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

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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 toward 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 wk postpartum. Such an expansion was not observed in non-Vγ9Vδ2 γδ T cells, which possessed a private TCR repertoire. Furthermore, only the Vγ9Vδ2 T cells differentiated into potent cytotoxic effector cells by 10 wk 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 bacillus Calmette–Guérin (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.

gammadelta | newborn | Vγ9Vδ2 | infant | TCR repertoire

Together with αβ T cell and B cells, γδ T cells have been conserved since the emergence of jawed vertebrates more than 450 million y ago and can play an important role in antimicrobial and antitumor 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 complementarity-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 midgestation 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) and 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 toward "adult-like" Vγ9Vδ2 T cells has already been initiated.

The tuberculosis (TB) vaccine bacillus Calmette–Guérin (BCG) contains phosphoantigens (23, 24) and is administered at birth in TB endemic settings. In adult nonhuman 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 wk) 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 toward 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.

Significance

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 with 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 antimicrobial effector T cells in order to combat infections in early life.

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The authors declare no competing interest.

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Data deposition: Fastq files of the TRG and TRD sequences reported in this paper have been deposited in the Sequence Read Archive (accession no. PRJNA624366).

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Results

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

Only the 10-wk-Old Vγ9Vδ2 TCR Repertoire Is Public and Fetal-Derived. Compared with 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 non-Vγ9Vδ2 γδ T cells sorted from 10-wk-old infants with the repertoire of their fetal and adult counterparts. The fetal TCR repertoire was characterized in blood collected at <30 (fetal) or at >37 wk (cord) of gestation (22). The 10-wk-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. 2 *A* and *B*, *Left*), in contrast to the adult repertoire. The 10-wk-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. 2 *A* and *B*, *Left*). A range of different clonotypes contributed to this high proportion of sharing among 10-wk-old infants (Fig. 2 *B*, *Left*); the most public clonotypes at 10 wk are shown in Table 1. Of relevance, the two most abundant clonotypes, CACDVLGDTDKLIF and CACDILGDTDKLIF, have been described to be highly prevalent in prethymic 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-wk-old non-Vγ9Vδ2 T cells was completely private (unique in each individual) like in adult non-Vγ9Vδ2 T cells, despite showing a significant level of sharing at the fetal stage (Fig. 2 *A* and *B*, *Right* and *SI Appendix*, Fig. S1 *A* and *B*).

An important feature in the detection of the developmental origin is the number of *N* additions used during the formation of CDR3 by V(D)J recombination (22). The 10-wk-old Vγ9Vδ2 CDR3δ repertoire possessed a low fetal-like level of *N* additions (Fig. 2 *C*, *Left*), which was again specific for the Vγ9Vδ2 T cells. Non-Vγ9Vδ2 T cells from the same infants showed a high, adult-like level of *N* additions in their CDR3δ sequences (Fig. 2 *C*, *Right*). In line with an important contribution of fetal-derived Vγ9Vδ2 T cells to the 10-wk-old Vγ9Vδ2 TCR repertoire was the relatively high usage of the fetal-like TRDJ2 and -3 segments at the expense of adult-like TRDJ1 (Fig. 2 *D*, *Left*). TRDJ2 and -3 are longer than TRDJ1 and therefore probably contribute to the maintenance of the CDR3δ length at 10 wk compared with adult Vγ9Vδ2 T cells (Fig. 2 *E*, *Left*), despite a lower number of

