Immunity to infection

Clinical

Research Article

Sepsis shapes the human γδ TCR repertoire in an age- and pathogen-dependent manner

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Sepsis affects 25 million children per year globally, leading to 2.9 million deaths and substantial disability in survivors. Extensive characterization of interactions between the host and bacteria in children is required to design novel preventive and therapeutic strategies tailored to this age group. Vγ9Vδ2 T cells are the first T cells generated in humans. These cells are defined by the expression of Vγ9Vδ2 T-cell receptors (TCRs, using the TRGV9 and TRDV2 gene segments), which react strongly against the prototypical bacterial phosphoantigen HMBPP. We investigated this reactivity by analyzing the TCR δ (TRD) repertoire in the blood of 76 children (0–16 years) with blood culture-proven bacterial sepsis caused by HMBPP-positive *Escherichia coli* or by HMBPP-negative *Staphylococcus aureus* or by HMBPP-negative *Streptococcus pneumoniae*. Strikingly, we found that *S. aureus*, and to a lesser extent *E. coli* but not *S. pneumoniae*, shaped the TRDV2 repertoire in young children (<2 years) but not in older children or adults. This dichotomy was due to the selective expansion of a fetal TRDV2 repertoire. Thus, young children possess fetal-derived Vγ9Vδ2 T cells that are highly responsive toward specific bacterial pathogens.

🖥 Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Sepsis contributes to a major burden of disease in early life. Worldwide, it is estimated that half of the 48.9 million annual cases of sepsis occur in children, leading to 2.9 million deaths [1]. This vulnerability in early life is not unexpected, given the predominantly naïve phenotype of neonatal immune cells and distinctive immunological challenges at birth and during childhood [2–4]. Attempts at modulating the immune system of newborns and young children to prevent or treat infections have yielded only limited benefits to date [3]. Therefore, extensive characterization of host–pathogen interactions in early life is required to develop novel preventive and therapeutic strategies [3].

γδ T cells express T-cell receptors (TCRs), each comprising a TRG (γ) and TRD (δ) chain, and are not restricted by classical MHC molecules, unlike conventional αβ T cells expressing αβ TCRs [5, 6]. Somatic rearrangements of the TRG and TRD loci take place during the development of γδ T cells from a common αβ/γδ T-cell precursor in the thymus, where variable (V), diversity (D, only for TRD), and joining (J) gene segments are used to form each a complete γ and δ chain, respectively [7]. The variability created during the V(D)J recombination is significantly enhanced by the junctional diversity, which includes the introduction of random nucleotides (N additions), a process mediated by the enzyme terminal deoxynucleotidyl transferase (TdT). The most variable domain, which includes these N additions and is usually accountable for antigen recognition, is termed the complementarity determining region 3 (CDR3) [8]. γδ T cells have been implicated in a wide range of immunological scenarios in health and disease, including bacterial and viral infections [5, 9]. In humans, γδ T cells can be divided into the innate-like phosphoantigen-reactive Vγ9Vδ2 T cells (V gene segments of the γ and δ chains encoded by TRGV9 and TRDV2 gene segments, respectively), and the more adaptive-like nonVγ9Vδ2 γδ T cells (e.g. Vδ1$^+$ γδ T cells) that can potentially react to a variety of antigen/ligand types [9–11]. "Phosphoantigens" are small phosphorus-containing molecules epitomized by the prototypical compound (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [12–14]. HMBPP is generated in the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid synthesis that is present in many bacteria but not in human cells [12]. As the biological activity of HMBPP is approximately 10,000 higher than that of the ubiquitous phosphoantigen isopentenyl pyrophosphate (IPP), the end product of both the MEP and the mevalonate pathway of isoprenoid synthesis, Vγ9Vδ2 T cells are thought to detect bacterial infections primarily via HMBPP [12, 14]. Of note, despite the innate-like reactivity of Vγ9Vδ2 T cells, the corresponding TRDV2-containing CDR3 repertoire is highly variable and it is not clear whether particular TRDV2-containing TCRs are more responsive than others toward particular infections [15].

γδ T cells are the first T cells generated in the vast majority of vertebrate species, including humans. During this early life period, they show a distinct thymic development compared with their counterparts in adults and can play an important role in viral and parasitic infection in early life [8, 16–29]. However, it is not

clear whether Vγ9Vδ2 T cells can react to invasive bacterial infection in children [23, 30–32]. As mice do not have Vγ9Vδ2 T cells [33], studying the role of these cells in vivo requires well-designed human cohorts. Here, we used high-throughput sequencing of the TRD repertoire to examine how Vγ9Vδ2 T cells respond in children with blood culture-proven sepsis. We focused on HMBPP-positive *Escherichia coli*, HMBPP-negative *Staphylococcus aureus*, and HMBPP-negative *Streptococcus pneumoniae*, which are major pathogens of sepsis in children [34, 35].

Strikingly, we found a substantial expansion of fetal-derived TRDV2 repertoire in children younger than two years infected with HMBPP-negative *S. aureus*, and, to a lesser extent, in children infected with HMBPP-positive *E. coli*. In contrast, infection with HMBPP-negative *S. pneumoniae* did not affect the TRDV2 repertoire. Children older than 2 years and adults infected with *S. aureus* or *E. coli* did not show these fetal-derived Vγ9Vδ2 T-cell expansions. Accordingly, the distinct thymic development of Vγ9Vδ2 T cells in the human fetus appears to have important functional consequences for the cellular immune response against invasive bacterial infections in early childhood.

Results

Patient characteristics

We analyzed the TRD repertoire in the blood of 76 children with blood culture-proven sepsis caused by *S. pneumoniae* ($n = 20$), *S. aureus* ($n = 36$), and *E. coli* ($n = 20$) (Table 1). The median postnatal age at sepsis onset was 48 months (IQR 20–82), 104 months (IQR 15–141), and 14 months (IQR 2–82) in *S. pneumoniae*, *S. aureus*, and *E. coli* sepsis, respectively (Table 1; Supporting Information Fig. S1). The proportion of patients with organ dysfunction during the course of infection was 35% (7/20), 31% (11/36), and 40% (8/20) for cases of *S. pneumoniae*, *S. aureus*, and *E. coli* sepsis, respectively (Table 1). None of the patients died. The median time between sepsis onset and sampling for analysis of the TRD repertoire via high throughput sequencing was similar across the different pathogen groups, with a median of 3–4 days (Table 1).

The TRD repertoire in children changes upon aging

Given the well-known impact of age on the development and function of many components of the immune system [36–39], we assessed the relationship between age and the TRD repertoire in children with blood culture-proven sepsis, distinguishing the etiologic agents *E. coli, S. aureus, and S. pneumoniae*. No clear differences in the occurrence of TRDV2 and TRDV1 gene segments were observed according to pathogen (Fig. 1A), but there was a common increase in the number of N additions among the TRDV2-containing CDR3 sequences, reaching almost 10 N additions at the age of 16 years (Fig. 1B, left panel). In contrast, the number of N additions among the TRDV1-containing CDR3

Table 1. Baseline demographics and clinical characteristics of children with blood culture-proven sepsis.

