

ARTICLE

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# A clonotypic $V\gamma 4J\gamma 1/V\delta 5D\delta 2J\delta 1$ innate $\gamma\delta$ T-cell population restricted to the $CCR6^+ CD27^-$ subset

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Here we investigate the TCR repertoire of mouse  $V\gamma 4^+$   $\gamma\delta$  T cells in correlation with their developmental origin and homeostasis. By deep sequencing we identify a high frequency of straight  $V\delta 5D\delta 2J\delta 1$  germline rearrangements without P- and N-nucleotides within the otherwise highly diverse *Trd* repertoire of  $V\gamma 4^+$  cells. This sequence is infrequent in  $CCR6^- CD27^+$  cells, but abundant among  $CCR6^+ CD27^-$   $\gamma\delta$  T cells. Using an inducible *Rag1* knock-in mouse model, we show that  $\gamma\delta$  T cells generated in the adult thymus rarely contain this germline-rearranged  $V\delta 5D\delta 2J\delta 1$  sequence, confirming its fetal origin. Single-cell analysis and deep sequencing of the *Trg* locus reveal a dominant CDR3 junctional motif that completes the TCR repertoire of invariant  $V\gamma 4^+ V\delta 5^+$  cells. In conclusion, this study identifies an innate subset of fetal thymus-derived  $\gamma\delta$  T cells with an invariant  $V\gamma 4^+ V\delta 5^+$  TCR that is restricted to the  $CCR6^+ CD27^-$  subset of  $\gamma\delta$  T cells.

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$\gamma\delta$  T cells use *Rag*-mediated V(D)J recombination to rearrange T-cell antigen receptors (TCRs) consisting of  $\gamma$  and  $\delta$  chains. In theory, the potential junctional diversity of  $\gamma\delta$  TCRs is in the range of  $10^{18}$ , and thus several orders of magnitudes higher than that of  $\alpha\beta$  TCR or Ig rearrangements<sup>1</sup>. However,  $\gamma\delta$  T cells are often regarded as innate T cells that use their TCR recombination machinery to generate identical  $\gamma\delta$  TCRs of limited diversity. This perception is based on the occurrence of a few prominent  $\gamma\delta$  T-cell subsets with no or little TCR junctional diversity, in which anatomical localization and function correlate with invariant  $\gamma\delta$  TCRs<sup>2,3</sup>. Specifically, the mouse skin epidermis contains a specialized  $\gamma\delta$  T-cell population of dendritic epidermal T cells (DETCs) with a fixed TCR composed of invariant  $V\gamma5J\gamma1C\gamma1$  and germline-rearranged  $V\delta1D\delta2J\delta2$  without P- or N-nucleotides (Tonegawa nomenclature)<sup>4,5</sup>. The same canonical  $V\delta1D\delta2J\delta2$  chain is employed in combination with an invariant  $V\gamma6J\gamma1C\gamma1$  TCR chain in interleukin (IL)-17-producing  $V\gamma6/V\delta1$  T cells. These  $V\gamma6/V\delta1$  cells were initially thought to be restricted to the uterus and the tongue<sup>6</sup>, but subsequently were also found in most other tissues including the lung<sup>7</sup>, liver<sup>8</sup>, dermis<sup>9,10</sup>, secondary lymphoid organs<sup>11</sup> and intestinal lamina propria<sup>12</sup>. Finally, IL-4-producing  $V\gamma1^+$   $\gamma\delta$  NKT cells with restricted  $V\delta6D\delta2J\delta1$  junctions and semi-invariant  $V\gamma1J\gamma4C\gamma4$  junctions are preferentially localized in the liver and spleen<sup>13,14</sup>. In contrast,  $\gamma\delta$  T cells circulating in the blood and secondary lymphoid organs mostly contain either  $V\gamma1$  or  $V\gamma4$  rearrangements and are thought to have highly diverse TCR repertoires<sup>3,15–18</sup>.

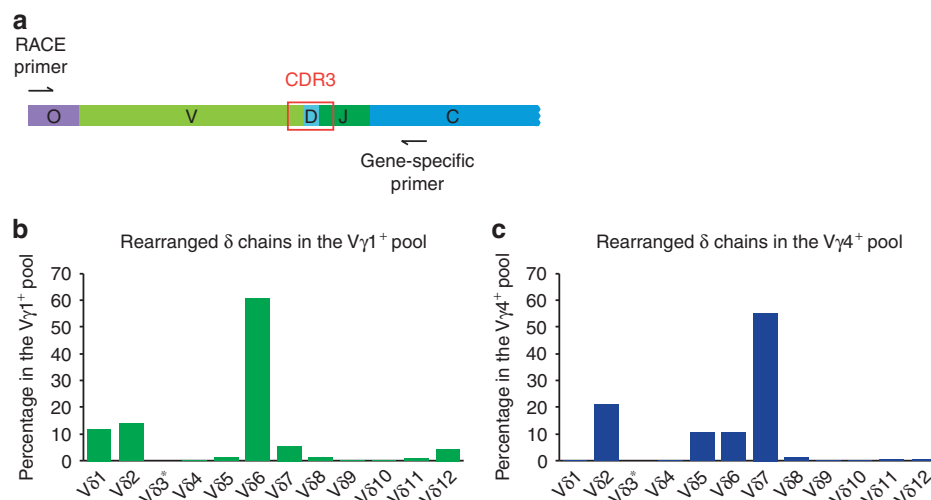
Recently,  $\gamma\delta$  T-cell populations grouped on the basis of the TCR  $\gamma$ -chain usage were included into the ImmGen transcriptome database<sup>19</sup>. The data suggested that  $\gamma\delta$  effector T-cell function correlated with  $\gamma\delta$  TCR usage also in  $V\gamma4^+$  T cells. Fetal thymic, and to a lesser extent splenic,  $V\gamma4^+$  T cells were recognized as  $\gamma\delta$  effector T cells associated with IL-17 production<sup>19</sup>. However, the pool of  $V\gamma4^+$  T cells is heterogeneous and contains both innate cells with IL-17-producing capacity as well as cells that are biased to interferon (IFN)- $\gamma$  production. These two populations can be segregated according to a  $CCR6^+CD27^-$  or  $CCR6^-CD27^+$  surface marker phenotype, respectively<sup>20–22</sup>. In addition,  $V\gamma4^+$  T cells