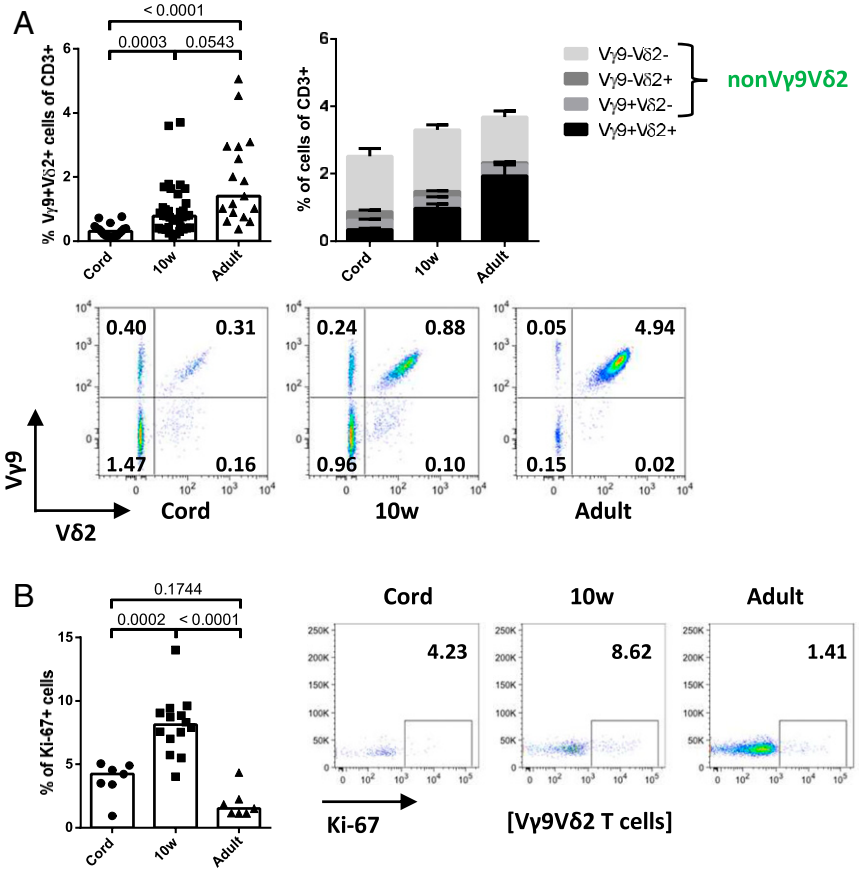

Fig. 1. Vγ9Vδ2 T cells expand early after birth. (*A*) Frequencies of the Vγ9+Vδ2+ subset in CD3+ cells (*Left*; bars indicate medians) and cumulative frequencies of Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2− γδ T cells, Vγ9−Vδ2+ γδ T cells, and Vγ9−Vδ2− γδ T cells among CD3+ cells (*Right*; error bars indicate mean ± SEM; $P > 0.5$ for the non-Vγ9Vδ2 γδ T cell subsets between 10-wk-old [10w] and cord) in cord ($n = 18$), 10-wk-old ($n = 36$), and adult ($n = 17$). Representative flow cytometry plots (*Bottom*) gated on γδ+CD3+ cells, percentages of γδ T cell subsets of CD3+ lymphocytes. (*B*) Percentage of Ki-67+ cells among Vγ9Vδ2 T cells (cord, adult, $n = 7$; 10w, $n = 14$). Bars indicate medians. Representative flow cytometry plots are shown (*Right*). Data are shown from independent donors (South African). *P* values are reported in the graphs.

