Classification: Biological Sciences - Immunology and Inflammation

Fetal public Vγ9Vδ2 T cells expand and gain potent cytotoxic functions early after birth

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Keywords:

γδ T cells, gammadelta, newborns, infant, human immunology, phosphoantigen, BCG, vaccination, TCR, CDR3 repertoire

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 human Vγ9Vδ2 T cells with a public/shared fetal-derived TCR repertoire expanded within 10 weeks postpartum. Such an expansion was not observed in the nonVγ9Vδ2 γδ T cells, which possessed a private TCR repertoire. Furthermore, only the Vγ9Vδ2 T cells differentiated into potent cytotoxic effector cells by 10 weeks of age, despite their fetal origin. Both the expansion of public fetal Vγ9Vδ2 T cells and their functional differentiation were not affected by newborn vaccination with the phosphoantigen-containing BCG vaccine. These findings suggest a strong and early priming of the public fetal-derived Vγ9Vδ2 T cells promptly after birth, likely upon environmental phosphoantigen exposure.

Significance statement

Early life immune responses have been described as suboptimal with neonates and infants being susceptible to infections. Vγ9Vδ2 T cells are the first T lymphocytes to be generated in the human fetus. Their T cell receptor-mediated responses to in vitro stimulation and their effector functions at birth are weaker compared to those in adults, possibly reflecting the need for tolerance in utero. However, here we show that upon transition to the prominent microbial exposure early after birth, public fetal-derived Vγ9Vδ2 T cells expand and differentiate into potent cytotoxic effector cells. Thus, they provide newborns with a first line of anti-microbial effector T cells in order to combat infections in early life.

Introduction

Together with αβ T cell and B cells, γδ T cells have been conserved since the emergence of jawed vertebrates more than 450 million years ago and can play an important role in anti-microbial and anti-tumor immunity(1–3). γδ T cells are the first T cells made during embryonic development 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(1, 4–10).

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(11, 12). 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 peripheral blood 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(13–15). This recognition of phosphoantigens allows Vγ9Vδ2 T cells to develop potent antimicrobial and anticancer responses(3, 13, 16–19). While Vγ9Vδ2 T cells are also abundant in the blood of mid-gestation fetuses, they represent only a small percentage of γδ T cells at birth(20, 21). Fetal 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 fetal counterparts(5, 20, 22). It is not clear, however, how Vγ9Vδ2 T cells respond to 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 Vγ9Vδ2 T cells found in the blood circulation early after birth are still 'fetal-like', or whether a switch towards 'adult-like' Vγ9Vδ2 T cells has already been initiated.

The tuberculosis (TB) vaccine Bacille Calmette-Guérin (BCG) contains phosphoantigens(23, 24) and is administered at birth in TB endemic settings. In adult non-human primate models (Vγ9Vδ2 T cells do not exist in rodents(25)), it has been shown that Vγ9Vδ2 T cells expand upon BCG vaccination, which correlated with protection against TB(26). Consistent with these observations, γδ T cell responses in human BCG-vaccinated adults and infants have been

reported(27–31). BCG can therefore 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) and possessed a public TCR repertoire which was related to their fetal origin. Furthermore, despite their fetal origin and in contrast to private γδ T cell subsets and conventional αβ T cells, Vγ9Vδ2 T cells showed a pronounced differentiation towards adult-like cytotoxic effector cells. Finally, this early and strong Vγ9Vδ2 T cell response was not altered by newborn BCG vaccination, suggesting an important role of environmental exposure in the expansion and functional differentiation of fetal-derived Vγ9Vδ2 T cells in early life.

Results

Vγ9Vδ2 T cells expand early after birth

First, we measured the abundance of Vγ9Vδ2 T cells and other γδ subsets (nonVγ9Vδ2) in peripheral blood of 10-week-old infants and compared it to cord and adult blood. The frequencies of Vγ9Vδ2 T cells were higher in 10-week-old infants and adults compared to cord (Fig. 1A), an observation that was specific for this γδ subset (Fig. 1A, right panel). Expression of the proliferation marker Ki-67 was highest in the 10-week old group, compared to both cord and adult blood, highlighting an active phase of proliferation early after birth (Fig. 1B).

Only the 10-week-old Vγ9Vδ2 TCR repertoire is public and fetal-derived

Compared to adult Vγ9Vδ2 T cells, fetal and cord blood Vγ9Vδ2 T cells respond poorly to microbial-derived phosphoantigens(5, 20, 32, 33). We investigated whether the expanded Vγ9Vδ2 T cells in infants were derived from fetal Vγ9Vδ2 T cells, or whether an 'adult-like' Vγ9Vδ2 developmental program was initiated immediately after birth. To answer this question, we compared the TCR repertoire of Vγ9Vδ2 and nonVγ9Vδ2 γδ T cells sorted from 10-week-old infants to the repertoire of their fetal and adult counterparts. The fetal TCR repertoire was characterized in blood collected at <30 (fetal) or at >37 weeks (cord) of gestation(22). The 10-week-old Vγ9Vδ2 TRD repertoire was highly shared between individuals, as demonstrated by the geometric mean of overlap frequencies (F) and number of clonotypes shared within the group (Fig. 2A-B left panels), in contrast to the adult repertoire. The 10-week-old TRD repertoire was even more shared (public) than that of cord blood and reached a similar level of overlap as observed for fetal Vγ9Vδ2 T cells (Fig. 2A-B, left panels). A range of different clonotypes contributed to this high proportion of sharing among 10-week-old infants (Fig. 2B, left panel); the most public clonotypes at 10 weeks are shown in Table 1. Of relevance, the two most abundant clonotypes, CACDVLGDTDKLIF and CACDILGDTDKLIF, have been described to be highly prevalent in pre-thymic fetal liver(34). Importantly, the high proportion of sharing within the TRD repertoire was specific for the Vγ9Vδ2 T cell subset. Indeed, the TRD repertoire of 10-week-old nonVγ9Vδ2 T cells was completely private (unique in each individual) like in adult nonVγ9Vδ2 T cells, despite showing a significant level of sharing at the fetal stage (Fig. 2A-B, right panels; Fig. S1A-B).