also comprise  $CD27^+CD45RB^{\text{high}}$  cells, a subset that readily produces IFN- $\gamma$  upon stimulation with IL-18 and IL-12 (ref. 23), similar to  $NK1.1^+$   $\gamma\delta$  T cells<sup>21</sup>. Moreover, the requirements for final differentiation into effector cells may vary between  $\gamma\delta$  T-cell types depending on their ontogeny<sup>24</sup>. For example, it was recently proposed that  $V\gamma4^+$  T cells but not  $V\gamma6^+$  T cells require extrathymic maturation for imprinting of skin-homing properties and acquisition of an IL-17-producing phenotype<sup>25</sup>.

To address the correlation of  $V\gamma4^+$  TCR and  $V\gamma4^+$  T-cell function in more detail, we performed a high-resolution analysis of the mouse  $\gamma\delta$  TCR repertoire. Focusing on  $V\gamma4^+$  T cells, rearranged *Trd* and *Trg* loci of the respective subsets were sequenced using 454 high-throughput sequencing technology. We found striking differences in functionally different subsets of  $V\gamma4^+$  T cells. Importantly, this study identifies invariant  $V\gamma4^+$   $V\delta5^+$  T cells as a novel innate T-cell population that is abundant only among  $CCR6^+CD27^-$   $\gamma\delta$  T cells, which are known for their IL-17-producing phenotype<sup>10,18,20–22,24,26,27</sup>.

## Results

**Highly diverse *Trd* repertoire in  $V\gamma1^+$  and  $V\gamma4^+$  cells.** Most  $\gamma\delta$  T cells in the blood and secondary lymphoid organs display a TCR that comprises either  $V\gamma1$  or  $V\gamma4$  rearrangements. In contrast to fetal  $V\gamma5^+$  DETCs or invariant  $V\gamma6^+$   $\gamma\delta$  T cells, which have a restricted TCR repertoire of very limited diversity, circulating  $V\gamma1^+$  or  $V\gamma4^+$  T cells are assumed to be far more polyclonal<sup>18</sup>. To investigate their TCR diversity in depth, we sorted these two major subsets of peripheral  $\gamma\delta$  T cells on the basis of a  $\gamma\delta$  T-cell-specific reporter fluorescence<sup>28</sup> and co-staining with monoclonal antibodies (mAbs) directed against their  $V\gamma1^+$  or  $V\gamma4^+$  TCR. Next, mRNA of these samples was amplified by rapid amplification of cDNA ends (RACE) using a gene-specific primer within the first exon of the constant gene segment of the *Trd* locus to amplify all rearranged VDJ combinations (Fig. 1a). We observed differentially biased recombination of *Trd* chains in  $V\gamma1^+$  and  $V\gamma4^+$  cells. Approximately 60% of the *Trd* chains from  $V\gamma1^+$  samples contained a  $V\delta6$  segment, followed by  $V\delta2$ ,  $V\delta1$ ,  $V\delta7$  and  $V\delta12$  (Fig. 1b). *Trd* chains from  $V\gamma4^+$  samples contained mainly rearrangements of  $V\delta7$  segments ( $\sim 55\%$ ),



**Figure 1 |  $V\delta$  gene segment usage among  $V\gamma1^+$  and  $V\gamma4^+$  cells from peripheral lymphoid organs.** (a) Schematic presentation of a rearranged *Trd* locus and primers for RACE PCR (O: SMARTer anchor oligonucleotide; V: variable, D: diversity, J: joining and C: constant gene segments). (b,c) Results of RACE PCR product sequencing. Bars indicate proportion of in-frame rearrangements of  $V\delta$  gene segments within the purified  $V\gamma1^+$  or  $V\gamma4^+$  cells pooled from the pLN and spleen of three *TrdH2BeGFP* mice. (\*:  $V\delta3$  is a pseudogene). For each  $V\gamma$  pool, at least 22,000 in-frame sequences were analysed. Data are representative of two independent experiments that gave similar results.

followed by V $\delta$ 2, V $\delta$ 5 and V $\delta$ 6 (Fig. 1c). This extends but is largely consistent with prior studies based on V $\delta$ -specific mAbs<sup>29</sup>. Further analysis of individual CDR3 sequences revealed a highly diverse repertoire in both V $\gamma$ 1<sup>+</sup> and V $\gamma$ 4<sup>+</sup> cells (Supplementary Fig. 1). A notable exception to broad *Trd* polyclonality was observed only in V $\delta$ 5 rearrangements within the pool of V $\gamma$ 4<sup>+</sup> cells, where one V $\delta$ 5<sup>+</sup>D $\delta$ 2<sup>+</sup>J $\delta$ 1<sup>+</sup> sequence was remarkably abundant (Supplementary Fig. 1).