	All sepsis cases (n = 76)	*Streptococcus pneumoniae* (n = 20)	*Staphylococcus aureus* (n = 36)	*Escherichia coli* (n = 20)	p-value[a]
Female sex	26 (34%)	5 (25%)	11 (31%)	10 (50%)	0.20
Age at sepsis, months	52 (9–126)	48 (20–82)	104 (15–141)	14 (2–82)	0.02
Comorbidity present[b]	26 (34%)	0	15 (42%)	11 (55%)	<0.001
Site of infection					<0.001
Pneumonia	16 (21%)	12 (60%)	4 (11%)	0	
Osteoarticular	14 (18%)	1 (5%)	13 (36%)	0	
Urinary tract	13 (17%)	0	1 (3%)	12 (60%)	
Central nervous system	8 (11%)	6 (30%)	0	2 (10%)	
Skin and soft tissue	8 (11%)	0	8 (22%)	0	
Other specific infection types[c]	3 (4%)	0	3 (8%)	0	
Admission to an intensive care unit	24 (32%)	9 (45%)	10 (28%)	5 (25%)	0.32
Number of organ dysfunctions[d]					0.04
0	50 (66%)	13 (65%)	25 (69%)	12 (60%)	
1	12 (16%)	1 (5%)	5 (14%)	6 (30%)	
>1	14 (18%)	6 (30%)	6 (17%)	2 (10%)	
Timing of sampling, days[e]	3 (2–6)	3 (3–6)	3 (3–5)	3 (2–5)	0.46

Note: Categorical variables are presented as frequencies (%), and continuous variables as median (interquartile range).
[a] p-value from Fisher's exact test for categorical variables and analysis of variance for continuous variables.
[b] Defined by the presence of chronic congenital or acquired medical conditions.
[c] Endocarditis and myositis.
[d] Defined according to reference #68.
[e] Number of days between sepsis onset and blood sampling for RNA analysis.

sequences was already at higher levels just after birth (mean of 15 N additions), as observed previously in cord blood [29, 40], and remained stable until the age of 16 years (Fig. 1B, right panel). Similarly, TRDJ3 use among TRDV2-containing CDR3 sequences was common early after birth, as reported previously [29, 40, 41], and declined steadily with age, whereas the opposite trend was observed for TRDJ1 (Fig. 1C). Thus, the TRD repertoire obtained from whole blood TRD sequencing in our pediatric sepsis cohort is consistent with previous observations on sorted γδ T cells [29, 40, 41]. Principal component analysis (PCA) of multiple TRD characteristics versus age identified two clusters, segregated as children aged <2 years and children aged ≥2 years (Supporting Information Fig. S1B and C; Fig. 1D). Subsequent analyses were conducted on the basis that young children (<2 years) might have different γδ T-cell responses than older children (≥2 years).

Sepsis caused by *S. aureus* and *E. coli*, but not by *S. pneumoniae*, shapes the TRD repertoire in young children

Zooming in on young children (<2 years) indicated that sepsis-related changes could be observed in the TRD repertoire according to the pathogen (Supporting Information Fig. S2). We, therefore, analyzed in more detail the characteristics of the TRD repertoire in young children (<2 years) infected by *E. coli, S. aureus*,

and *S. pneumoniae* and children of the same age who never had any invasive bacterial infection (Fig. 2; Table 1; Supporting Information Fig. S1B). In order to further minimize the potential effect of age on the TRD data within this age group, we analyzed in parallel the effect of pathogen (uninfected controls, *E. coli, S. aureus*, and *S. pneumoniae*) with age as a covariate using the analysis of covariance (ANCOVA; Supporting Information Table S1). The TRDV2 gene segment was used more commonly in children infected with *S. aureus* compared with uninfected children and children infected with *S. pneumoniae* or *E. coli* (Fig. 2A, left panel; Supporting Information Table S1) and associated with a lower number of N additions in the corresponding CDR3 (Fig. 2B, left panel; Supporting Information Table S1). Of note, TRDV2 usage and a corresponding number of N additions in the *E. coli* group were highly variable (Fig. 2A and B, left panels) and were inversely correlated (Fig. 2C). No difference in N additions was detected among TRDV1- and TRDV3-containing CDR3 (Fig. 2B, middle and right panel; Supporting Information Table S1), highlighting the specificity of the TRDV2 response. As the difference in N additions among TRDV2-containing CDR3 did not result in a difference in CDR3 length (Fig. 2D), we wondered whether differential TRDJ segment use could contribute to the minimization of the CDR3 length differences. Indeed, the TRDJ3 gene segment paired more commonly with the TRDV2 gene segment in the *S. aureus* group and, to a lesser extent, in the *E. coli* group than in the *S. pneumoniae* and control groups (Fig. 2E; Supporting Infor-
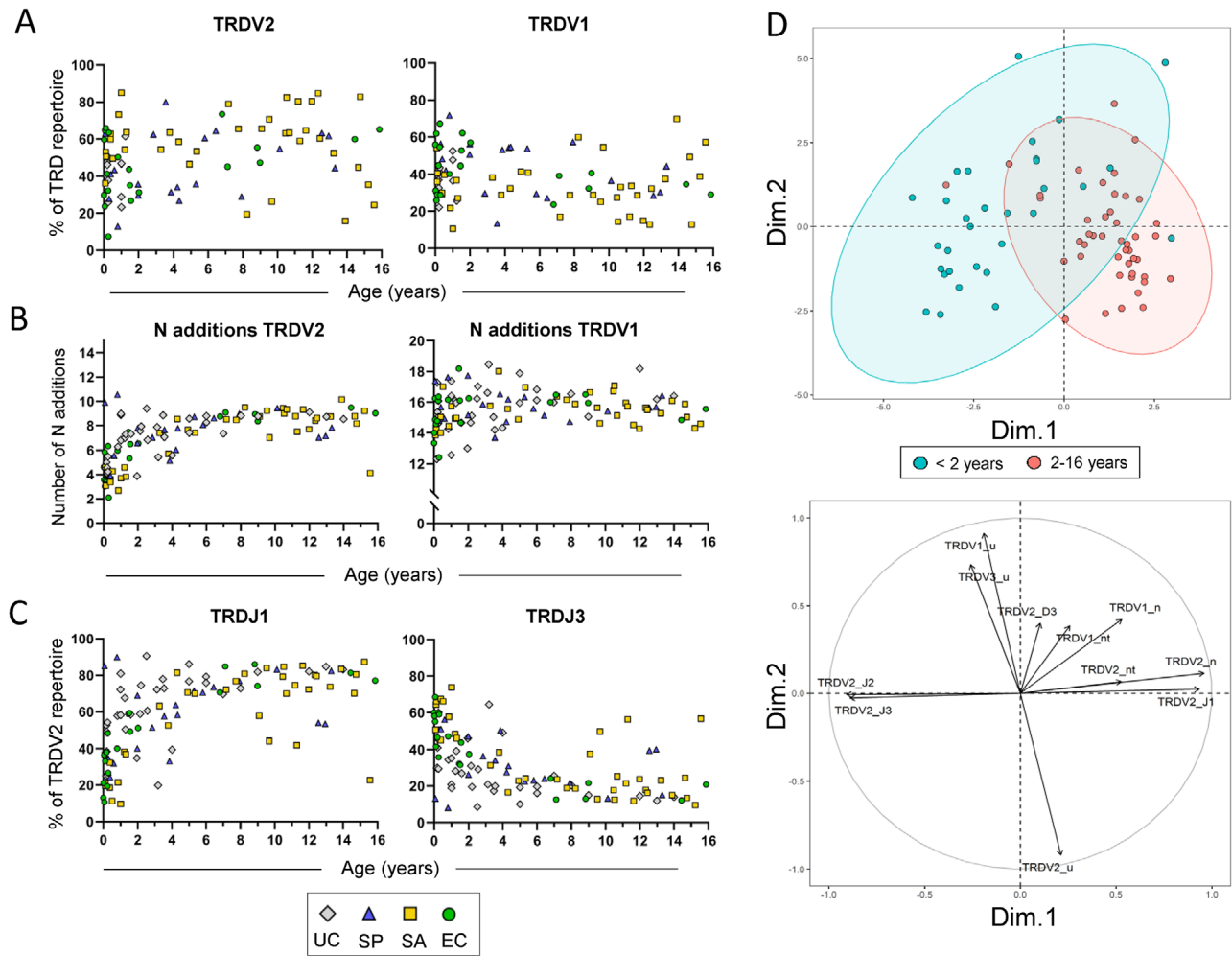
Figure 1. The TRD repertoire in children changes with age. (A) TRDV2 (left panel) and TRDV1 (right panel) usage by age. (B) Number of N additions from TRDV2- and TRDV1-containing CDR3 sequences by age. Each dot represents the weighted mean of an individual sample. (C) Percent frequency of TRDV2 sequences containing a TRDJ1 (left panel) or a TRDJ3 (right panel) segment by age. (D) Cumulative contribution of 11 parameters in a principal component analysis of the CDR3δ repertoires by age (see Methods section for more details). Top: PCA with two-dimensional representation based on the first two principal components. Blue dots represent young children (<2 years, *n* = 31), and red dots represent older children (≥2 years, *n* = 45). Bottom: arrows indicate the projection of each original variable onto these principal components. The length of each arrow indicates the magnitude of contribution to the explained variance. Variables are labeled at the tip of each arrow for identification (TRDV2_D3 = % of TRDV2 sequences containing TRDD3; TRDV2_J1, TRDV2_J2, TRDV2_J3 = % of TRDV2 sequences containing TRDJ1 or TRDJ2 or TRDJ3; TRDV1_u, TRDV2_u, TRDV3_u = % of TRDV1 usage, % of TRDV2 usage, % of TRDV3 usage (of total TRD); TRDV1_n, TRDV2_n = mean number of N additions in TRDV1 or TRDV2 sequences; TRDV1_nt, TRDV2_nt = mean CDR3 nucleotide length in TRDV1 or TRDV2 sequences). UC, uninfected control (*n* = 10 in A and *n* = 33 in B, C); SP, *S. pneumoniae* (*n* = 20 in A–C); SA, *S. aureus* (*n* = 36 in A–C); EC, *E. coli* (*n* = 20 in A–C). Uninfected control samples were obtained from datasets available in the public domain (see Materials and methods section for more details). Each dot represents a distinct biological replicate.