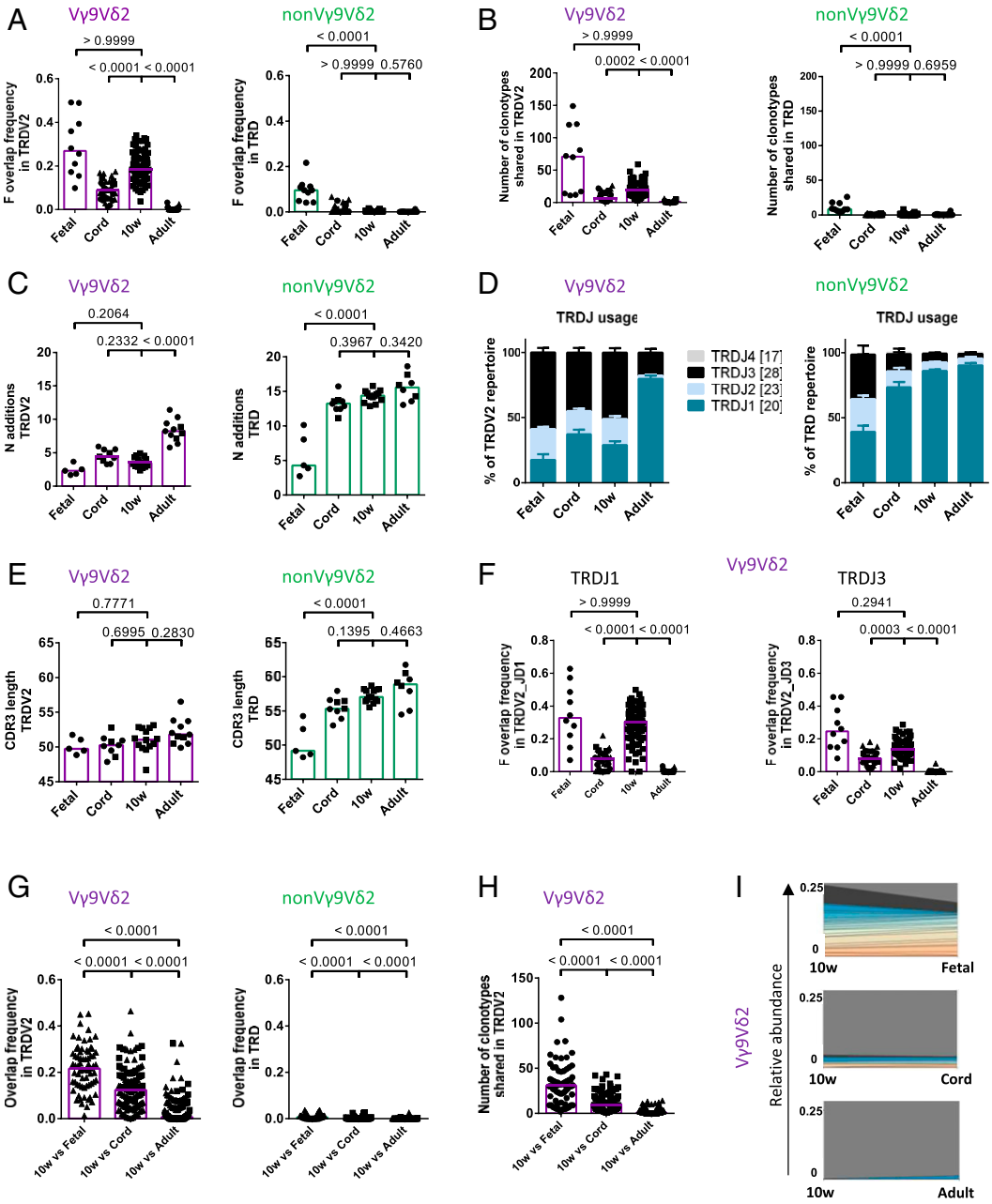

Fig. 2. Only the 10-wk-old Vγ9Vδ2 TCR repertoire is public and fetal-derived. (*A*–*E*) Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (*Left*) and non-Vγ9Vδ2 γδ T cells (*Right*), derived from fetal ($n = 5$, Belgian), cord ($n = 9$, Belgian, 6, South African, 3), 10w ($n = 14$, South African), and adult ($n = 11$ for Vγ9Vδ2, Belgian, 8, South African, 3; $n = 8$ for non-Vγ9Vδ2, Belgian, 5, South African, 3) blood. (*A*) Comparison of the 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 mean ± 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 the geometric mean of relative overlap frequencies (*F* metrics by VDJ tools) within pairs of fetal, cord, 10w, or adult blood donors in the TRDJ1 repertoire (*Left*) and TRDJ3 repertoire (*Right*) 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; $n = 8$ for non-Vγ9Vδ2, Belgian, 5, South African, 3) blood. Each dot represents the *F* value of a pair of samples. (*G* and *H*) Description of the CDR3 TRD repertoire of sorted Vγ9Vδ2 T cells (*Left*) and non-Vγ9Vδ2 γδ T cells (*Right*), 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; $n = 8$ for non-Vγ9Vδ2, Belgian, 5, South African, 3) blood. (*G*) Relative abundance of the 10w repertoire overlapping with the 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 (*Left*) for 10w and (*Right*) for fetal, cord, or adult. The other shared clonotypes are represented in dark gray. The rest of the repertoire (that is thus nonoverlapping) is represented in light gray. Note that only up to 25% of the repertoire is shown. Data are shown from independent donors (from Belgium in round symbols, from South Africa in square symbols, and pair comparisons including both Belgian and South African samples in triangle symbols). Bars indicate medians (*A*–*C* and *E*–*H*). *P* values are reported in the graphs.

Table 1. Most shared CDR3δ clonotypes among 10-wk-old 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: number of *N* additions incorporated into the nucleotype(s) encoding each clonotype; occurrences, /14: number of donors where the clonotype was detected (out of 14); median abundance, %: median percentage of the repertoire in the 14 10w donors.

*Of the two nucleotypes encoding this clonotype, one is germline and one includes one *N* addition.

N additions (Fig. 2*C*). TRDJ usage of the non-Vγ9Vδ2 T cells was similar in infants and adults but different from fetal cells (Fig. 2 *D*, *Right*). Of note, the high proportion of the 10-wk-old Vγ9Vδ2 TRD repertoire sharing was not directly associated with the preferential usage of TRDJ3. Indeed, TRDJ1-containing CDR3δ sequences of 10-wk-old Vγ9Vδ2 T cells showed even a higher degree of overlap than TRDJ3-containing CDR3δ sequences (Fig. 2*F*). To investigate the fetal origin of the 10-wk-old Vγ9Vδ2 T cells more directly, we examined the level of sharing between the 10-wk-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. 2 *G*, *Left* and Fig. 2*H*). Once more, this was highly specific for the Vγ9Vδ2 subset, and as such sharing was not observed between 10-wk-old and fetal non-Vγ9Vδ2 T cells (Fig. 2 *G*, *Right* and *SI Appendix*, Fig. S1 *C* and *D*). The high sharing between fetal and 10-wk-old Vγ9Vδ2 T cells was due to a relatively high number of clonotypes and not just a few abundant fetal ones (Fig. 2 *H* and *I*). The lower overlap found in cord (Fig. 2*A*) 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 wk with the expansion of (fetal-derived) Vγ9Vδ2 T cells (*SI Appendix*, Fig. S1*E*). Results of analyzing the CDR3γ repertoire (*SI Appendix*, Fig. S2) were similar to those of the CDR3δ repertoire (Fig. 2), with the main exception that the adult TRGV9 was largely public (*SI Appendix*, Fig. S2), in line with previous studies (22, 35, 36).