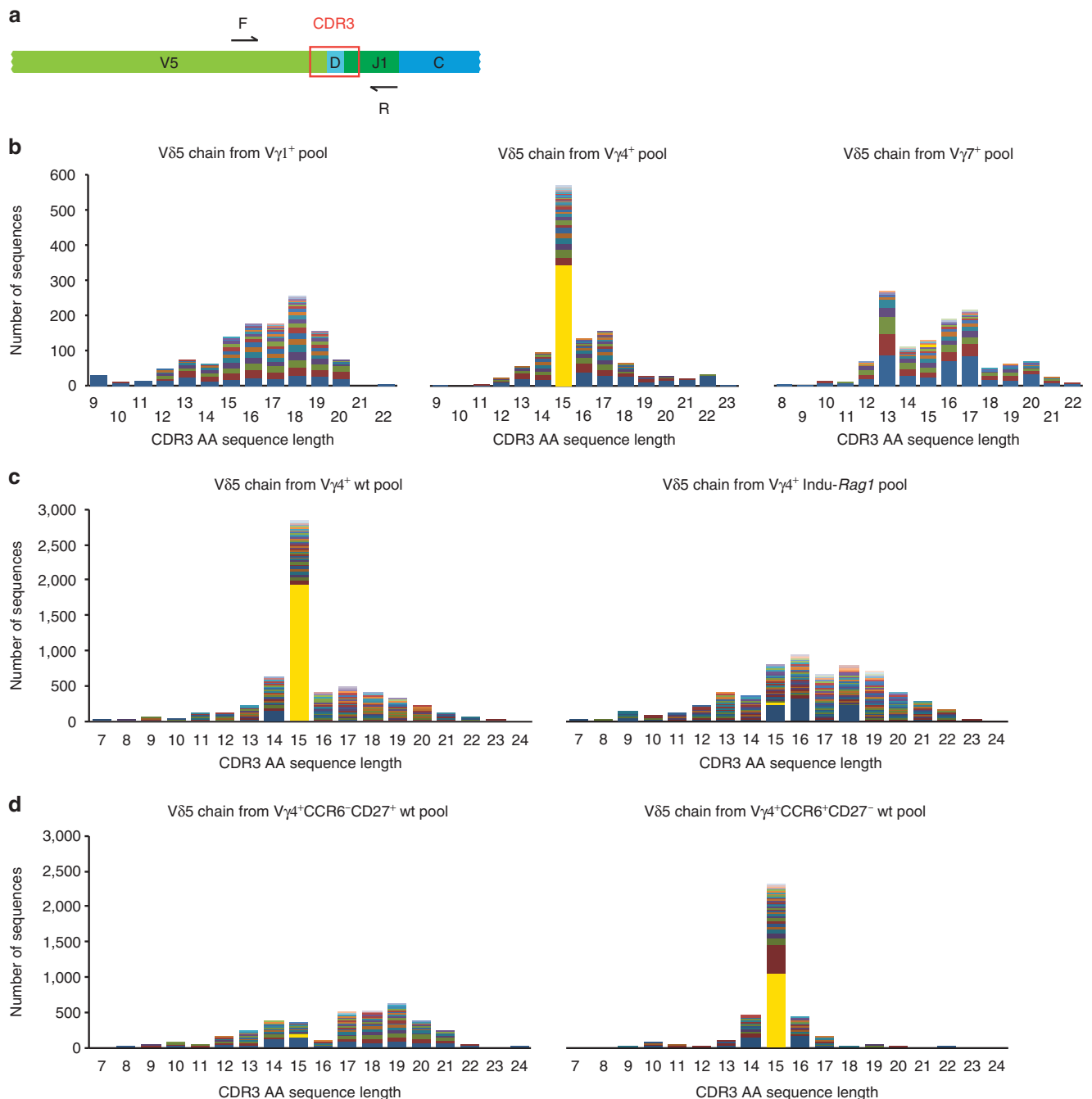
**Abundant V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangements in V $\gamma$ 4<sup>+</sup> cells.** In order to further investigate the occurrence of abundant V $\delta$ 5D $\delta$ 2J $\delta$ 1 sequence in  $\gamma\delta$  T cells, we applied primers that specifically amplified CDR3 regions located between V $\delta$ 5 and J $\delta$ 1 segments (Fig. 2a). With this independent approach, we confirmed the data obtained by RACE analysis. On the amino-acid level, ~30% of V $\delta$ 5<sup>+</sup> *Trd* sequences in V $\gamma$ 4<sup>+</sup> cells isolated from secondary lymphoid organs showed a unique arrangement of V $\delta$ 5, D $\delta$ 2 and J $\delta$ 1 segments, whereas this combination was absent in V $\gamma$ 1<sup>+</sup> T cells, and represented less than 0.3% of V $\delta$ 5<sup>+</sup> *Trd* sequences in intestinal V $\gamma$ 7<sup>+</sup> cells (Fig. 2b). Importantly, the V $\delta$ 5, D $\delta$ 2 and J $\delta$ 1 segments were directly rearranged without P- and N-nucleotides (hereafter called germline rearrangements) in more than 95% of these canonical *Trd* sequences, while other nucleotide sequences coding for the same CDR3 amino-acid sequence were uncommon (Table 1). Next, we hypothesized that these canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 sequences originated from V $\gamma$ 4<sup>+</sup>  $\gamma\delta$  T cells that had developed in the fetal thymus, similar to the invariant *Trd* chains found in V $\gamma$ 5<sup>+</sup> DETCs and in V $\gamma$ 6<sup>+</sup>  $\gamma\delta$  T cells. Their TCRs share canonical N-nucleotide-lacking V $\delta$ 1D $\delta$ 2J $\delta$ 2 rearrangements<sup>4–6</sup>. To this end, we sequenced the V $\delta$ 5 repertoire of V $\gamma$ 4<sup>+</sup>  $\gamma\delta$  T cells sorted from either wild-type *TcrdH2BeGFP* control mice (wt) or from *Indu-Rag1* mice<sup>30</sup> crossed with *TcrdH2BeGFP* mice. In the latter, deficient V(D)J recombination had been restored in the adult stage by tamoxifen-induced cre recombinase expression. Only ~1% of the canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangements were found when only adult  $\gamma\delta$  T-cell development had been possible in *Indu-Rag1* mice as compared with control V $\gamma$ 4<sup>+</sup>  $\gamma\delta$  T cells with more than 30% of such sequences (Fig. 2c). These results suggest that the large majority of canonical V $\gamma$ 4<sup>+</sup>/V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells are generated early in ontogeny, presumably in the embryonic thymus.