mation Table S1), likely explaining the observed CDR3 length conservation, given that TRDJ3 contains eight more nucleotides than TRDJ1 [29]. When following the pattern of N additions and TRDJ1/TRDJ3 usage with increasing age in the <2-year-old children, it can be seen that, in contrast to the other groups, these remain stable in the *S. aureus* group (Supporting Information Fig. S2). There was also an increase in the use of the TRDD3 gene segment (13 nucleotides) in the *S. aureus* group compared with the other groups at the expense of the shorter TRDD1 (8 nucleotides) and TRDD2 gene segments (9 nucleotides; Supporting Information Fig. S3A; Supporting Information Table S1). Note that the ANCOVA results (Supporting Information Table S1, post

hoc analyses with pairwise comparisons) confirm that the analyses performed by investigating the samples pooled per study group (Fig. 2) are not confounded by age.

To explore the possibility that specific infections might lead to the expansion of common CDR3 sequences, we investigated the level of sharing within the TRD repertoires associated with each pathogen. This analysis revealed strikingly increased sharing of the TRDV2 repertoire among young children (<2 years) infected with *S. aureus* compared with age-matched uninfected controls and children infected with *S. pneumoniae* or *E. coli* (Fig. 3A and B, left panel). The *E. coli* group showed an increase in TRDV2 sharing compared with *S. pneumoniae* and control groups (Fig. 3A
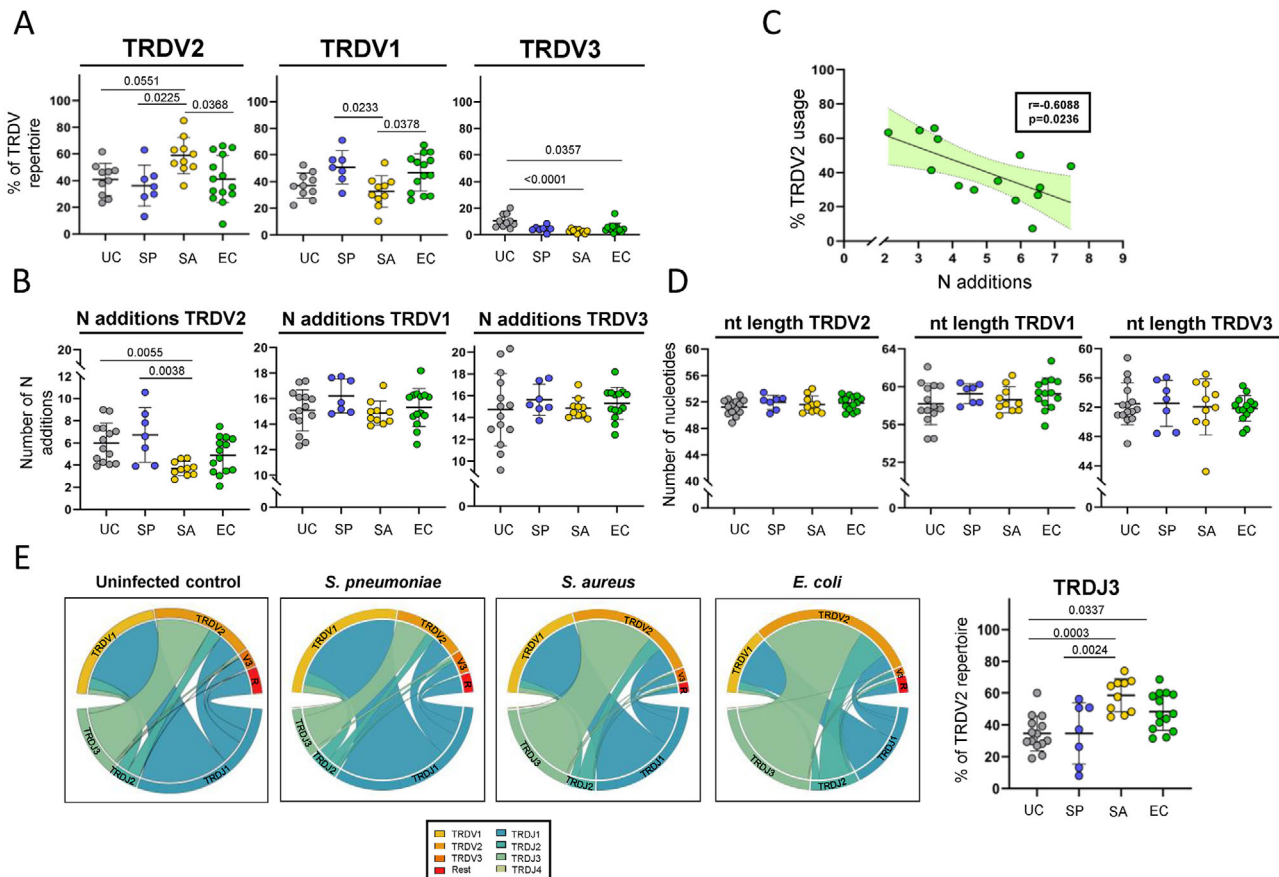
Figure 2. Sepsis induced by *S. aureus* or *E. coli* but not by *S. pneumoniae* shapes the TRD repertoire in young children. (A) Percent frequency of TRDV2-, TRDV1-, and TRDV3-containing CDR3 sequences from the TRD repertoires of (un)infected young children (<2 years). (B) Mean number of N additions (each dot represents the weighted mean of an individual sample) from TRDV2-, TRDV1-, and TRDV3-containing CDR3 sequences from the TRD repertoires of (un)infected young children (<2 years). (C) Mean number of TRDV2-containing CDR3 sequences and percent frequency of TRDV2-containing sequences from the TRD repertoires of young children (<2 years) infected with *E. coli*. (D) Mean nucleotide length of TRDV2-, TRDV1-, and TRDV3-containing CDR3 sequences from the TRD repertoires of (un)infected young children (<2 years). (E) Left: representative circoplots showing TRDV-TRDJ paired use in (un)infected young children (<2 years). The width of a link corresponds to the rearrangement frequency of a given V/J pairing. Right: percent frequency of sequences containing a TRDJ3 segment in the TRDV2 repertoires of young children (<2 years). (A, B, D, E) Data were analyzed using the Kruskal–Wallis test with Dunn's multiple comparison test. Error bars indicate mean ± SEM. (C) Data were analyzed using Spearman's correlation. 95% confidence intervals are highlighted in light green. UC, uninfected control (*n* = 10 in A and *n* = 14 in B, D, E); SP, *S. pneumoniae* (*n* = 7 in A, B, D, E); SA, *S. aureus* (*n* = 10 in A, B, D, E); EC, *E. coli* (*n* = 14 in A–E). Each dot represents a distinct biological replicate.