In summary, TCR sequencing indicates that a large fraction of the early postnatal expanded infant Vγ9Vδ2 T cells are derived from <30-wk 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. Ten-week-old Vγ9Vδ2 T cells were highly activated compared with cord Vγ9Vδ2 T cells and, more surprisingly, also compared with adult Vγ9Vδ2 T cells (Fig. 3*A* and *SI Appendix*, Fig. S3*A*). In addition, at 10 wk they started to gradually differentiate by losing the expression of CD27 and CD28 (Fig. 3*B*). However, this did not lead to a significant increase in fully differentiated cells, as observed for adult Vγ9Vδ2 T cells (Fig. 3 *B*, *Right* and *SI Appendix*, Fig. S3*B*).

Aside from their TCRs, Vγ9Vδ2 T cells can also use natural killer receptors (NKRs) to recognize target cells (37). We verified the expression of a series of NKRs and found that 10-wk-old Vγ9Vδ2 T cells specifically showed increased expression of the inhibitory NKR NKG2A compared with cord blood, while other T cell subsets and other NKRs (CD161, KLRG1, CD158a/b, NKG2C) did not show such an expression pattern (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S3*C*). 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. 3*D*).

A major function of Vγ9Vδ2 T cells in adults is the 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 wk, despite their relatively limited differentiation (*SI Appendix*, Fig. S3*B*), the Vγ9Vδ2 T cells coexpressed these cytotoxic mediators at adult-like levels (Fig. 3 *E*–*G*). Interestingly, while all of 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. 3 *E* and *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 only reached high levels of expression in adults (Fig. 3 *G* and *H*). Granzyme A mediates killing of target cells by a different mechanism from 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. 3*I*), as observed previously in fetal (<30-wk gestation) Vγ9Vδ2 T cells (20). This expression further increased in 10-wk-old Vγ9Vδ2 T cells and remained highly restricted to the Vγ9Vδ2 T cell subset (Fig. 3*I*). In addition, the per-cell