**Canonical rearrangements in CCR6<sup>+</sup>CD27<sup>-</sup>  $\gamma\delta$  T cells.** Next, we tested whether V $\gamma$ 4<sup>+</sup> cells with canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangements were contributing to the pool of IL-17-producing V $\gamma$ 4<sup>+</sup>  $\gamma\delta$  T cells, as these were recently discovered to develop exclusively before birth and subsequently persist in adult mice as self-renewing, long-lived cells<sup>11</sup>. Such IL-17-producing effector  $\gamma\delta$  T cells are contained within a population of  $\gamma\delta$  T cells identified by a CCR6<sup>+</sup>CD27<sup>-</sup> surface marker phenotype<sup>20–22</sup>. Therefore, we sorted V $\gamma$ 4<sup>+</sup> T cells from peripheral lymph nodes (pLNs) and spleen of adult *TcrdH2BeGFP* mice into CCR6<sup>-</sup>CD27<sup>+</sup> and CCR6<sup>+</sup>CD27<sup>-</sup> subsets, sequenced their *Trd* loci and compared them with each other. Clearly, canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangements were highly enriched in the CCR6<sup>+</sup>CD27<sup>-</sup> subset (Fig. 2d). Thereby, the corresponding CDR3 amino-acid motif ASGYIGGIRATDKLV from CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells was principally but not exclusively encoded by V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangements unmodified by P- or N-nucleotides (Fig. 3). Together, these results suggest that CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells have a distinct TCR repertoire that comprises canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells. The data further support the hypothesis that IL-17-producing

CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells are principally generated within a functional wave of embryonic  $\gamma\delta$  T-cell development<sup>11,18</sup>.

**Ontogeny and organ distribution of V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup> T cells.** To further explore the potential fetal thymic origin of canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup> V $\gamma$ 4<sup>+</sup> T cells, we sorted V $\gamma$ 4<sup>+</sup> T cells derived from the thymus at different stages during ontogeny and sequenced the *Trd* repertoire of V $\delta$ 5–J $\delta$ 1 amplicons (Fig. 4a). The invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1 sequence was already abundant in the fetal thymic *Trd* repertoire of V $\gamma$ 4<sup>+</sup> T cells on embryonic day 18 (E18, 15% of all V $\delta$ 5 sequences), but also persisted in juvenile (11–17%) or adult thymi (7% at 4 months; Fig. 4a and Supplementary Fig. 2). These data further support the hypothesis that IL-17-producing  $\gamma\delta$  T cells, in particular with the invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1 clonotype, are already generated in the fetal thymus, but later persist as resident cells in the juvenile and adult thymus<sup>11,24</sup>. Moreover, these findings are consistent with a previous ontogeny study as described in (ref. 31). However, invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup> T cells were even more abundant in peripheral lymphoid organs than in the thymus, suggesting peripheral homeostatic expansion of the subset. Notably, invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup> *Trd* sequences made up 43% of all V $\delta$ 5 sequences of V $\gamma$ 4<sup>+</sup> T cells in pLNs and 27% in the spleen (Fig. 4b). Because TCR repertoire as well as the developmental requirements of V $\gamma$ 4<sup>+</sup> T cells vary along with location<sup>25</sup>, we investigated different peripheral anatomical sites separately. By deep sequencing of V $\delta$ 5–J $\delta$ 1 amplicons we examined the TCR repertoire of V $\gamma$ 4<sup>+</sup> T cells derived from other organs including the lung, skin and liver (Fig. 4b and Supplementary Fig. 3). It turned out that the invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1 sequence also dominated the *Trd* repertoire of V $\gamma$ 4<sup>+</sup> T cells in peripheral tissues such as the skin (27%), lung (15%) and liver (25%), albeit to a lesser extent than in pLNs. Across all samples derived from the pLN and spleen, the V $\delta$ 5D $\delta$ 2J $\delta$ 1 clonotype made up ~35% of the V $\delta$ 5 repertoire in V $\gamma$ 4<sup>+</sup> T cells. Since ~10% of V $\gamma$ 4<sup>+</sup> T cells used the V $\delta$ 5 segment (as shown by RACE analyses in Fig. 1), these constitute 3.5% clonotypic cells of all V $\gamma$ 4<sup>+</sup> T cells. Thus, with V $\gamma$ 4<sup>+</sup> T cells making up to 50% of all  $\gamma\delta$  T cells derived from the pLN<sup>32</sup>, the actual rate of clonotypic invariant V $\delta$ 5D $\delta$ 2J $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells is 1.5–2% among all  $\gamma\delta$  T cells. This remarkably high frequency is in the range of invariant IL-17A-producing V $\gamma$ 6V $\delta$ 1<sup>+</sup> T cells that constitute 2–4% among all  $\gamma\delta$  T cells in secondary lymphoid organs<sup>33,34</sup>. Nevertheless, only moderate expansion of this subset in peripheral tissues as compared with the thymus underlines their genuine innate nature.