and B, left panel). This increased TRDV2 sharing in the *S. aureus* and *E. coli* groups was independent of any associated increase in TRDV2 sequences with zero N additions or TRDV2 sequences paired with the TRDJ3 gene segment in the shared repertoire (Supporting Information Fig. S3B–D), consistent with increased sharing in both the TRDV2-TRDJ3 repertoire and the TRDV2-TRDJ1 repertoire (Fig. 3C). No increase in TRDV1 and TRDV3 sharing was observed in any of the bacterial infections studied here (Fig. 3B, middle and right panel), again highlighting the specificity of the TRDV2-containing γδ TCR response against specific bacterial infections at this age.

Overall, the TRD repertoire in young children (<2 years) was profoundly shaped by invasive infection with *S. aureus* and, to a variable extent, with *E. coli*, whereas no such influence was observed following invasive infection with *S. pneumoniae*.

Shaping of the TRD repertoire by sepsis is specific for young children

Next, we quantified a series of TRD features in children aged less than two years and in children aged between two and 16 years to assess the influence of age at the time of infection (Fig. 4; Supporting Information Fig. S1B and C). Striking differences were observed as a function of age (<2 years versus 2–16 years) in the *S. aureus* and *E. coli* groups for sharing across the TRDV2 repertoire (Fig. 4A), N additions (Fig. 4B), and TRDJ use (Fig. 4C).

To extend this observation, we analyzed the TRD repertoire of adults with acute peritonitis [42]. Cases were stratified according to pathogen into infection with HMBPP-negative bacteria (that are not *S. aureus*), *S. aureus*, or HMBPP-positive bacteria (Fig. 4, Supporting Information Table S2). These adults displayed TRD repertoire characteristics that were essentially identical to those
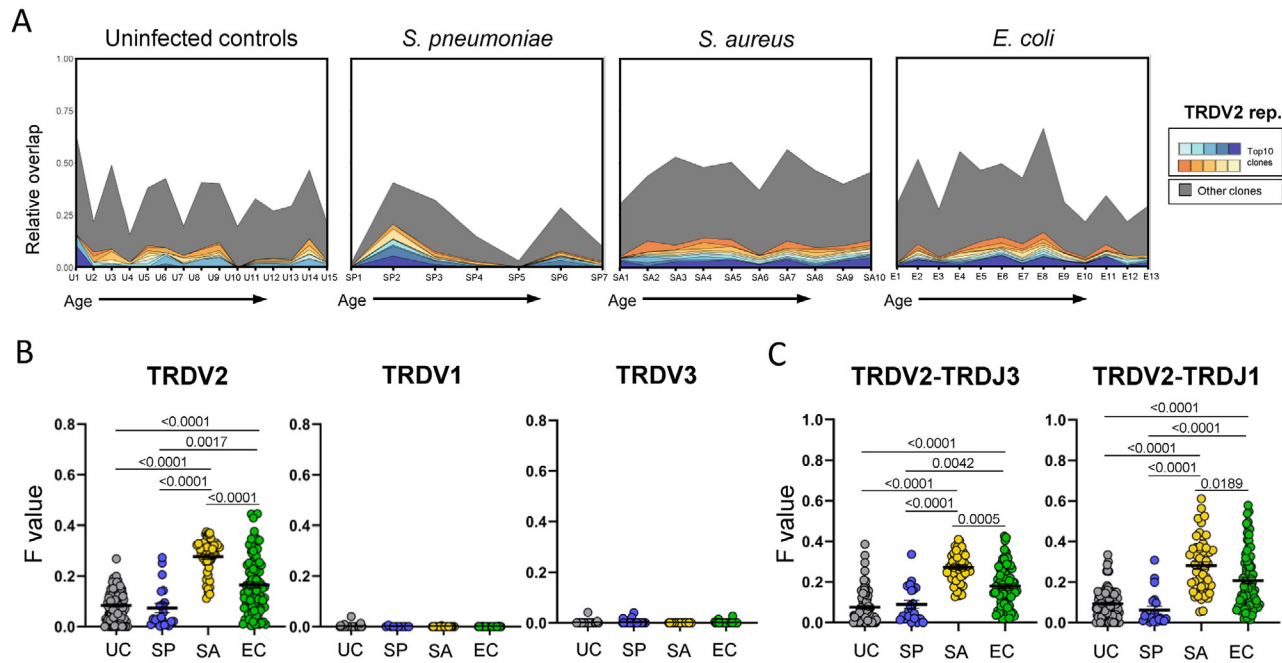
Figure 3. The TRDV2 repertoire of young children with *S. aureus* or *E. coli* sepsis is highly public. (A) Longitudinal tracking of shared TRDV2 sequences among young children (<2 years) by pathogen or uninfected controls. The frequencies of the top 10 most abundant shared clonotypes in each group are plotted in distinct colors, and the remaining fractions of shared TRDV2 sequences are plotted in dark grey at the top. Each tick on the X-axis corresponds to an individual in ascending order by age. The relative frequency of a particular sequence or group of sequences in the total TRDV2 repertoire of each individual is shown on the Y-axis. (B) Comparison of the geometric mean of relative overlap frequencies (F metrics by VDJ tools, see Methods for more details) between pairs of children infected with the same pathogen (or uninfected controls) for the TRDV2, TRDV1, and TRDV3 repertoires. (C) Comparison of the geometric mean of relative overlap frequencies (F metrics by VDJ tools) between pairs of children infected with the same pathogen (or uninfected controls) for the TRDV2-TRDJ1 and TRDV2-TRDJ3 repertoires. (B, C) Each dot represents the F value of a pair of samples. Data were analyzed using the Kruskal–Wallis test with Dunn's multiple comparisons test. Error bars indicate mean ± SEM. UC, uninfected control (n = 14 in B, C); SP, *S. pneumoniae* (n = 7 in B, C); SA, *S. aureus* (n = 10 in B, C); EC, *E. coli* (n = 14 in B, C).