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(22). The 10-week-old Vγ9Vδ2 CDR3δ repertoire possessed a low fetal-like level of N additions (Fig. 2C, left

panel), which was again specific for the Vγ9Vδ2 T cells. NonVγ9Vδ2 T cells from the same infants showed a high adult-like level of N additions in their CDR3δ sequences (Fig. 2C, right panel). In line with an important contribution of fetal-derived Vγ9Vδ2 T cells to the 10-week Vγ9Vδ2 TCR repertoire, was the relative high usage of the fetal-like TRDJ2-3 segments at the expense of adult-like TRDJ1 (Fig. 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 adult Vγ9Vδ2 T cells (Fig. 2E, left panel), despite a lower number of N additions (Fig. 2C). TRDJ usage of the nonVγ9Vδ2 T cells was similar in infants and adults, but different to fetal cells (Fig. 2D, right panel). Of note, the high proportion of 10-week-old Vγ9Vδ2 TRD repertoire sharing 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 overlap than TRDJ3-containing CDR3δ sequences (Fig. 2F). To investigate the fetal origin of the 10-week-old Vγ9Vδ2 T cells more directly, we examined the level of sharing between the 10-week-old CDR3δ repertoire and the other groups and observed that around 30% is similar to the fetal, while the overlap with the cord and adult repertoire was significantly lower (Fig. 2G, left panel, Fig. 2H). Once more, this was highly specific for the Vγ9Vδ2 subset, as such sharing was not observed between 10-week-old and fetal nonVγ9Vδ2 T cells (Fig. 2G, right panel, Fig. S1C-D). The high sharing between fetal and 10-week-old Vγ9Vδ2 T cells was due to a relatively high number of clonotypes and not just a few abundant fetal ones (Fig. 2H-I). The lower overlap found in cord (Fig. 2A) was in line with a higher diversity estimation (D25; percentage of unique clonotypes required to account for 25% of total repertoire) which was reduced at 10-weeks with the expansion of (fetal-derived) Vγ9Vδ2 T cells (Fig. S1E). Results of analyzing the CDR3γ repertoire (Fig. S2) were similar to those of the CDR3δ repertoire (Fig. 2), with the main exception that the adult TRGV9 was largely public (Fig. S2), in line with previous studies(22, 35, 36).

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

Fetal-derived Vγ9Vδ2 T cells get activated and become highly cytotoxic rapidly after birth

Next, we investigated whether fetal-derived Vγ9Vδ2 T cells expanded early after birth were functionally mature. 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. 3A, Fig. S3A). In addition, at 10 weeks they started to gradually differentiate by losing the expression

of CD27 and CD28 (Fig. 3B). However, this did not lead to a significant increase in fully differentiated cells, as observed for adult Vγ9Vδ2 T cells (Fig. 3B right panel, Fig. S3B).

Aside from their TCR, Vγ9Vδ2 T cells can also use NK receptors (NKR) to recognize target cells(37). We verified the expression of a series of NKR and found that 10-week old Vγ9Vδ2 T cells specifically showed increased expression of the inhibitory NKR NKG2A compared to cord blood, while other T cell subsets and other NKR (CD161, KLRG1, CD158a/b, NKG2C) did not show such expression pattern (Fig. 3C-D; Fig. S3C). NKG2D, an important activating NKR for γδ T cells, including the Vγ9Vδ2 subset(37), was already highly expressed by cord blood Vγ9Vδ2 T cells and was not further increased after birth (Fig. 3D).

A major function of Vγ9Vδ2 T cells in adults is killing of infected and cancer cells(3, 16, 18). We evaluated the cytotoxic potential of the Vγ9Vδ2 T cells in detail by analyzing different cytotoxic mediators that each play a different role in the killing machinery(38). At birth, Vγ9Vδ2 T cells lacked the expression of granzyme B and perforin, a combination that is known to efficiently kill infected cells via apoptosis(38). Strikingly, at 10-weeks, despite their relatively limited differentiation (Fig. S3B), the Vγ9Vδ2 T cells co-expressed these cytotoxic mediators at adult-like levels (Fig. 3E-G). Interestingly, while all the T cell subsets in adults expressed granzyme B and perforin, in early life expression of these markers was restricted to the Vγ9Vδ2 T cell subset (Fig. 3E-F). Granulysin can mediate specific killing of intracellular and extracellular microbes(39, 40). In contrast to perforin and granzyme B, granulysin was almost absent in early life and reached only high levels of expression in adults (Fig. 3G-H). Granzyme A mediates killing of target cells by a different mechanism than granzyme B, and is known to have alternative roles to cytotoxic activity(38, 41, 42). Granzyme A was already expressed in cord specifically by Vγ9Vδ2 T cells (Fig. 3I), as observed previously in fetal (<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. 3I). In addition, the per-cell expression was even higher in infant compared to adult Vγ9Vδ2 T cells (Fig. 3G, Fig. S3D).

In summary, 10-week-old Vγ9Vδ2 T cells are highly activated and express a particular pattern of cytotoxic mediators that is clearly different from Vγ9Vδ2 T cells at birth (high perforin and granzyme B), as well as from adult Vγ9Vδ2 T cells (absence of granulysin, higher granzyme A).

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(1–3), 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. 4A-B). Vγ9Vδ2 T cells were the main producers of IFNγ in early life, while in adults other T cells expressed IFNγ as well (Fig. 4A). While the percentage of Vγ9Vδ2 expressing IFNγ and TNFα remained stable at 10 weeks (compared to cord blood), 10-week-old Vγ9Vδ2 T cells expressed much more IFNγ (but not TNFα) per cell (Fig. 4A, right panel, Fig 4B right panel, Fig. S3E). 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. 4C), which are known to be important for IFNγ production in γδ T cells(43).
Thus, the cytokine expression capacity (IFNγ, TNFα and associated transcription factors) of Vγ9Vδ2 T cells is mainly programmed before birth while the IFNγ levels per Vγ9Vδ2 T cell is highly increased early after birth.

Phosphoantigen-reactivity remains stable early after birth

It is known that fetal and cord blood-derived Vγ9Vδ2 T cells show a significantly reduced response towards phosphoantigens such as the microbial-derived (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)(13, 20, 32, 33), compared to adult-derived Vγ9Vδ2 T cells. However, it is not clear whether this response would change early after birth(5). HMBPP induced comparable low levels of IFNγ in infant Vγ9Vδ2 T cells as observed in cord blood (Fig. 4D), while the higher response of adult Vγ9Vδ2 was confirmed (Fig. 4D). Further, treatment with zoledronic acid, which leads to intracellular isopentenyl pyrophosphate (IPP) accumulation and depends on different accessory leukocyte populations than HMBPP(33, 44), also did not lead to a higher IFNγ production in 10-week-old Vγ9Vδ2 T cells compared to cord (Fig. 4E). Thus, upon functional differentiation early after birth, the fetal-derived Vγ9Vδ2 T cells do not show an increase of their phosphoantigen-reactivity towards adult-like levels.

BCG vaccination at birth does not alter the TCR repertoire nor functional differentiation of Vγ9Vδ2 T cells

Next, we investigated whether the expansion and associated activation and functional maturation of 10-week-old Vγ9Vδ2 T cells could be influenced by vaccination at birth with BCG, a known Vγ9Vδ2 T cell activator(23, 24, 26, 45) which can also expand cord blood Vγ9Vδ2 T cells in vitro(32). Thus, we compared the function and repertoire of Vγ9Vδ2 T cells from 10-week-old infants who received BCG vaccination at birth (BCG+) with infants who did not receive vaccination at birth (BCG-).
To our surprise, frequencies of Vγ9Vδ2 T cells were not increased in BCG+ compared to BCG-infants (Fig. 5A, top panel). This was confirmed by a very similar proliferation rate ex-vivo (Fig.