**Decreased TCR diversity in CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells.** Next, we asked whether TCR diversity in noninvariant V $\gamma$ 4<sup>+</sup> V $\delta$ 5<sup>+</sup> T cells among the CCR6<sup>+</sup>CD27<sup>-</sup> subset was also different to CCR6<sup>-</sup>CD27<sup>+</sup> V $\gamma$ 4<sup>+</sup> T cells (Fig. 5). Within an equally sized pool of in-frame amino-acid sequences obtained from both populations, CCR6<sup>-</sup>CD27<sup>+</sup> V $\gamma$ 4<sup>+</sup> T cells had a higher proportion of unique V $\delta$ 5 rearrangements that were present only once in the tested sample (singletons), while CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells showed fewer singletons (Fig. 5a). Consequently, samples from CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells contained a higher frequency of sequences that were detected several times. In Fig. 5b, in which all sequences are represented according to their actual frequency among all sequences, such clones were considered as expanded (1–1.99%) or highly expanded ( $\geq$  2%). To quantify the TCR diversity of the respective two  $\gamma\delta$  T-cell subsets, we calculated the effective number of sequences using the Shannon index, which considers the number of observed individual sequences as well their abundance in the repertoire<sup>35,36</sup>. It turned out that the CCR6<sup>+</sup>CD27<sup>-</sup> subset was three times less



**Figure 2 | One Vδ5Dδ2Jδ1 sequence is dominant in CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> T cells.** (a) Schematic presentation of a rearranged *Trd* locus showing the position of representative primers used for amplification of variable *Trd* regions between Vδ5 and Jδ1, F: forward primer, R: reverse primer. (b) CDR3 amino-acid sequence length distribution and composition for in-frame rearrangements of Vδ5–Jδ1 chain in Vγ1<sup>+</sup> T cells (left panel), and Vγ4<sup>+</sup> T cells (middle panel) isolated from a pool of pLN and spleen, or Vγ7<sup>+</sup> intestinal IELs (right panel), of three *TcrdH2BeGFP* mice in each subset. (c) In-frame rearrangements of Vδ5–Jδ1 chains in Vγ4<sup>+</sup> T cells pooled from the pLN and spleen of four *TcrdH2BeGFP* mice (wt; left panel) or four tamoxifen-induced *Indu-Rag1* × *TcrdH2BeGFP* mice (right panel). Genomic DNA from 10,000 Vγ4<sup>+</sup> T cells was used as template. For each subset, 5,870 sequences were analysed. (d) In-frame rearrangements of Vδ5–Jδ1 chains in CCR6<sup>-</sup>CD27<sup>+</sup> (left panel) versus CCR6<sup>+</sup>CD27<sup>-</sup> (right panel) Vγ4<sup>+</sup> T cells pooled from the pLN and spleen of three *TcrdH2BeGFP* mice. cDNA from 4,800 CCR6<sup>-</sup>CD27<sup>+</sup> or CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> cells was used as template. For each subset, 3,552 sequences were analysed. Individual in-frame-rearranged CDR3 amino-acid sequences are separated by colours. Data are representative of at least two independent experiments that gave similar results.

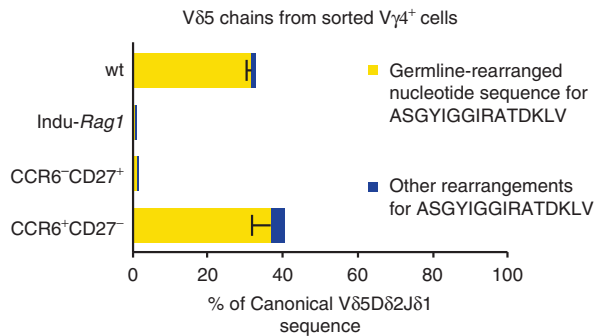
diverse than CCR6<sup>-</sup>CD27<sup>+</sup> cells, and still two times less diverse when the dominant canonical Vδ5Dδ2Jδ1 sequence was excluded from the equation (Fig. 5c). Qualitatively, we observed essentially no overlapping Vδ5 sequences between two independently analysed samples of CCR6<sup>-</sup>CD27<sup>+</sup> Vγ4<sup>+</sup> cells. The only

exception was the canonical sequence that was found repeatedly, albeit at low frequency (Fig. 5d, left panel). In contrast, two independent samples of CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> T cells showed an overlap of 29 *Trd* sequences, beside the canonical Vδ5Dδ2Jδ1 sequence (Fig. 5d, right panel). Accordingly, the similarity of two