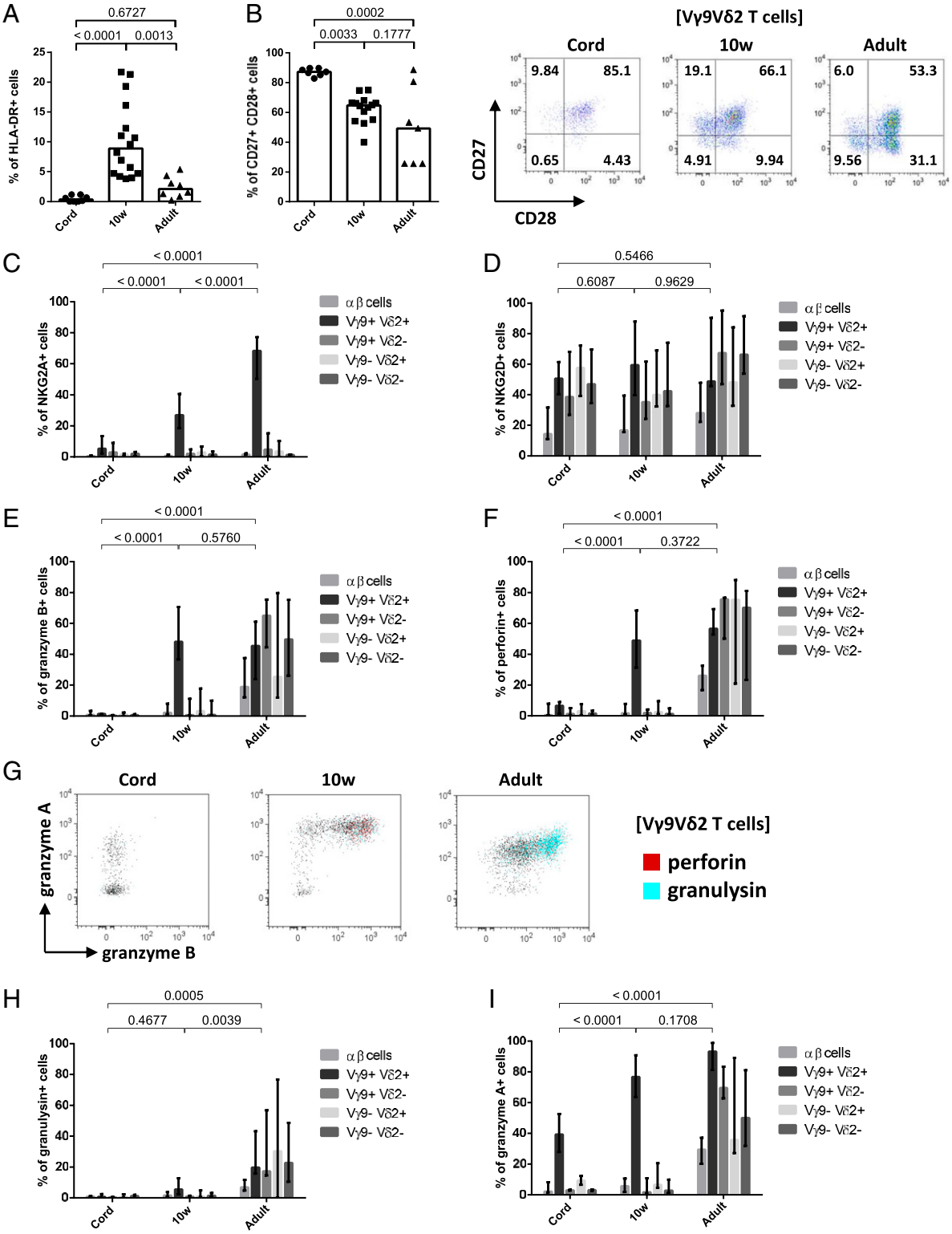

Fig. 3. Vγ9Vδ2 T cells are activated and become cytotoxic rapidly after birth. (*A*) Percentages of activated (HLA-DR+) cells among Vγ9Vδ2 T cells (cord, adult, $n = 8$; 10w, $n = 16$). (*B*) Percentages of naive (CD27+CD28+) Vγ9Vδ2 T cells (*Left*) and representative flow plots (*Right*). (*C–F*) Percentages 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 the following. (*C*) NKG2A in cord ($n = 4$), 10w ($n = 8$ to 10), and adult ($n = 3$ or 4). (*D*) NKG2D in cord ($n = 4$ or 5), 10w ($n = 8$ to 10), and adult ($n = 4$ or 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 coexpression flow cytometry plots of granzyme A, granzyme B, perforin, and granulysin in cord, 10w, and adult Vγ9Vδ2 T cells. (*H* and *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 the following. (*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 are shown from independent donors (South African). Bars indicate medians (*A* and *B*). Error bars indicate medians ± interquartile range (IQR) (*E*, *F*, *H*, and *I*). *P* values are reported in the graphs.

expression was even higher in infant compared with adult Vγ9Vδ2 T cells (Fig. 3*G* and *SI Appendix*, Fig. S3*D*).

In summary, 10-wk-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 and 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 interferon γ (IFNγ) and tumor necrosis factor α (TNFα) (1–3), we explored this effector capacity with strong short-term stimulation by phorbol 12-myristate 13-acetate (PMA) and ionomycin. We observed high expression of the two cytokines by cord, 10-wk-old, and adult Vγ9Vδ2 T cells (Fig. 4 *A* and *B*). Vγ9Vδ2 T cells were the main producers of IFNγ in early life, while in adults other T cells expressed IFNγ as well (Fig. 4*A*). While the percentage of Vγ9Vδ2 T cells expressing IFNγ and TNFα remained stable at 10 wk (compared with cord blood), 10-wk-old Vγ9Vδ2 T cells expressed much more IFNγ (but not TNFα) per cell (Fig. 4 *A*, *Right*, Fig. 4 *B*, *Right*, and *SI Appendix*, Fig. S3*E*). 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. 4*C*), 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 toward phosphoantigens such as the microbial-derived (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (13, 20, 32, 33), compared with 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. 4*D*), while the higher response of adult Vγ9Vδ2 was confirmed (Fig. 4*D*). Further, treatment with zoledronic acid, which leads to intracellular isopentenyl pyrophosphate accumulation and depends on different accessory leukocyte populations from HMBPP (33, 44), also did not lead to a higher IFNγ production in 10-wk-old Vγ9Vδ2 T cells compared with cord (Fig. 4*E*). Thus, upon functional differentiation early after birth, the fetal-derived Vγ9Vδ2 T cells do not show an increase of their phosphoantigen reactivity toward 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-wk-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-wk-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 with BCG− infants (Fig. 5 *A*, *Top*). This was confirmed by a very similar proliferation rate ex vivo (Fig. 5 *A*, *Bottom*). We further investigated the shaping of the repertoire in detail early after birth (Fig. 1 *B*–*H*), which may shed light on how the TCR repertoire changed in response to a specific stimulus (BCG) (17). Overall, TCR diversity (Fig. 5 *B* and *C*), number of *N* additions (Fig. 5*D*), usage of J segment (Fig. 5*E*), CDR3 length (Fig. 5*F*), and level of repertoire overlap (Fig. 5 *G* and *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 and *SI Appendix*, 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 with 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 10 wk of birth, Vγ9Vδ2 T cells from healthy infants were already expanding. Furthermore, TCR repertoire analysis indicated a preferential expansion of early fetal-derived (<30 wk 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-wk-old Vγ9Vδ2 T cells, previously described to be highly abundant and shared in prethymic livers of 6/7-wk 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 non-Vγ9Vδ2 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 repertoires of both Vγ9Vδ2 and non-Vγ9Vδ2 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 (interleukin [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 coincubated 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 and 10 y, possibly reflecting expansion of adult-like Vγ9Vδ2 T cells (21). Compared with samples collected >1 y after birth, the number of samples collected at <1 y was more limited in this study, possibly explaining the lack of observation of 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 y 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 who were born prematurely.