observed in older children (≥2 years; Fig. 4), confirming the specificity of changes observed in the TRD repertoires of young children (<2 years) upon invasive bacterial infection with *S. aureus* or *E. coli*.

Shaping of the TRD repertoire in young children during invasive bacterial infection is due to expansion of a fetal TRD repertoire

Fetal and adult Vγ9Vδ2 T cells develop distinctly, a process that is reflected in the TRD repertoire [29, 40]. As some of the TRD changes driven by *S. aureus* and, to a lesser extent, by *E. coli* (Fig. 2 and 3) were reminiscent of a fetal TRD repertoire (e.g. low numbers of N additions and frequent use of TRDJ3 and TRDD3), we compared the TRD repertoires from the different pathogen groups with fetal TRD repertoires (<30 weeks gestation). This analysis revealed that the blood TRD repertoires obtained from young children (<2 years) infected with *S. aureus* were shared to a greater extent with the fetal blood Vγ9Vδ2 TRD repertoires than the blood TRD repertoires obtained from age-matched uninfected children and young children (<2 years) infected with *S. pneumoniae* (Fig. 5A and B). Relatively high numbers of overlap-

ping clonotypes rather than just a few abundant fetal clonotypes were responsible for this shared landscape (Fig. 5A–C). Again, the *E. coli* group showed a more variable degree of sharing with borderline significance compared with the *S. pneumoniae* and control groups (Fig. 5B and C). Accordingly, fetal Vγ9Vδ2 T cells appeared to be highly responsive to invasive bacterial infection, especially with *S. aureus*, resulting in a restructuring of the TRD repertoire in young children (<2 years).

Discussion

In this study, we show that in early childhood (<2 years) but not in older children and adults, fetal-derived Vγ9Vδ2 TCR clonotypes expand following invasive bacterial infections and that they do this in a pathogen-dependent manner.

Vγ9Vδ2 T cells derived from <30 weeks gestation fetal blood [43], peripheral blood from preterm newborns [44], umbilical cord blood from full-term newborns [15, 45, 46], or peripheral blood from infants aged 10 weeks [40] react poorly in vitro against phosphoantigens including HMBPP. This relative lack of reactivity may be due to specific regulatory mechanisms that predominate in early life, such as those mediated by the inhibitory
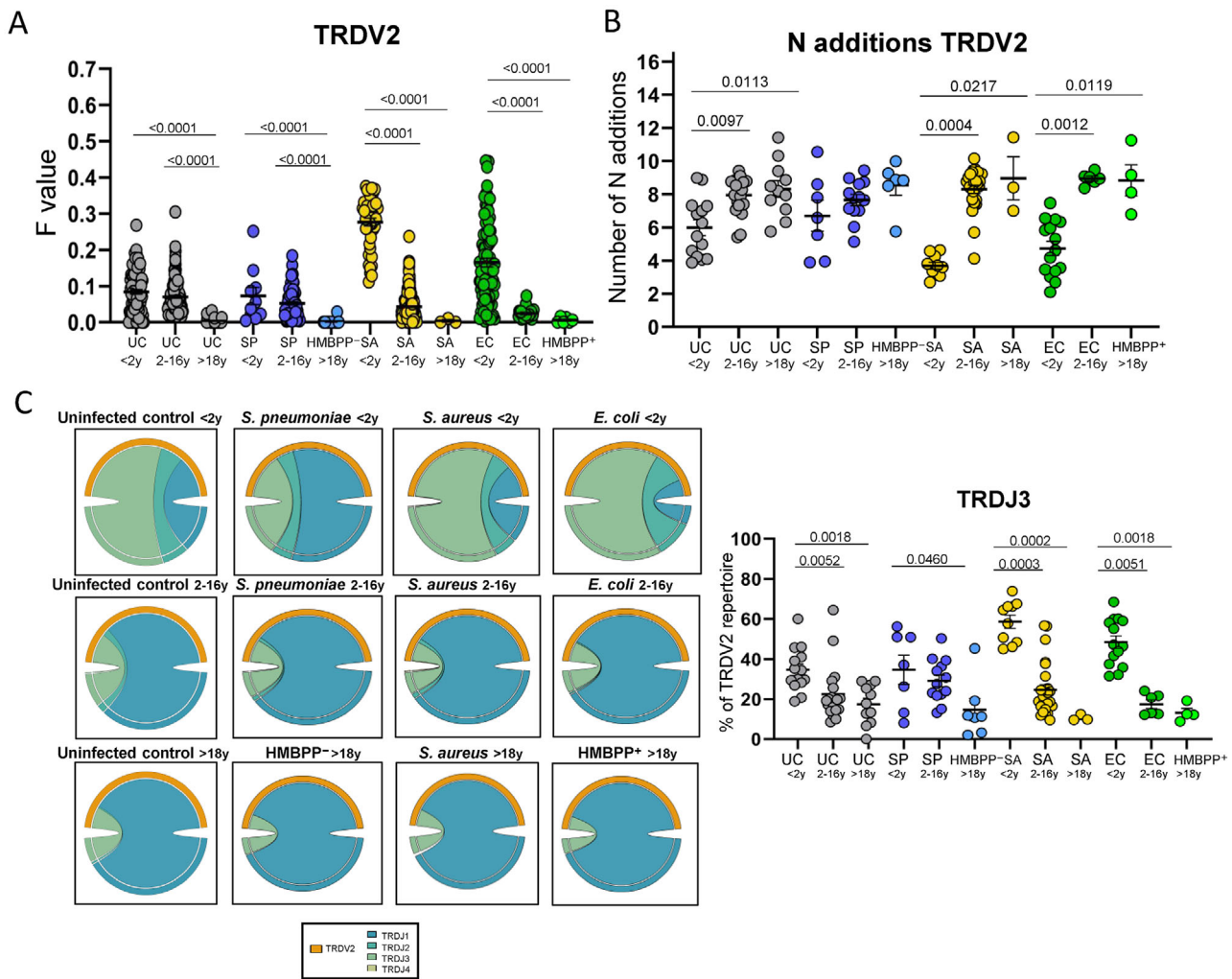
Figure 4. TRD repertoire shaping by sepsis is specific for young children. (A) Overlap frequencies (F metrics by VDJ tools) for the TRDV2 repertoire between pairs of uninfected and infected individuals belonging to the same study group (young children (<2 years), older children (≥2, between 2 and 16 years), and adults (>18 years). (B) Mean number of N additions (each dot represents the weighted mean of an individual sample) for TRDV2-containing CDR3 sequences from the TRD repertoires of the different study groups. (C) Left: representative circoplots showing TRDV2-TRDJ paired use according to age. The width of a link corresponds to the rearrangement frequency of a given V/J pairing. Right: percent frequency of sequences containing a TRDJ3 segment in the TRDV2 repertoires according to age. (A–C) Data were analyzed using the Kruskal–Wallis test with Dunn's multiple comparisons test. Error bars indicate mean ± SEM. UC, uninfected control; SP, *S. pneumoniae*; SA, *S. aureus*; EC, *E. coli*; HMBPP⁻, pathogens negative for HMBPP; HMBPP⁺, pathogens positive for HMBPP. In B and C, each dot represents a distinct biological replicate. In A–C, the number of samples in the distinct groups is UC = 14, SP = 7, SA = 10, EC = 14 in less than 2 years, UC = 18, SP = 13, SA = 26, EC = 6 in 2–16 years, and UC = 11, HMBPP- = 7, SA = 3, HMBPP+ = 4 in >18 years.