5A, bottom panel). We further investigated the shaping of the repertoire in detail early after birth (Fig. 1B-H), which may shed light on how the TCR repertoire changed in response to a specific stimulus (BCG)(17). Overall, TCR diversity (Fig. 5B-C), number of N additions (Fig. 5D), usage of J segment (Fig. 5E), CDR3 length (Fig. 5F), and level of repertoire overlap (Fig. 5G-H) were not significantly different between BCG- and BCG+ infants.

A detailed analysis of the phenotype and effector functions of infant Vγ9Vδ2 T cells indicated that BCG vaccination did not influence the rapid and striking differentiation of the neonatal Vγ9Vδ2 T cells early after birth (Fig. 6, Fig. S4).

Overall, these data suggest that the expansion of public fetal-derived Vγ9Vδ2 T cells and their functional differentiation early after birth, most likely upon environmental (phosphoantigen) exposure, are not altered by the administration of the phosphoantigen-containing vaccine BCG at birth.

Discussion

Compared to adult blood Vγ9Vδ2 T cells, cord blood Vγ9Vδ2 T cells show only a limited expansion upon in vitro phosphoantigen stimulation(5, 20, 32, 33). Despite this, we show here that within ten weeks of birth Vγ9Vδ2 T cells from healthy infants were already expanding. Furthermore, TCR repertoire analysis indicated a preferential expansion of early fetal-derived (<30 weeks of gestation) clonotypes, showing a high level of sharing. Consistent with this was the identification of CACDVLGDTDKLIF and CACDILGDTDKLIF among the top shared TRD sequences in 10-week-old Vγ9Vδ2 T cells, previously described to be highly abundant and shared in pre-thymic livers of 6/7-week gestation fetuses(34). Hence, it appears that public Vγ9Vδ2 clonotypes, derived from fetal thymus(22) and/or fetal liver(34), are maintained until birth and show a preferential expansion upon birth. In sharp contrast, the other blood γδ T cell subsets (grouped as nonVγ9Vδ2 T cells, not responding to phosphoantigens), which also showed TCR repertoire overlap at the fetal stage, became private at term delivery and did not expand early after birth. In adults, the TRD repertoire of both Vγ9Vδ2 and nonVγ9Vδ2 T cells were private, consistent with other studies(35, 36, 46). A major potential source of phosphoantigens in the first weeks after birth is the developing microbiota(47). Indeed, HMBPP, 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 fetal/cord Vγ9Vδ2 T cells in vitro(13, 22, 47–49). The high phosphoantigen-activation threshold early after birth may be reduced by factors such as innate cytokines (IL-18, IL-23) that are then highly expressed(50). Indeed, fetal/cord blood Vγ9Vδ2 T cells show an increased in vitro phosphoantigen responsiveness when co-incubated with IL-18 or IL-23, they show high expression of the IL-18 receptor and the expression of the IL-23 receptor is induced upon phosphoantigen exposure(20, 33, 44). 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(21). 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(6, 36, 51) 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 until 6 years with a series of nongenetic determinants (prenatal maternal lifestyle-related or immune-mediated determinants, birth characteristics and bacterial/viral exposure–related determinants)(52). Interestingly, among the 26 different determinants investigated, only 'premature rupture of membranes' was found to be associated with Vγ9Vδ2 T cell levels(52). At premature gestation times Vγ9Vδ2 T cells are the main subset, while at term-delivery Vδ1+ γδ T cells are predominant(20, 53). Since we show here that fetal-derived Vγ9Vδ2 T cells expand immediately upon delivery, 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 (nonVγ9Vδ2), at 10 weeks after birth the public Vγ9Vδ2 T cells were activated and differentiated towards high expression of cytotoxic mediators (perforin, granzyme B, granzyme A). While variable perforin expression has been described in pediatric Vδ2+ cells(5) (containing both the Vγ9+Vδ2+ and Vγ9-Vδ2+ subset(5, 20, 36, 51)), 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(38). Therefore, their co-expression in 10-week-old Vγ9Vδ2 T cells suggests that these cells are potent cytotoxic effectors against (phosphoantigen-generating) infections early after birth. Granzyme A, which is highly expressed by 10-week-old Vγ9Vδ2 T cells, at levels even higher than in adults, can induce a different cell death pathway than granzyme B(38). Furthermore, granzyme A produced by Vγ9Vδ2 T cells promotes inhibition of mycobacterial growth in macrophages(42). 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. Unlike perforin and granzyme B expression, the cytokine expression capacity (IFNγ, TNFα and associated transcription factors) of Vγ9Vδ2 T cells was mainly programmed before birth. Among NKR, NKG2A was highly upregulated early after birth on the cell surface Vγ9Vδ2 T cells, which can be triggered by phosphoantigen exposure(54). The similar expression patterns of the cytotoxic mediators perforin/granzyme B and the inhibitory NKR NKG2A in 10-week old Vγ9Vδ2 T cells suggest that NKG2A signaling could regulate 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(40). As opposed to cord-and 10-week Vγ9Vγ2 T cells, granulysin was highly expressed by adult Vγ9Vδ2 T cells. Furthermore, adult Vγ9Vδ2 T cells clearly showed a higher

response towards HMBPP compared to 10-week and cord blood Vγ9Vδ2 T cells. These specific features of Vγ9Vδ2 T cells in adult blood circulation may be due to their distinct development compared to fetal-derived Vγ9Vδ2 T cells(22).

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 by BCG vaccination in early life (29–31), but these studies did not consider age-matched unvaccinated controls. A possible explanation for the absence of BCG-induced effects on infant Vγ9Vδ2 T cells is that the expansion due to the sudden microbial phosphoantigen exposure at birth (including the developing microbiome) overrides a possible effect of BCG administration detectable by 10 weeks of age. This could explain why clear expansions of Vγ9Vδ2 T cells can be seen in non-human primates (NHP) (in clean facilities) upon vaccination with BCG(26). Moreover, intravenous administration (instead of the routine intradermal administration practiced in humans) and the higher dosage of BCG vaccine used in NHP studies (26, 55) seem to favor the activation of immune cells as demonstrated recently by Darrah and colleagues(56). In this study, there was an increase of peripheral Vγ9+ γδ T cells only after high-dose intravenous BCG administration (and not intradermal), which was notably transient(56). In addition, the distinct development of fetal and adult Vγ9Vδ2 T cells may contribute to different responses to vaccination with BCG, depending on the age of the vaccinated donors(22, 27, 28). Of note, γδ T cells have been increasingly recognized as important players in vaccine-mediated protection from infection(57). As our study shows that fetal-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)(58, 59), 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, fetal-derived Vγ9Vδ2 T cells expressing public/shared TCRs specifically expand and differentiate to a cytotoxic subset with functions closer to those seen in adults than the fetal counterparts. This differentiation is not affected by BCG vaccination at birth, a strong γδ stimulus, which is likely due to

prominent environmental exposure. This post-natal polyclonal burst of Vγ9Vδ2 T cells combined with strong functional maturation shapes an innate T cell subset in newborns that may be important to fight infections at a time when the conventional (memory) αβ T cell response is not fully active(60).