**Table 1 | Most frequent Vδ5 CDR3 nucleotide sequences that encode the canonical Vδ5 amino-acid sequence.**

Vδ5 (5'-3')	P	N1	Dδ1	Dδ2 (5'-3')	N2	P	Jδ1 (5'-3')	Frequency
<b>ASGY</b>				<b>IGGIR</b>			<b>.TDKLV</b>	<b>Germline</b>
gcctcggggtat				atcggaggatacag			ctaccgacaaactcgtc	95-98%
gcctcggggtat				atcggaggatacag	g	g	ctaccgacaaactcgtc	0.9-1.4%
gcctcggggtat				atcggaggatacag..	cg		..accgacaaactcgtc	0.3-0.6%
gcctcggggtat				atcggaggatacag..	t	g	ctaccgacaaactcgtc	0.2%
gcctcggggtat	ata			...ggaggatacag			ctaccgacaaactcgtc	0-0.9%
gcctcggg....			....atat	atcggaggatacag			ctaccgacaaactcgtc	0-0.4%
gcctcggggtat.		c		atcggaggatacag..	g	g	ctaccgacaaactcgtc	0-0.3%
gcctcggg....		ttat		atcggaggatacag			ctaccgacaaactcgtc	0-0.2%

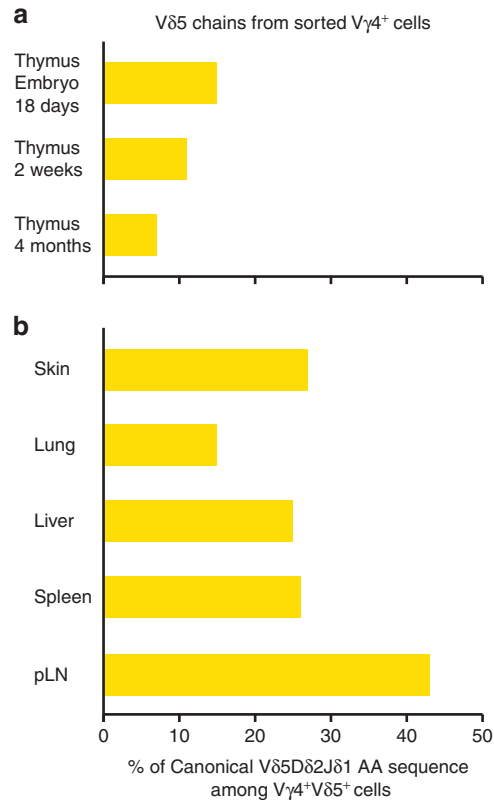
The germline sequences of related gene segments are demonstrated in red. P and N refer to P- and N-nucleotides, respectively.



**Figure 3 | Frequency of a canonical Vδ5Dδ2Jδ1 CDR3 sequence.** Bar graphs show percentages of the canonical CDR3 amino-acid sequence ASGYIGGIRATDKLV among in-frame Vδ5 sequences derived from the indicated Vγ4+ T-cell populations. Yellow bars represent dominant rearrangements of germline-Vδ5, -Dδ2 and -Jδ1 segments without P- and N-nucleotides. Error bars show s.e.m. Blue bars represent the sum of all other nucleotide sequences coding for ASGYIGGIRATDKLV. Data are pooled from two independent experiments that gave similar results.

independent samples, calculated as the Morisita–Horn index, was low (0.003) for CCR6-CD27+ and high (0.889) for CCR6+CD27- Vγ4+ T cells. In conclusion, CCR6+CD27- Vγ4+ T cells, which are known for their IL-17-production capacity, display TCR repertoires of limited diversity and a considerable TCR sequence overlap between independent samples derived from individual mice. This is consistent with the view that these cells develop as a population of prewired innate effector cells with limited TCR diversity in the fetal thymus to persist and expand after birth.