receptor PD1 [47], which are thought to prevent harmful activation of cytotoxic and Th1-biased Vγ9Vδ2 T-cell responses in the developing fetuses and young children. These features are likely related to (tolerance) requirements of the fetal immune system, which involves a distinct thymic development [26, 29, 37]. Here we show that invasive bacterial infection with *S. aureus* or *E. coli* alters the TRD repertoire of Vγ9Vδ2 T cells in young children (<2 years) indicating that they respond to these pathogens. We further propose that such reshaping of the TRD repertoire in vivo reflects conditions that allow Vγ9Vδ2 T cells to bypass immunological tolerance [46, 48]. In addition, we found that fetal-derived Vγ9Vδ2 T-cell clonotypes expand preferentially during invasive bacterial

infection, consistent with the expression of high-affinity TCRs. The demonstration of such differential affinity between fetal and adult Vγ9Vδ2 T-cell clonotypes [29] is complicated because there is no clearly defined ligand structure for the CDR3 loops of the Vγ9Vδ2 TCRs [13, 49, 50]. In premature infants, sepsis has been associated with increased frequencies of γδ T cells, albeit without the identification of a responsive subset at the level of Vδ1 versus Vδ2 TCRs [23]. Although Vδ1 T cells are abundant in cord blood and expand numerically in adults with sepsis [51], our data suggest that Vδ1 T cells do not react in young children with invasive bacterial infection and that reactivity within the γδ T-cell compartment is driven mainly by fetal Vγ9Vδ2 T cells early after birth. We
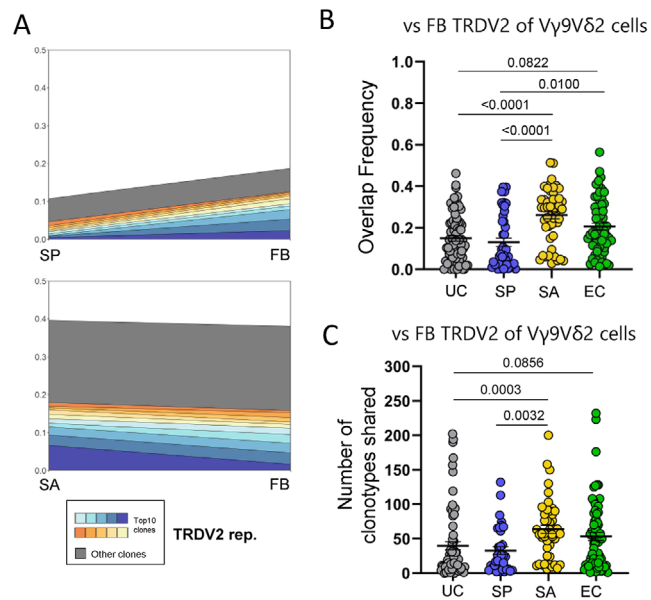
Figure 5. Sepsis induced by *S. aureus* or *E. coli* but not by *S. pneumoniae* results in the expansion of a fetal TRD repertoire in young children. (A) Representative shared clonotype abundance plots for TRDV2 repertoires in young children (<2 years) infected with SP or SA versus one fetal TRDV2 repertoire from flow-sorted Vγ9Vδ2 T cells. The frequencies of the top 10 most abundant shared clonotypes are plotted at the bottom in distinct colors, and the remaining fractions of shared TRDV2 sequences are plotted in dark grey at the top. (B) Relative abundance of the TRDV2 repertoires in young children (<2 years) infected with different pathogens (or uninfected controls) overlapping with the TRDV2 repertoire of flow-sorted fetal Vγ9Vδ2 T cells. Each dot represents a pair comparison. (C) Number of clonotypes shared between the TRDV2 repertoires of young children (<2 years) and the TRDV2 repertoire of flow-sorted fetal Vγ9Vδ2 T cells. Each dot represents a pair comparison. (B, C) Data were analyzed using the Kruskal–Wallis test with Dunn's multiple comparisons test. Error bars indicate mean ± SEM. UC, uninfected control (*n* = 14 in B, C); SP, *S. pneumoniae* (*n* = 7 in B, C); SA, *S. aureus* (*n* = 10 in B, C); EC, *E. coli* (*n* = 14 in B, C).

propose that postnatal thymic production [29, 52] is the source of most Vγ9Vδ2 T cells in the periphery after 2 years of age and that fetal thymus-derived Vγ9Vδ2 T cells become too dilute to expand appreciably in response to bacterial infection. In contrast, the Vγ9Vδ2 repertoire in premature infants is likely constituted primarily by fetal thymus-derived Vγ9Vδ2 T cells [26, 29], indicating that Vγ9Vδ2 T cells at this developmental stage may be highly responsive to systemic bacterial infection.