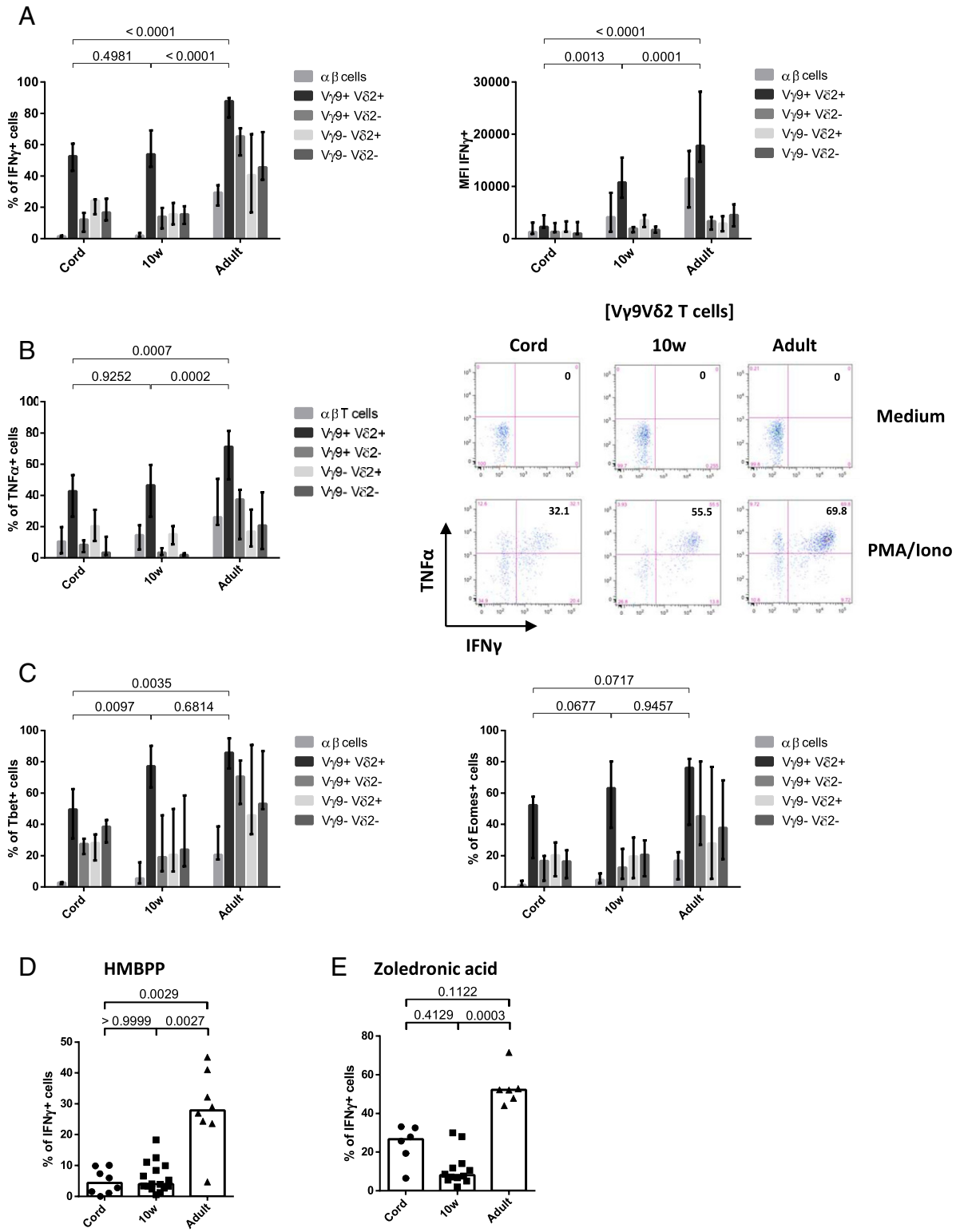

Fig. 4. Cytokine expression capacity of Vγ9Vδ2 T cells is mainly determined before birth. (*A*–*C*) Flow cytometry data on αβ T cells, Vγ9+Vδ2+ γδ T cells, Vγ9+Vδ2− γδ T cells, Vγ9−Vδ2+ γδ T cells, and Vγ9−Vδ2− γδ T cells. (*A*) IFNγ expression after 4-h PMA-ionomycin stimulation: percentage of positive cells (*Left*; cord, adult, $n = 12$; 10w, $n = 24$) and median fluorescence intensity (MFI) (*Right*; cord, adult, $n = 4$; 10w, 8). (*B*) TNFα expression after 4-h PMA-ionomycin stimulation: percentage of positive cells (*Left*; cord, $n = 8$; 10w, $n = 17$; adult, $n = 9$) and representative coexpression flow plots of IFNγ and TNFα in cord, 10w, and adult Vγ9Vδ2 T cells (*Right*). (*C*) Ex vivo expression of T-bet (*Left*) and Eomes (*Right*) (cord, adult, $n = 7$ or 8; 10w, $n = 14$ to 16). (*D*) Percentage of IFNγ+ cells among Vγ9Vδ2 T cells after stimulation with the phosphoantigen HMBPP (3 d or overnight, in the presence of IL-2; cord, adult, $n = 8$; 10w, $n = 16$). Values derived from the "medium+IL-2" condition are subtracted. (*E*) Percentage of IFNγ+ cells among Vγ9Vδ2 T cells after stimulation with zoledronic acid (3 d, in the presence of IL-2; cord, adult, $n = 6$; 10w, $n = 12$). Values derived from the medium+IL-2 condition are subtracted. Data are shown from independent donors (South African). Error bars indicate medians ± IQR (*A*–*C*). Bars indicate medians (*D* and *E*). *P* values are reported in the graphs.