Materials and Methods

Study Populations

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 intradermally (Danish 1331 strain, Statens Serum Institut, Denmark) at birth as is routine in South Africa (BCG+, median age 65 days, min 56 – max 77), and another group not vaccinated with BCG at birth (BCG-, median age 67.5 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 newborns (cord blood) and adults from the same community (all independent donors).

Newborns, 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.

Whole blood was collected in CPT tubes or heparinized polypropylene tubes. Peripheral blood mononuclear cells (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 fetal blood. Since the fetal blood samples originated from Belgium (no access to South-African fetal 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 (fetal, cord and adult blood) analyzed here were previously described(22). Briefly, samples included fetal 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 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.

Methods

Flow cytometry, sorting and cell cultures, TCRγ (TRG) and TCRδ (TRD) high-throughput sequencing and statistical analysis are described in SI Appendix, Supplemental Methods.

Data availability

Fastq files of TRG and TRD sequences are deposited under SRA accession code PRJNA624366.

Acknowledgements

We are grateful to the mothers and infants that participated to this study. We are also grateful for the key contribution of Ms Bernadette Pienaar, Study Coordinator at SATVI, who has prematurely passed. We thank the GIGA Genomics platform (Latifa Karim and Wouter Coppieters) for their outstanding technical support. This work was supported by the FNRS (J.0078.13), the European Regional Development Fund (FEDER Immunomarquers) and an ARC (Action de Recherche Concertée) grant of the ULB. MP is supported by the FNRS (FRIA), Fonds Van Buuren-Jaumotte-Demoulin and Fonds Hoguet. This article is published with the support of the Fondation Universitaire of Belgium.

Author contributions

MP and TD designed and undertook experiments; MSh, LB, HV, NK, HA, MSt, WAH, TJS and EN were involved in clinical design and execution, including blood collection and storage; MP, TD and DV processed and interpreted data; TD, TJS and EN revised the manuscript; MP and DV wrote the manuscript and DV designed the study.

Competing interests

The authors declare no competing interests.

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Figure legends:

Figure 1

Vγ9Vδ2 T cells expand early after birth.

A. Frequencies of the Vγ9+Vδ2+ subset in CD3+ cells (left panel, bars indicate medians) and cumulative frequencies 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; p>0.5 for the nonVγ9Vδ2 γδ T cell subsets between 10w and cord) in cord (n=18), 10-week-old (10w, n=36) and adult (n=17). Representative flow cytometry 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=7, 10w n=14). Bars indicate medians. Representative flow cytometry plots (right panel).

Data shown from independent donors (South-African). P values are reported on graphs.

Figure 2

Only the 10-week-old Vγ9Vδ2 TCR repertoire is public and fetal-derived.

A-E. Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (left panels) and nonVγ9Vδ2 γδ T cells (right panels), derived from fetal (n=5, Belgian), cord (n=9, Belgian=6, South-African=3), 10w (n=14, South-African) and adult (n=11 for Vγ9Vδ2, Belgian=8, South-African=3 and n=8 for nonVγ9Vδ2, Belgian=5, South-African=3) blood.

A. Comparison of geometric mean of relative overlap frequencies (F metrics by VDJ tools) within pairs of fetal, cord, 10w or adult blood donors, each dot represents the F value of a pair of samples.
B. Number of clonotypes shared within pairs of fetal, cord, 10w or adult blood donors, 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 fetal, cord, 10w and or adult blood donors, in TRDJ1 repertoire (left panel) and TRDJ3 repertoire (right panel) of sorted Vγ9Vδ2 T cells derived from fetal (n=5, Belgian), cord (n=9, Belgian=6, South-African=3), 10w (n=14, South-African) and adult (n=11 for Vγ9Vδ2, Belgian=8, South-African=3 and n=8 for nonVγ9Vδ2, Belgian=5, South-African=3) blood. Each dot represents the F value of a pair of samples.

G-H. Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (left panels) and nonVγ9Vδ2 γδ T cells (right panels), derived from fetal (n=5, Belgian), cord (n=9, Belgian=6, South-African=3), 10w (n=14, South-African) and adult (n=11 for Vγ9Vδ2, Belgian=8, South-African=3 and n=8 for nonVγ9Vδ2, Belgian=5, South-African=3) blood.

G. Relative abundance of the 10w repertoire overlapping with fetal, cord or adult repertoire. Each dot represents a pair comparison.
H. Number of clonotypes shared between the 10w repertoire and fetal, cord or adult repertoire in Vγ9Vδ2 T cells. Each dot represents a pair comparison.
I. Representative shared clonotype abundance plots for one 10w Vγ9Vδ2 TRD repertoire versus one fetal (top), one cord (middle) and one adult (bottom) Vγ9Vδ2 TRD repertoire. The shared top 20 clonotypes between two samples are each represented in a distinct color. The frequency of these clonotypes in each sample is represented on the left side for 10w and on right side for fetal, cord, or adult. The other shared clonotypes are represented in dark grey. The rest of the repertoire (that is thus non-overlapping) is represented in light grey. Note that only up to 25% of the repertoire is shown.

Data shown from independent donors (from Belgium in round symbols, from South-Africa in square symbols, pair comparisons including both Belgian and South-African samples in triangle symbols). Bars indicate medians (a-c, e-h, j). *P*-values are reported on graphs.

Figure 3

Vγ9Vδ2 T cells are activated and become cytotoxic rapidly after birth.

A. Percentages of activated (HLA-DR+) cells among Vγ9Vδ2 T cells (cord, adult n=8, 10w n=16).
B. Percentages of naive (CD27+CD28+) Vγ9Vδ2 T cells (left panel) and representative flow plots (right panel).

C-F. Percentages of αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells expressing:

C. NKG2A in cord (n=4), 10w (n=8-10) and adult (n=3-4).
D. NKG2D in cord (n=4-5), 10w (n=8-10) and adult (n=4-5).
E. granzyme B in cord (n=7), 10w (n=14) and adult (n=7).
F. perforin in cord (n=5), 10w (n=10) and adult (n=5).
G. Representative co-expression flow cytometry plots of granzyme A, granzyme B, perforin and granulysin in cord, 10w and adult Vγ9Vδ2 T cells.

H-I. Percentages of αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2- γδ T cells, Vγ9-Vδ2+ γδ T cells and Vγ9-Vδ2- γδ T cells expressing:

H. granulysin in cord (n=7), 10w (n=14) and adult (n=7).
I. granzyme A in cord (n=7), 10w (n=14) and adult (n=7).

Data shown from independent donors (South-African). Bars indicate medians (a,b). Error bars indicate medians ± IQR (e-f, h-i). P values are reported on graphs.

Figure 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 (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=7-8, 10w=14-16).
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 zoledronic acid (3 days, in presence of IL-2; cord, adult n=6, 10w=12). Values derived from 'medium+IL-2' condition are subtracted.

Data shown from independent donors (South-African). Error bars indicate medians ± IQR (a-c). Bars indicate medians (d-e). P values are reported on graphs.