The well-annotated cohort of children with microbiologically documented bloodstream infection in the Swiss Pediatric Sepsis Study (SPSS) allowed us to determine the influence of pathogen type on the repertoire of Vγ9Vδ2 TCRs. HMBPP, a metabolite from the MEP pathway (also known as the nonmevalonate pathway) of isoprenoid synthesis in certain bacteria and parasites, is the most potent natural phosphoantigen [12, 14, 53]. Indeed, HMBPP activates Vγ9Vδ2 T cells approximately 10,000 times more efficiently than the ubiquitous phosphoantigen IPP, providing an innate-like signal of infection to the immune system [12, 14, 54]. As such, it was unexpected that the most robust Vγ9Vδ2 T-cell response,

at least as quantified in molecular terms, was observed in children infected with HMBPP-negative *S. aureus* rather than with HMBPP-positive *E. coli*. The reasons for this observation remain obscure. The superantigen staphylococcal enterotoxin A (SEA), which is known to bind specific Vβ chains, can also bind the Vγ9 chain [55], potentially activating Vγ9Vδ2 T cells in a manner akin to human-derived BTN2A1 [56, 57]. However, the concentrations of SEA required to induce the proliferation of Vγ9-expressing γδ T-cell clones were several orders of magnitude higher than those required to induce a response in αβ T-cell clones, questioning the relevance of this observation in vivo [55]. More recently, it has been suggested that Vγ9Vδ2 T cells can recognize *S. aureus*-infected dendritic cells, at least in part via the expressed TCR [58]. Of note, fetal-like TRD expansions (mouse TRDV4 [9]) have also been observed in a murine model of *S. aureus* skin infection, where they were associated with protection [59]. Accordingly, despite the lack of homology between the TRD repertoires in humans and mice [9, 33], a public/fetal-like TRD response against *S. aureus* infection appears to be a conserved mechanism across species in mammalian evolution. Our observations regarding fetal-derived Vγ9Vδ2 T-cell expansions in the *S. aureus* and *E. coli* groups are not explained by the presence of a single or a limited subset of TRDV2 sequences; instead, these expansions were polyclonal. While this observation may reflect an increased sensitivity of fetal TCR sequences toward specific pathogenic encounters, we cannot discount the possibility that this expansion is driven by specific (cytokine) stimuli from *E. coli* and *S. aureus* infections to which fetal-derived Vγ9Vδ2 T cells might be more sensitive. A similar phenomenon, where increased cytokine responsiveness rather than specific TCR engagement drives immune expansion, has also been described in early-life CD8$^+$ αβ T cells [60, 61].

This study has some limitations. Sampling from the SPSS was limited to blood RNA at a single time point. Therefore, we could not perform functional studies or investigate the time course of γδ TCR repertoire changes. A very recent study found that both IFNγ$^+$ and CD83$^+$ Vγ9Vδ2 T cells were increased upon sepsis in a cohort of preterm infants, highlighting phenotypically diverse responses of fetal-derived Vγ9Vδ2 T cells upon bacterial infection [62]. As different pathogens affect different age groups [34], children infected by *S. pneumoniae*, *E. coli*, and *S. aureus* were not perfectly age-matched. To address this limitation, we performed an ANCOVA and included a control group of children <2 years who never had any invasive bacterial infection. The sample size did not allow to assess the impact of potential confounding factors, such as previous viral infections and comorbidities, on the γδ TCR repertoire.

In summary, we have shown that young children (<2 years) but not older children and adults harbor fetal-derived Vγ9Vδ2 T cells that are highly responsive to specific bacterial pathogens in vivo. This finding challenges the idea that immune cells in early life are immature versions of their adult counterparts. We propose that fetal Vγ9Vδ2 T cells are held in check by various tolerance mechanisms, which are broken upon invasive infection with

specific pathogens unleashing their strong reactivity, at a time preceding the development of fully functional αβ T-cell memory. This knowledge can guide further studies investigating the immune system in early childhood and potentially inform the development of new therapeutic approaches to improve the health of young children.

Materials and methods

Patients and samples

This multicenter study was based on a subset of the prospective observational SPSS [34]. Children aged between 0 and 16 years presenting with blood culture-proven bacterial sepsis were recruited in the ten major pediatric hospitals in Switzerland between September 2011 and December 2015. Sepsis was defined by the presence of positive blood cultures in children with a systemic inflammatory response syndrome, according to the 2005 pediatric consensus definition [63]. After having obtained written informed consent, we collected arterial or venous blood from the children. Whole arterial or venous blood was collected in PAXgene Blood RNA Tubes, which were subsequently cryopreserved at –80°C. The study was approved by Swissethics (KEK-029/11). Written informed consent was obtained in all cases in accordance with the principles of the Declaration of Helsinki.

Children infected with *E. coli, S. aureus*, or *S. pneumoniae* as the etiologic agent of sepsis were selected from the observational SPSS. Patients with conditions that might have influenced the number and/or the activation status of γδ T cells, such as primary immune deficiency, systemic immunosuppression related to cancer therapy, autoimmune or inflammatory disease, transplantation, cystic fibrosis, and congenital infection with cytomegalovirus [18], were excluded from the study. High-throughput TRD sequencing data from healthy children who never had any invasive infection were obtained from previous studies: 10 children from Italy [19] and 24 children from Germany [64, 65, 66]. Fetal blood TRD repertoire data (5 samples, Belgium) were obtained similarly from a dataset available in the public domain [40].

Vγ9Vδ2 T cells were also isolated for TRD repertoire analysis from the peritoneal effluent of 14 dialysis patients presenting with acute peritonitis due to infection with either HMBPP-positive or HMBPP-negative bacteria, as listed in Supporting Information Table S2. The recruitment of peritoneal dialysis patients was approved by the South East Wales Local Ethics Committee (04WSE04/27), and the study was registered with the UK Clinical Research Network Study Portfolio (#11838, PERIT-PD). Patients with acute peritonitis (day 1) were admitted to the University Hospital of Wales. The diagnosis of acute peritonitis was based on the presence of abdominal pain and cloudy peritoneal effluent with >100 white blood cells/mm^3 and a positive microbiological culture. Written informed consent was obtained from all participants in accordance with the principles of the Declaration

of Helsinki. High-throughput TRD sequencing data from Vγ9Vδ2 T cells of 11 healthy adult volunteers (8 adults from Belgium and 3 from South Africa) were obtained from a previous study [40].