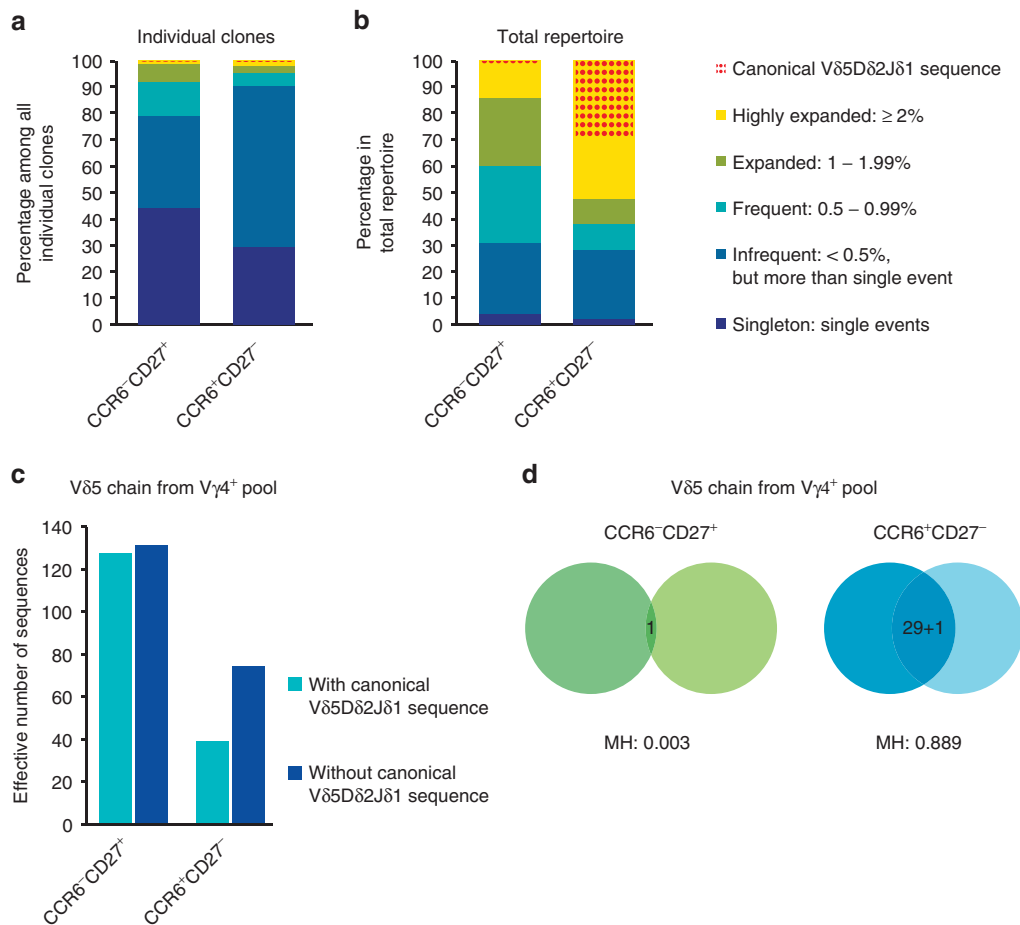
**The corresponding Vγ4 repertoire.** To complement the *Trd* repertoires, we next determined the respective Vγ4 repertoires of sorted Vγ4+ T-cell populations. Sequencing was performed using a Vγ4-specific primer in combination with a consensus primer amplifying Jγ1, Jγ2 and Jγ3 (Fig. 6). Consistent with previous findings<sup>2,37</sup>, overall diversity of the rearranged *Trg* loci was lower than at *Trd* loci, and more than 99% of Vγ4 rearrangements involved Jγ1. Analysis of CDR3 amino-acid sequence length distribution and composition of Vγ4 chain repertoires demonstrated an expanded Vγ4 motif, SYGXYSSGFHKV. Notably, this dominant motif was abundant in Vγ4+ T cells derived from wt and *Indu-Rag1* mice (Fig. 6a) as well as in CCR6-CD27+ and CCR6+CD27- Vγ4+ T cells



**Figure 4 | Frequency of a canonical Vδ5Dδ2Jδ1 CDR3 sequence in thymus ontogeny and in peripheral tissues.** Bar graphs show percentages of the canonical CDR3 amino-acid sequence ASGYIGGIRATDKLV among in-frame Vδ5 sequences derived from the indicated fluorescence-activated cell sorting-sorted Vγ4+ T-cell populations. (a) Frequency of the canonical CDR3 sequence in the thymus of day E18 old embryos, 2-week-old pups and 4-month-old adult mice. (b) Frequency of canonical CDR3 sequence in the skin, lung, liver, spleen and pLN. Data represent eight individual samples from one 454-sequencing run.

(Fig. 6b). This is in contrast to our findings for the canonical Vδ5Dδ2Jδ1 *Trd* locus rearrangement, which was exclusively abundant in fetal thymus-derived CCR6+CD27- γδ T cells. Nevertheless, the Vγ4 repertoire of the IL-17-producing CCR6+CD27- subset was even more focused towards the SYGXYSSGFHKV consensus CDR3 sequence than CCR6-CD27+ Vγ4+ T cells (Fig. 6b). Among all Vγ4 chain sequences analysed, a leucine residue was most frequently found





**Figure 5 | TCR diversity of CCR6<sup>-</sup>CD27<sup>+</sup> versus CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> cells.** (a) Proportion of 309 individual sequences of CCR6<sup>-</sup>CD27<sup>+</sup> Vγ4<sup>+</sup> cells, or 276 individual sequences of CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> cells, among 3,552 Vδ5 sequences of each subset. Each individual sequence is counted only once regardless of abundance. (b) Proportion of total sequences from the same pool as a, considering the abundance. For a, b, data shown are from one representative of two independent experiments. (c) Diversity measured as ‘effective number of sequences’ (e to the power of the Shannon index) for the same data set of 3,552 CDR3 amino-acid sequences in each subset, including the canonical sequence (light blue), or after eliminating the canonical sequence from the repertoires (dark blue). (d) Venn diagrams display overlap between the 100 most frequent CDR3 amino-acid sequences in similar populations from two independent experiments. Numbers indicate overlapping individual sequences. Similarity between the repertoires from two independent experiments was calculated using the Morisita–Horn index (MH). The MH index reveals the degree of similarity by analysing individual sequences considering their frequency among the total repertoire, scoring from 0 for completely dissimilar subsets to 1 for identical subsets.