Figure 5

Neonatal BCG vaccination does not shape of the 10-week-old Vγ9Vδ2 TCR repertoire.

A. Frequencies of the Vγ9+Vδ2+ subset in 10-week-old unvaccinated infants (10w BCG-) or vaccinated infants (10w BCG+) CD3+ cells (top panel; n=18) and percentage of Ki-67+ cells among Vγ9Vδ2 T cells (bottom panel; n=7)).

B-H. Comparison of the CDR3 TRDV2 (top row) and TRGV9 (bottom row) repertoire of sorted Vγ9Vδ2 T cells derived from 10w BCG- (n=7) and 10w BCG+ (n=7) 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 colors 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) within pairs of BCG- or pairs of BCG+ infants; each dot represents the F value of a pair of samples.
H. Relative abundance of the BCG- or BCG+ repertoire overlapping with fetal. Each dot represents a pair comparison.

Data shown from independent donors (South-African). 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.

Figure 6

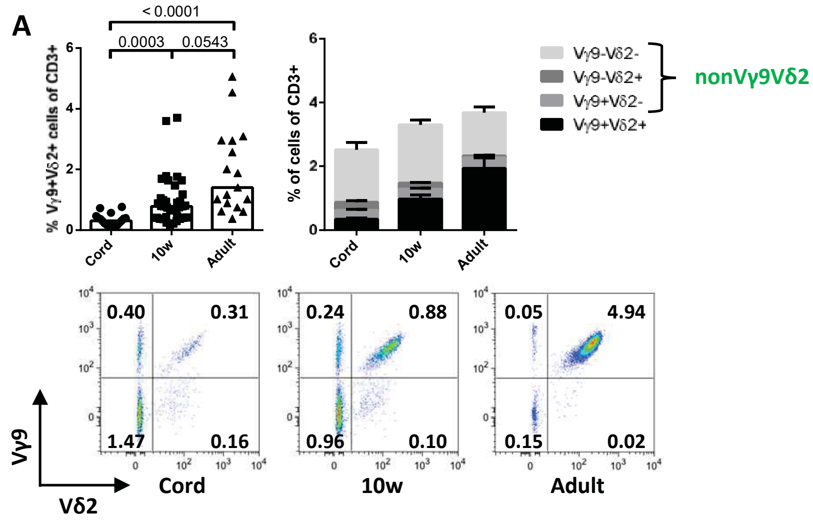
Neonatal BCG vaccination does not influence the functional differentiation of 10-week-old Vγ9Vδ2 T cells.

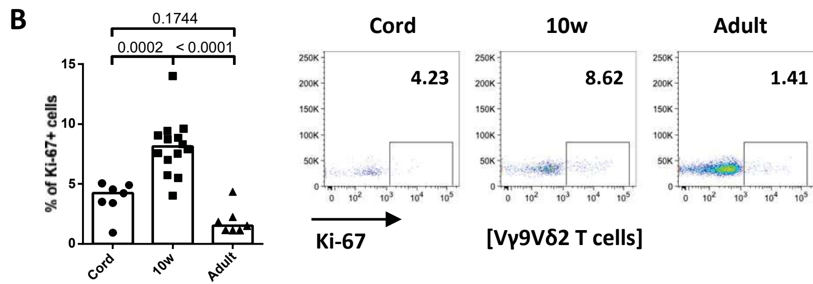
PCA analysis profiling of Vγ9Vδ2 T cells derived from cord, 10-week-old BCG-, 10-week-old BCG+ and adult blood (n=5), based on the percentage of: HLA-DR+, CD27-CD28-, CD27-CD28+, CD27+CD28-, CD27-CD28-, T-bet+, Eomes+, granzyme A+, granzyme B+, perforin+, granulysin+, IFNγ+ and TNFα+ cells. Data shown from independent donors (South-African).

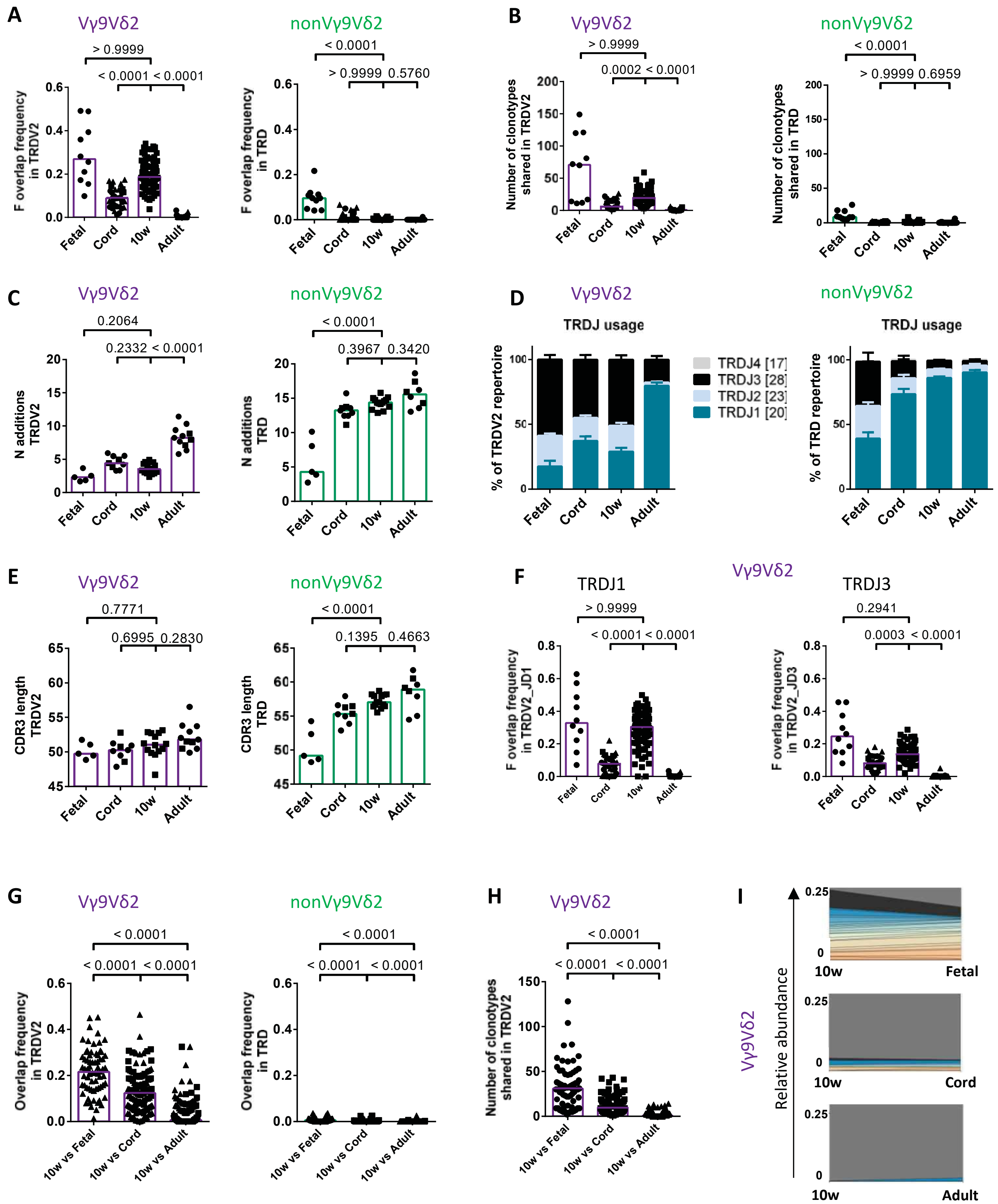
Table 1: The most shared CDR3δ clonotypes among 10-week-old (10w) Vγ9Vδ2 T cells.