RNA isolation and high-throughput sequencing

RNA was extracted from PAXgene Blood RNA Tubes (SPSS) using a PAXgene Blood RNA Kit (PreAnalytiX). The eluted RNA was subsequently concentrated to a volume of 14 μL volume using an RNeasy MinElute Cleanup Kit (Qiagen). This concentrated RNA was prepared for downstream TCR amplification. As functional TCR-γ rearrangements can be found in all peripheral blood T cells [67], we focused our analysis on TCR-δ chains, given the unseparated nature of our starting template. cDNA synthesis was conducted through a template-switch anchored reverse-transcription PCR. This employed a primer specific to the human constant delta gene segment (TRDC) with the sequence 5′-GTAGAATTCCTTCACCAGACAAG-3′, a template-switch adaptor with the sequence 5′-AAGCAGTGGTATCAACGCA-GAGTACATrGrGrG-3′, and SuperScript II reverse transcriptase (Thermo Fisher Scientific). The cDNA was then purified using AMPure XP Beads (Agencourt). Amplification of the TRD region was achieved using an additional PCR incorporating another TRDC-specific primer (5′-*GTCTCGTGGGCTCGGAGA-TGTGTATAAGAGACAGA*CGGATGGTTTGGTATGAGGCTGACTTCT-3′, adapter sequence italicized) and a primer complementary to the template-switch adaptor (5′-*TCGTCGGCAGCGTCAGA-TGTGTATAAGAGACAGA*AGCAGTGGTATCAACGCAG-3′, (adapter sequence italicized) in combination with a KAPA real-time library amplification kit (Kapa Biosystems). Amplification products were purified using AMPure XP Beads (Agencourt) and subjected to an index PCR incorporating sequencing adapters in combination with a Nextera XT index kit (Illumina). The final products were purified again using AMPure XP Beads (Agencourt) and sequenced at the GIGA Center (University of Liege, Belgium) using an Illumina MiSeq platform with MiSeq v3 600 cycles kit (ref: MS-102-3003).

γδ T cells were also sorted via flow cytometry from the cloudy effluent sampled from peritoneal dialysis patients presenting with acute peritonitis. Briefly, peripheral blood mononuclear cells were isolated from peritoneal dialysis fluid via density gradient centrifugation using Lymphoprep (Axis-Shield). Cells were then stained with the following reagents: anti-CD14–V500 (clone M5E2), anti-CD19–V500 (clone HIB19), anti-CCR7–PE-Cy7 (clone 3D12), and anti-Vδ2–PE (clone B6) from BD Biosciences; anti-CD4–PE-Cy5.5 (clone S3.5) and LIVE/DEAD Fixable Aqua from Thermo Fisher Scientific; anti-CD3–APC/Fire 750 (clone SK7), anti-CD8–BV711 (clone RPA-T8), anti-CD27–BV421 (clone M-T271), anti-CD45RA–PE-Dazzle 594 (clone HI100), anti-TCRγδ–PE-Cy5 (clone B1), and anti-Vα7.2–BV605 (clone 3C10) from BioLegend; anti-CD161–APC (clone 191B8) from Miltenyi Biotec; and anti-CXCR3–FITC (clone 49801) from R&D Systems. Viable T cells expressing Vδ2 were identified in the CD3$^+$ lineage and sorted into RNAlater (Thermo Fisher Scientific) using

a custom-built FACSAria II (BD Biosciences) equipped with FACS-Diva software version 8.0 (BD Biosciences). TCR sequencing was performed by iRepertoire (MiSeq v2 500 cycle flow cell).

TRD repertoire analysis

CDR3 sequences were extracted from raw FASTQ files after aligning the reads to reference V, D, and J genes from TRD loci in the GenBank database using the MiXCR software version 3.0.12 [68]. CDR3 reads were assembled using the "assemble" function, and TRDD segments were assigned using the "OcloneFactoryParameters.dParameters.absoluteMinScore = 10" option based on three consecutive nucleotides from the international ImMunoGeneTics information system [69]. CDR3 sequences were further analyzed using VDJtools software version 1.2.1 [70]. Out-of-frame sequences and nonfunctional TRD segments were excluded from the analysis. Note that the TRDV gene usage was not calculated for uninfected children from the Ravens cohort (2020) [64], because in that cohort specific primers for TRDV genes were used (multiplex PCR) which can potentially result in a different TRDV content compared with the unbiased approach using a primer targeting the template-switch adaptor as in the current study; for other TRD sequencing read-outs, this has no potential influence. *F*-values representing the geometric mean of relative overlap frequencies between a pair of samples were calculated using the "CalcPairwiseDistances" function. PCA was performed using FactoMineR version 2.7 in R [71] with the following variables: % of TRDV2-TRDD3-containing sequences, % of TRDV2-TRDJ1-containing sequences, % of TRDV2-TRDJ2-containing sequences, % of TRDV2-TRDJ3-containing sequences, % of TRDV1 use, % of TRDV2 use, % of TRDV3 use, mean number of N additions in TRDV1 sequences, mean number of N additions in TRDV2 sequences, mean nucleotide length in TRDV1 sequences, and mean nucleotide length in TRDV2 sequences. All variables were scaled to a similar range using Z-score normalization prior to PCA. Results were visualized using FactoExtra version 1.0.7 in R. Circoplots were generated using circlize version 0.4.15 in R [72].

Statistics

All statistical analyses except the ANCOVA were performed using Prism version 8.0.2 (GraphPad). To control for the effect of age on the different TCR repertoire variables in children aged under 2 years, we conducted an ANCOVA using the rstatix (v0.7.2) and emmeans (v1.10.0) R packages. We verified that the assumptions of the model (normality of residuals, homogeneity of variances, linearity) were met. Subsequently, we fitted the model and performed pairwise comparisons between groups using estimated marginal means. The results of the analysis (ANCOVA table, pairwise comparisons, and means of dependent variables before and after covariate adjustment) are presented in Supporting Information Table S1.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

Author contributions: Eric Giannoni, David A. Price, Matthias Eberl, Luregn J. Schlapbach, and David Vermijlen conceived and supervised the study. Eric Giannoni, Philipp K.A. Agyeman, Donald J. Fraser, and Luregn J. Schlapbach led patient recruitment and acquisition of clinical data. Guillem Sanchez Sanchez, Eric Giannoni, Isoline Verdebout, Maria Papadopoulou, Moosa Rezwani, Raya Ahmed, Kristin Ladell, Kelly L. Miners, and James E. McLaren designed and performed the experiments; Guillem Sanchez Sanchez, Eric Giannoni, and David Vermijlen analyzed and interpreted the data. Eric Giannoni, Matthias Eberl, and Luregn J. Schlapbach acquired funding. Eric Giannoni, Guillem Sanchez Sanchez, and David Vermijlen wrote and revised the manuscript. All coauthors reviewed and revised the manuscript.

Data availability statement: FASTQ files of TRD sequences are deposited in the Sequence Read Archive (SRA) under accession no. PRJNA1033066.

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Abbreviations: **CDR3**: complementarity determining region 3 · **HMBPP**: (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate · **MEP**: 2-*C*-methyl-D-erythritol 4-phosphate · **PCA**: principal component analysis · **TCRs**: T-cell receptors · **TdT**: terminal deoxynucleotidyl transferase

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