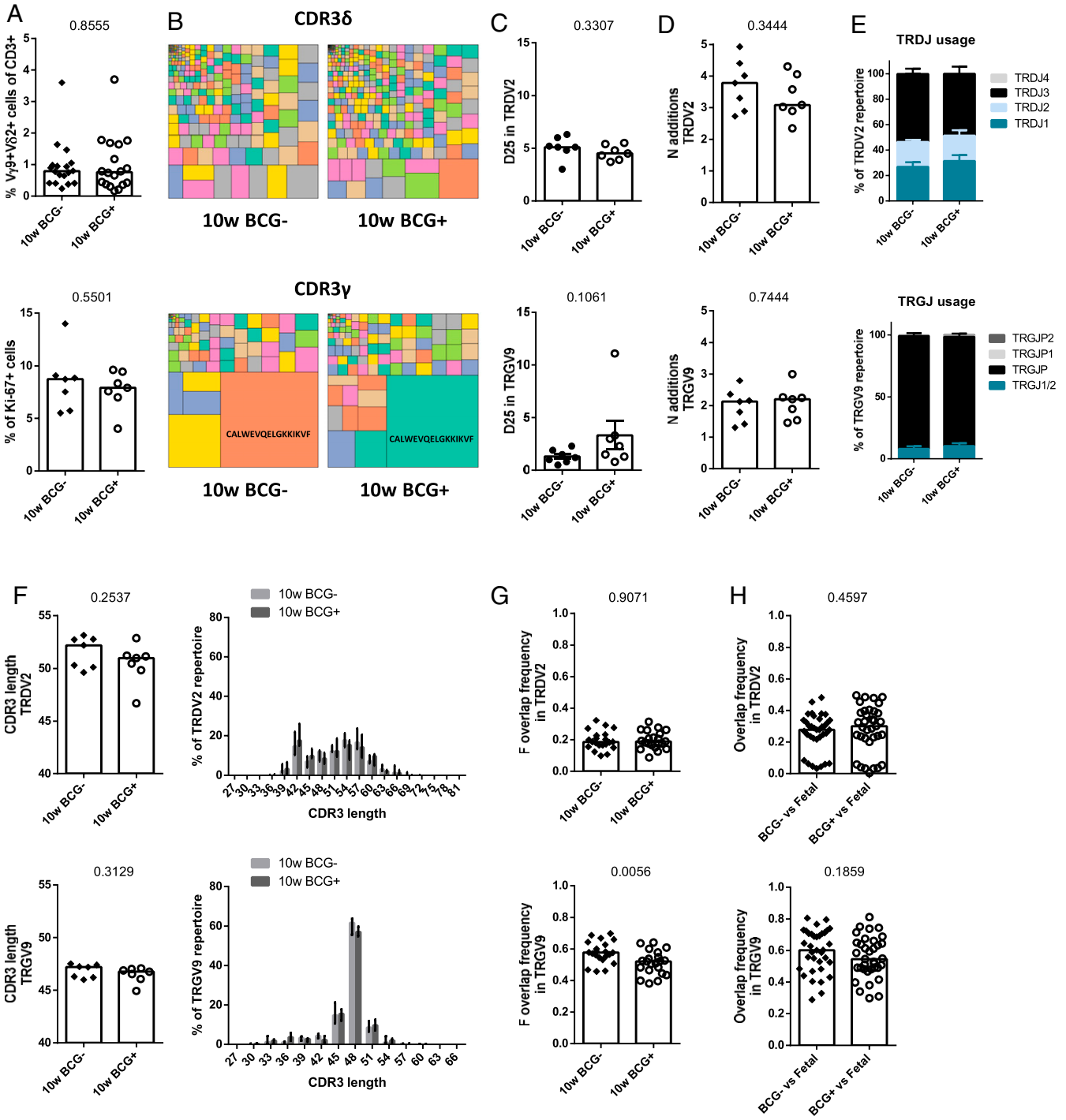

Fig. 5. Neonatal BCG vaccination does not shape the 10-wk-old Vγ9Vδ2 TCR repertoire. (*A*) Frequencies of the Vγ9+Vδ2+ subset in 10-wk-old unvaccinated infants (10w BCG−) or vaccinated infants (10w BCG+) CD3+ cells (*Top*; $n = 18$) and percentage of Ki-67+ cells among Vγ9Vδ2 T cells (*Bottom*; $n = 7$). (*B–H*) Comparison of the CDR3 TRDV2 (*Top*) and TRGV9 (*Bottom*) 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 *D*25 values (percentage of unique clonotypes required to account for 25% of the 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*); frequency of repertoire per CDR3 length (*Right*). (*G*) Comparison of the 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 are shown from independent donors (South African). Bars indicate medians (*A*, *C*, *D*, and *F* [*Left*], *G*, and *H*). Error bars indicate medians ± IQR (*E* and *F*, *Right*). *P* values are reported in the graphs.