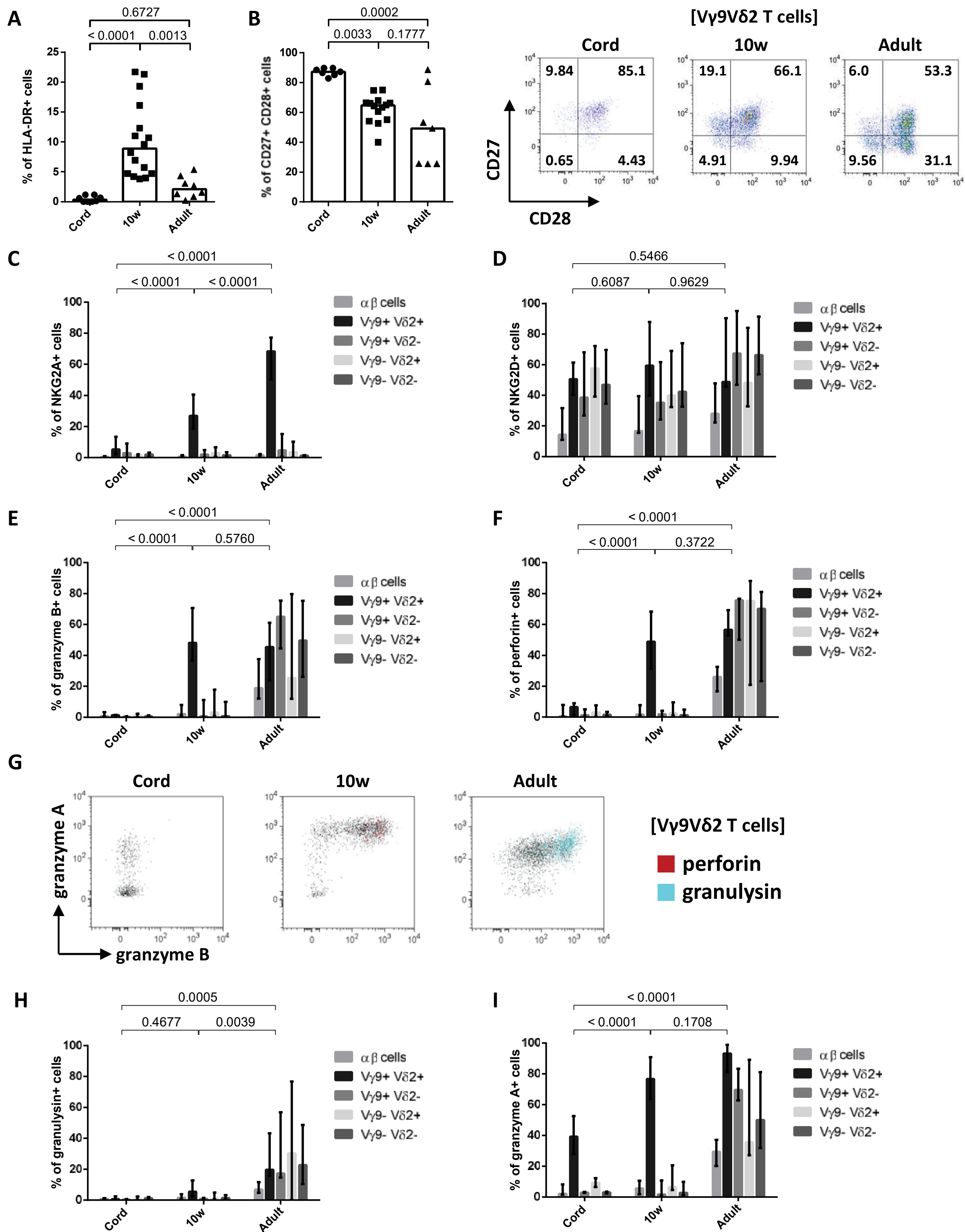
CDR3 clonotype (aa)	Number of N additions	Occurrences (/14)	Median Abundance
CACDILGDTDKLIF	0	13	3.298%
CACDTVLGDTWDTRQMFF	0	13	0.858%
CACDTVLGDSSWDTRQMFF	0	13	0.337%
CACDVLGDTDKLIF	0	12	2.598%
CACDILGDTAQLFF	0	10	0.694%
CACDTWGYTDKLIF	1/0*	10	0.572%
CACDTWGTDKLIF	0	10	0.258%
CACDTLGDTDKLIF	0	9	0.250%
CACDILGDTLTAQLFF	0	9	0.222%
CACDSTGGYSWDTRQMFF	0	9	0.146%
CACDTAGGYSWDTRQMFF	1	9	0.047%
CACDTVGDTDKLIF	1	8	0.388%
CACDNTGGYSWDTRQMFF	1	8	0.122%
CACDTWGMTAQLFF	0	8	0.116%
CACDTWGSSWDTRQMFF	0	8	0.094%
CACDVLGDLTAQLFF	0	8	0.078%
CACDTWDTRQMFF	0	8	0.024%
CACDTVLGDTDKLIF	0	8	0.003%

Clonotypes detected in more than 50% of the 10w donors are shown. CDR3 clonotype (aa): amino acid CDR3 sequence; number of N additions incorporated in the nucleotype(s) encoding each clonotype; occurrences: number of donors where the clonotype was detected (out of 14); median abundance: median percentage of repertoire in the fourteen 10w donors. *Of the two nucleotypes encoding this clonotype, one is germline and one includes one N addition. (aa: amino acid).


