyr+sulf</td>
<td>At birth</td>
<td>3 weeks</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>5 days</td>
</tr>
<tr>
<td>I-Toxo-011</td>
<td>FACS-lysed</td>
<td>F</td>
<td>28 gw</td>
<td>29 gw</td>
<td>Pyr+sulf</td>
<td>At birth</td>
<td>3 weeks</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>1 day</td>
</tr>
<tr>
<td>I-Toxo-012</td>
<td>FACS-lysed</td>
<td>F</td>
<td>34 gw</td>
<td>34 gw</td>
<td>spyrr</td>
<td>At birth</td>
<td>3 weeks</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>1 day</td>
</tr>
<tr>
<td>I-Toxo-024</td>
<td>FACS-lysed</td>
<td>M</td>
<td>34 gw</td>
<td>35 gw</td>
<td>spyrr</td>
<td>2 months</td>
<td>2 months</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>99 days</td>
</tr>
<tr>
<td>I-Toxo-072</td>
<td>FACS-lysed</td>
<td>M</td>
<td>At delivery</td>
<td>no</td>
<td>no</td>
<td>At birth</td>
<td>At birth</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>93 days</td>
</tr>
<tr>
<td>I-Toxo-075</td>
<td>FACS-lysed</td>
<td>F</td>
<td>26 gw</td>
<td>28 gw</td>
<td>Pyr+sulf</td>
<td>In utero</td>
<td>At birth</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>29 days</td>
</tr>
<tr>
<td>I-Toxo-080</td>
<td>FACS-lysed/PBMC</td>
<td>M</td>
<td>24 gw</td>
<td>26 gw</td>
<td>spyrr</td>
<td>4 months</td>
<td>4 months</td>
<td>Pyr+sulf+FA</td>
<td>retinitis</td>
<td>511 days</td>
</tr>
<tr>
<td>I-Toxo-085</td>
<td>FACS-lysed/PBMC</td>
<td>F</td>
<td>35 gw</td>
<td>36 gw</td>
<td>spyrr</td>
<td>At birth</td>
<td>1 month</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>73 days</td>
</tr>
<tr>
<td>I-Toxo-090</td>
<td>PBMC</td>
<td>F</td>
<td>12 gw</td>
<td>no</td>
<td>no</td>
<td>At birth</td>
<td>3 months</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>434 days</td>
</tr>
<tr>
<td>I-Toxo-106</td>
<td>PBMC</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>Pyr+sulf</td>
<td>7 days</td>
<td>15 days</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>82 days</td>
</tr>
<tr>
<td>I-Toxo-108</td>
<td>PBMC</td>
<td>F</td>
<td>36 gw</td>
<td>37 gw</td>
<td>spyrr</td>
<td>At birth</td>
<td>15 days</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>755 days</td>
</tr>
<tr>
<td>I-Toxo-122</td>
<td>PBMC</td>
<td>M</td>
<td>27 gw</td>
<td>28 gw</td>
<td>Pyr+sulf (only 1 month)</td>
<td>15 days</td>
<td>21 days</td>
<td>Pyr+sulf+FA</td>
<td>No</td>
<td>78 days</td>
</tr>
<tr>
<td>I-Toxo-132</td>
<td>PBMC</td>
<td>F</td>
<td>33 gw</td>
<td>-</td>
<td>no</td>
<td>At birth</td>
<td>15 days</td>
<td>pyr+sulf</td>
<td>No</td>
<td>65 days</td>
</tr>
<tr>
<td>I-Toxo-135</td>
<td>PBMC</td>
<td>M</td>
<td>22 gw</td>
<td>26 gw</td>
<td>spyrr</td>
<td>At birth</td>
<td>15 days</td>
<td>pyr+sulf</td>
<td>No</td>
<td>59 days</td>
</tr>
<tr>
<td>I-Toxo-137</td>
<td>PBMC</td>
<td>M</td>
<td>25 gw</td>
<td>27 gw</td>
<td>spyrr/pyr+sulf</td>
<td>At birth</td>
<td>15 days</td>
<td>pyr+sulf</td>
<td>Retinitis</td>
<td>67 days</td>
</tr>
</tbody>
</table>

gw: gestation week; “-“:no information; pyr: pyrimethamine; sulf: sulfodiazine; spyrr: spyramicin; FA: folic acid. All deliveries were term deliveries (> 37 gw).