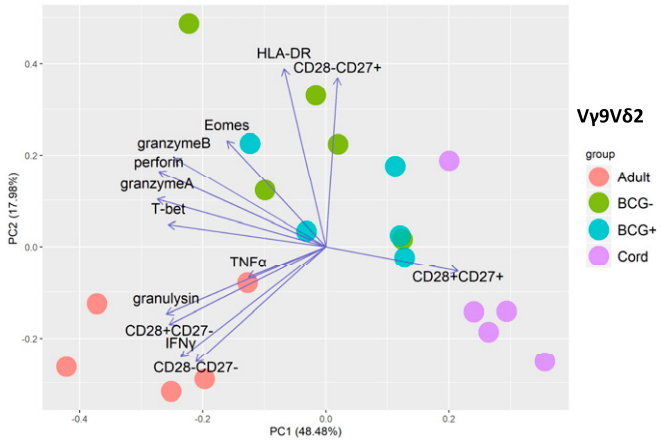

Fig. 6. Neonatal BCG vaccination does not influence the functional differentiation of 10-wk-old Vγ9Vδ2 T cells. Principal-component analysis (PCA) profiling of Vγ9Vδ2 T cells derived from cord, 10-wk-old BCG−, 10-wk-old BCG+, and adult blood (n = 5), based on the percentage of HLA-DR+, CD27+CD28+, CD27−CD28+,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

In contrast to the private γδ T cell subsets (non-Vγ9Vδ2), at 10 wk after birth the public Vγ9Vδ2 T cells were activated and differentiated toward 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+ subsets (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 coexpression in 10-wk-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-wk-old Vγ9Vδ2 T cells, at levels even higher than in adults, can induce a different cell-death pathway from 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-wk-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 NKRs, NKG2A was highly up-regulated early after birth on the cell surface of 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-wk-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-wk-old Vγ9Vγ2 T cells, granulysin was highly expressed by adult Vγ9Vδ2 T cells. Furthermore, adult Vγ9Vδ2 T cells clearly showed a higher response toward HMBPP compared with 10-wk-old 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 with 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-wk-old Vγ9Vδ2 T cells with regard 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 wk of age. This could explain why clear expansions of Vγ9Vδ2 T cells can be seen in nonhuman primates (NHPs) (in clean facilities) upon vaccination with BCG (26). Moreover, intravenous (i.v.) 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 i.v. 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 independent 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 nonspecific 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 2 mo 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 postnatal 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-wk-old infants with those in cord and adult blood. The 10-wk-old infants consisted of two groups, one vaccinated with BCG intradermally (Danish 1331 strain, Statens Serum Institut) at birth as is routine in South Africa (BCG+, median age 65 d, minimum 56 to maximum 77), and another group not vaccinated with BCG at birth (BCG−, median age 67.5 d, minimum 61 to maximum 86). In those not vaccinated at birth, BCG vaccine was administered at 10 wk 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 (reference 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, and suspicion of TB or known household contact with TB patients.

Exclusion criteria for infants included BCG vaccination before planned blood collection at 10 wk 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 wk of life,

isoniazid therapy during the first 10 wk of life, any chronic disease in the first 10 wk of life, any acute disease during the 2 wk before blood collection, infants born before 37 wk of gestation (preterm) and those with low birth weight (<2,500 g), 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 mo, any acute or chronic illness, history of TB disease, and pregnant or lactating females.

Whole blood was collected in CPT tubes or heparinized polypropylene tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from blood, cryopreserved, and shipped to Belgium for further analysis.

For the CDR3 repertoire analysis, 10-wk-old Vγ9Vδ2 T cells were compared with fetal blood. Since the fetal blood samples originated in 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-wk-old, and 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 to 30 wk of gestation), approved by the Hôpital Erasme ethics committee; umbilical cord blood after delivery (vaginal) (39- to 41-wk term delivery) with the approval of University Hospital Center Saint-Pierre; and adult peripheral blood, approved by the ethics committee of Centre Hospitalier Universitaire de Tivoli, La Louvière. PBMCs were isolated from blood and cryopreserved for subsequent experiments.

Methods. Flow cytometry, sorting and cell cultures, TRG and TRD high-throughput sequencing, and statistical analysis are described in *SI Appendix, Methods*.

Data Availability. Fastq files of TRG and TRD sequences are deposited in the Sequence Read Archive under accession no. PRJNA624366 (61).

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