A
< 0.0001
0.0003
0.0543
% Vγ9+Vδ2+ cells of CD3+
Cord
10w
Adult
% of cells of CD3+
Vγ9-Vδ2-
Vγ9-Vδ2+
Vγ9+Vδ2-
Vγ9+Vδ2+
nonVγ9Vδ2
0.40
0.31
1.47
0.16
0.24
0.88
0.96
0.10
0.05
4.94
0.15
0.02
Vγ9
Vδ2
Cord
10w
Adult


B
0.1744
0.0002
< 0.0001
% of Ki-67+ cells
Cord
10w
Adult
Cord
10w
Adult
4.23
8.62
1.41
Ki-67
[Vγ9Vδ2 T cells]


A
Vγ9Vδ2
nonVγ9Vδ2
F overlap frequency in TRDV2
F overlap frequency in TRD
> 0.9999
< 0.0001
< 0.0001
> 0.9999
0.5760
Fetal
Cord
10w
Adult
B
Number of clonotypes shared in TRDV2
Number of clonotypes shared in TRD
0.0002
0.6959
C
N additions TRDV2
N additions TRD
0.2064
0.2332
0.3967
0.3420
D
TRDJ usage
% of TRDV2 repertoire
% of TRD repertoire
TRDJ4 [17]
TRDJ3 [28]
TRDJ2 [23]
TRDJ1 [20]
E
CDR3 length TRDV2
CDR3 length TRD
0.7771
0.6995
0.2830
0.1395
0.4663
F
TRDJ1
TRDJ3
F overlap frequency in TRDV2_JD1
F overlap frequency in TRDV2_JD3
0.2941
0.0003
G
Overlap frequency in TRDV2
Overlap frequency in TRD
10w vs Fetal
10w vs Cord
10w vs Adult
H
I
Relative abundance
Vγ9Vδ2


A
B
C
D
E
F
G
H
I
[Vγ9Vδ2 T cells]
Cord
10w
Adult
CD27
CD28
% of HLA-DR+ cells
% of CD27+ CD28+ cells
% of NKG2A+ cells
% of NKG2D+ cells
% of granzyme B+ cells
% of perforin+ cells
% of granulysin+ cells
% of granzyme A+ cells
granzyme A
granzyme B
perforin
granulysin
αβ cells
Vγ9+ Vδ2+
Vγ9+ Vδ2-
Vγ9- Vδ2+
Vγ9- Vδ2-
0.6727
< 0.0001
0.0013
0.0002
0.0033
0.1777
0.5466
0.6087
0.9629
0.5760
0.3722
0.0005
0.4677
0.0039
0.1708
9.84
85.1
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4.43
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4.91
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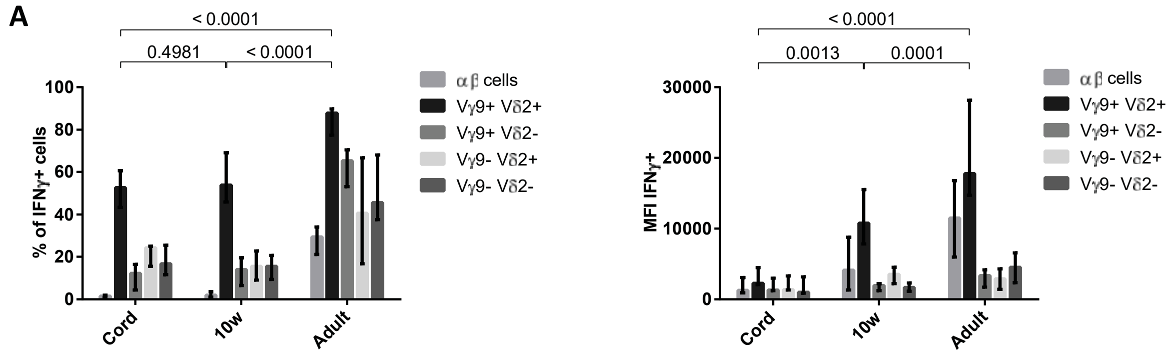
6.0
53.3
9.56
31.1

A



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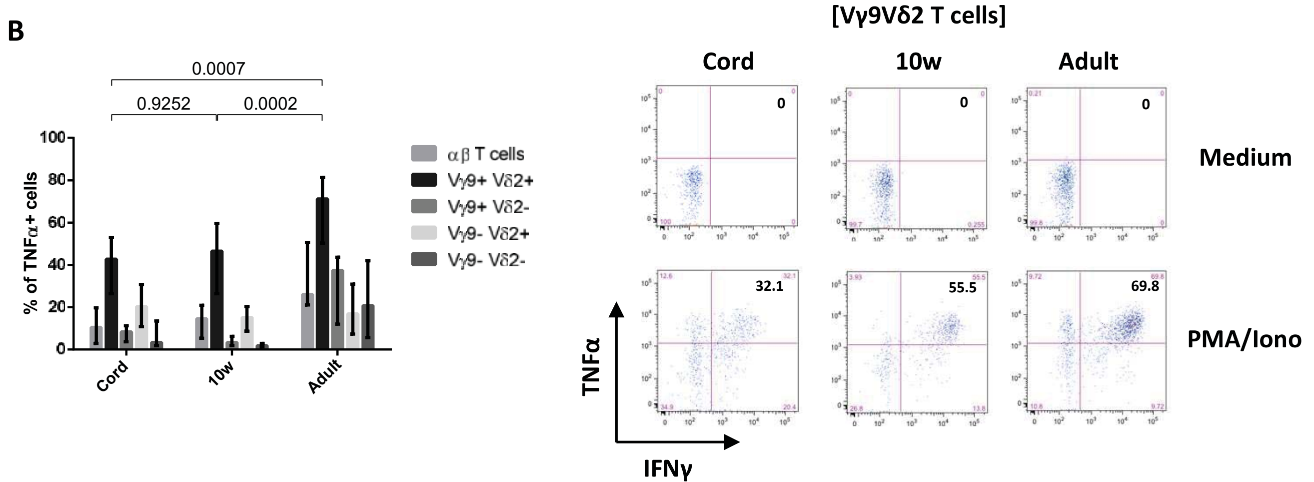
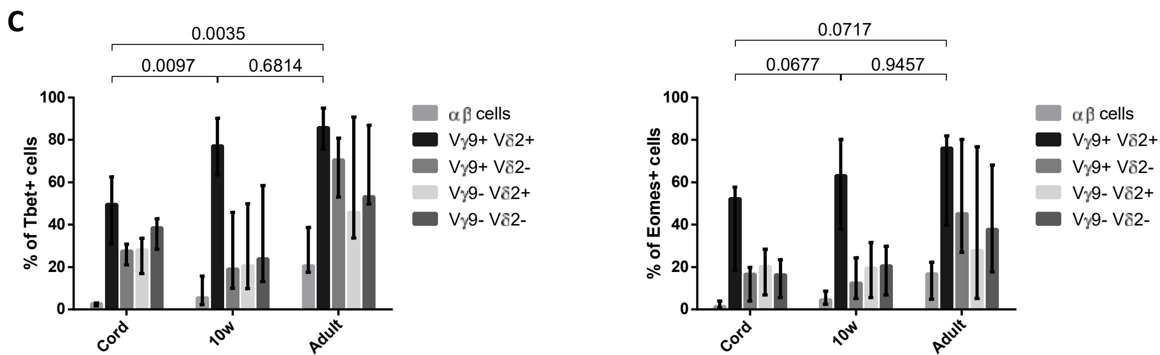
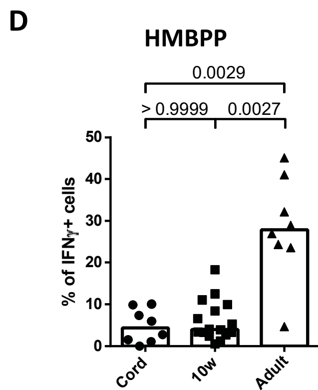


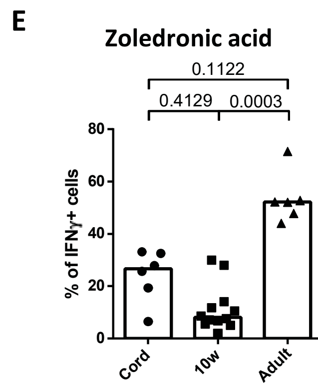
C

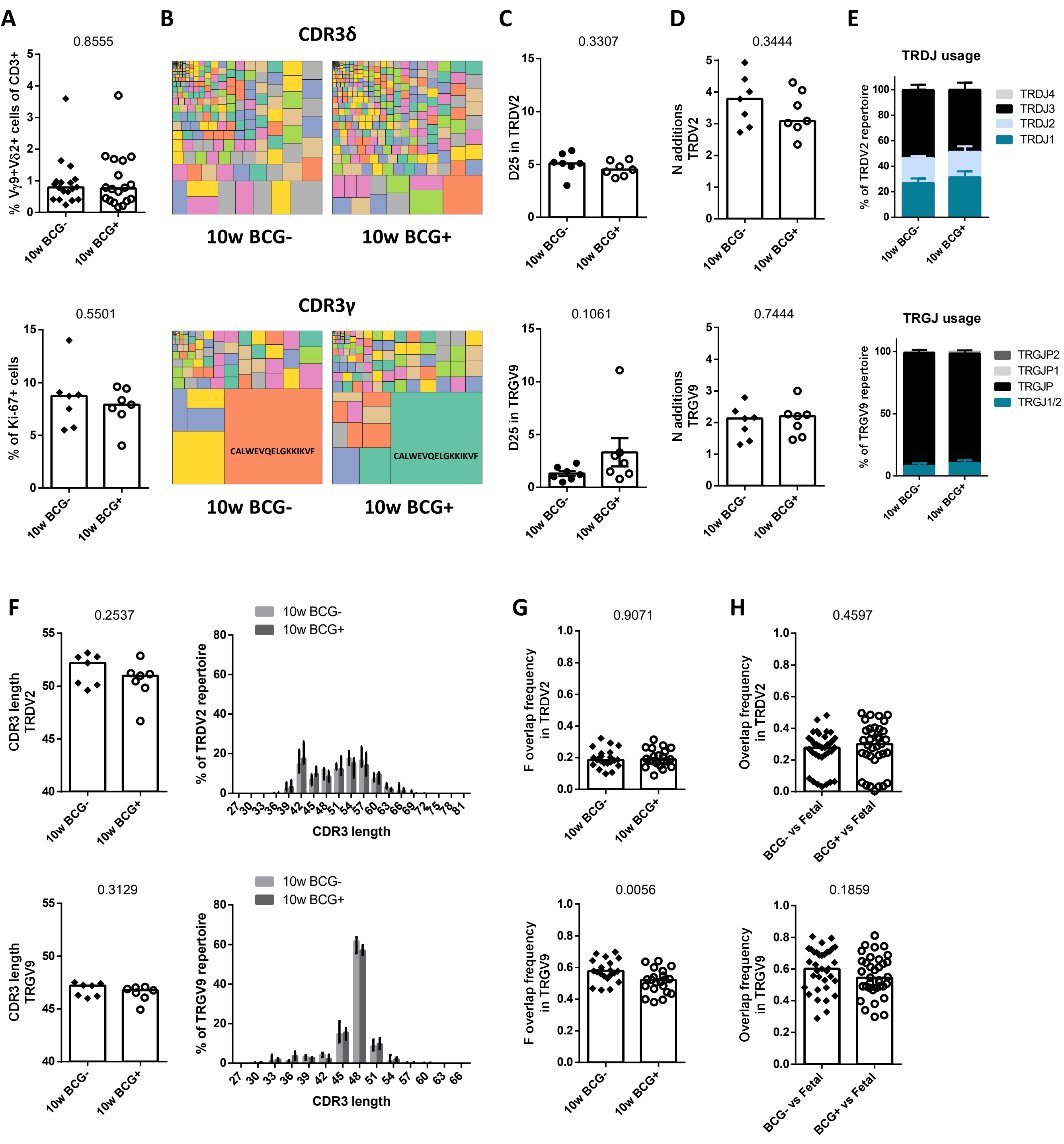


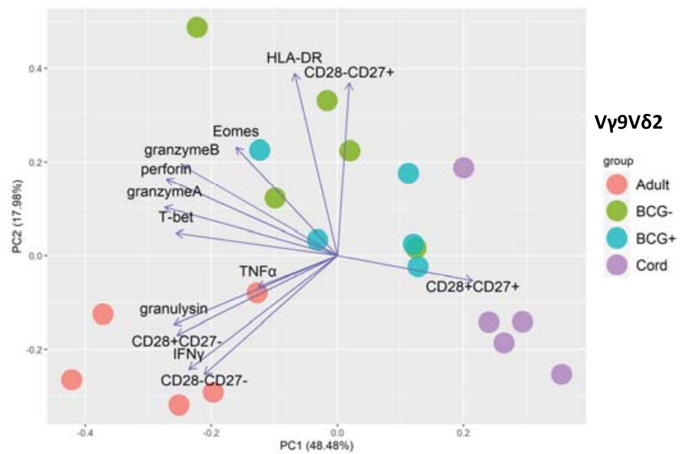
D



E




A
B
C
D
E
F
G
H
0.8555
% Vγ9+Vδ2+ cells of CD3+
10w BCG-
10w BCG+
CDR3δ
CDR3γ
0.3307
D25 in TRDV2
0.3444
N additions TRDV2
TRDJ usage
% of TRDV2 repertoire
TRDJ4
TRDJ3
TRDJ2
TRDJ1
0.5501
% of Ki-67+ cells
CALWEVQELGKKIKVF
0.1061
D25 in TRGV9
0.7444
N additions TRGV9
TRGJ usage
% of TRGV9 repertoire
TRGJP2
TRGJP1
TRGJP
TRGJ1/2
0.2537
CDR3 length TRDV2
% of TRDV2 repertoire
CDR3 length
0.9071
F overlap frequency in TRDV2
0.4597
Overlap frequency in TRDV2
BCG- vs Fetal
BCG+ vs Fetal
0.3129
CDR3 length TRGV9
% of TRGV9 repertoire
0.0056
F overlap frequency in TRGV9
0.1859
Overlap frequency in TRGV9



Vγ9Vδ2
group
Adult
BCG-
BCG+
Cord
HLA-DR
CD28-CD27+
Eomes
granzymeB
perforin
granzymeA
T-bet
TNFα
granulysin
CD28+CD27-
IFNγ
CD28-CD27-
CD28+CD27+
PC1 (48.48%)
PC2 (17.98%)