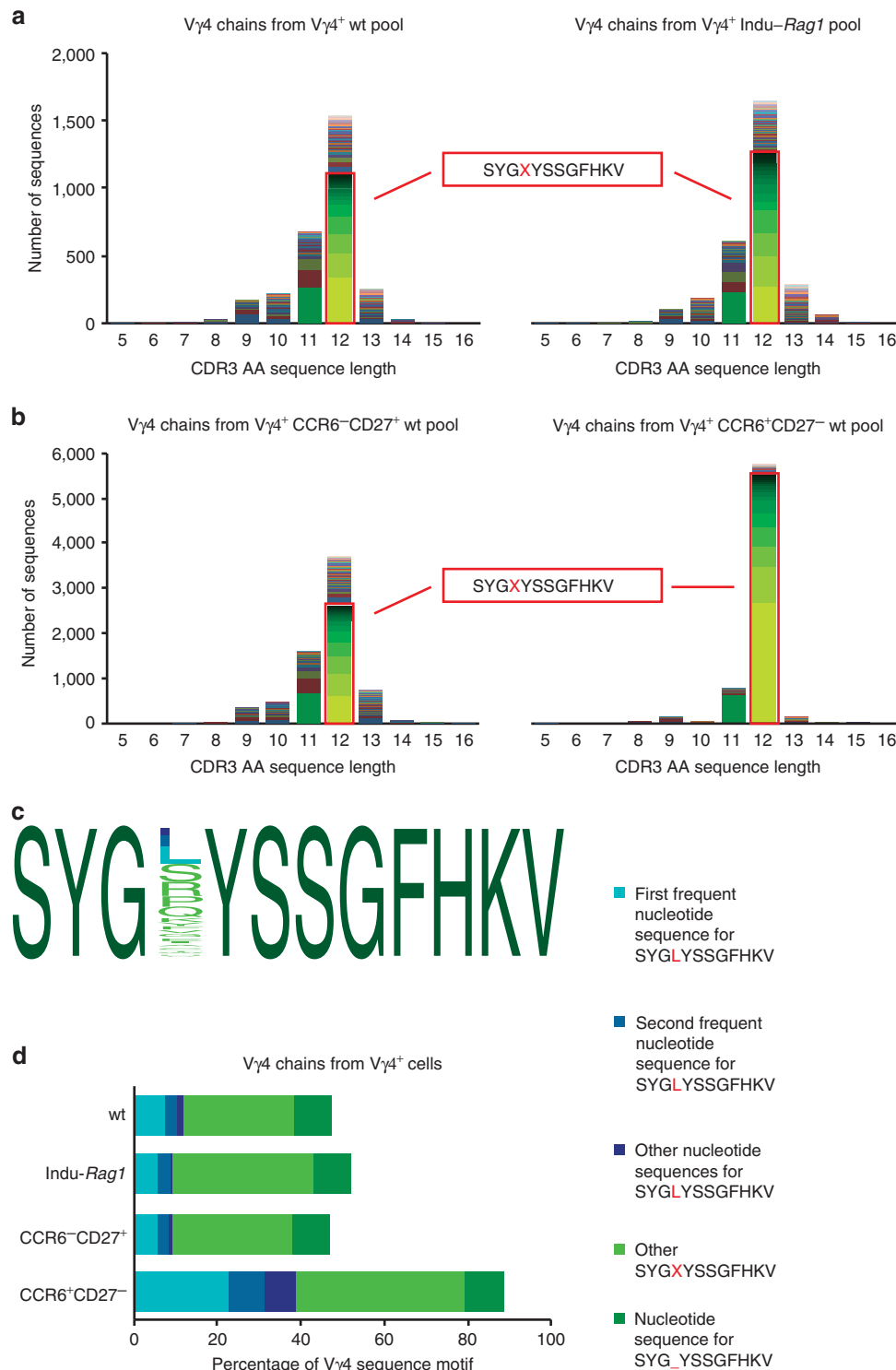
at the variable position X of this CDR3 motif, followed by serine, arginine, proline and subsequently all other amino acids (Fig. 6c). While a stop codon within the germline Vγ4 segment precludes true germline rearrangements, the two most frequent nucleotide sequences coding for SYGLYSSGFHKV were still rather germline-like as they both lacked N-nucleotides (Table 2). Together, these two sequences accounted for one-third of all in-frame sequences obtained from the CCR6<sup>+</sup>CD27<sup>-</sup> subset of Vγ4<sup>+</sup> T cells (Fig. 6d). In conclusion, sequence analyses of the corresponding Vγ4 repertoire is consistent with the view that rearrangement at the γ and δ TCR loci is differentially controlled. Furthermore, the presence of the semi-invariant Vγ4 sequences also in CCR6<sup>-</sup>CD27<sup>+</sup> Vγ4<sup>+</sup> T cells and in T cells from *Indu-Rag1* mice would alone not be sufficient for any potential positive selection of IL-17-producing CCR6<sup>+</sup>CD27<sup>-</sup> subset of Vγ4<sup>+</sup> T cells.

**Invariant Vδ5Dδ2Jδ1 chains pair with a canonical Vγ4 chain.** Finally, we sought to identify the corresponding TCR γ and TCR δ chain pairs that constituted the TCR heterodimer of

IL-17-producing CCR6<sup>+</sup>CD27<sup>-</sup> Vγ4<sup>+</sup> T cells. To this end, we performed single-cell PCR from cDNA of sorted Vγ4<sup>+</sup>CCR6<sup>+</sup>CD27<sup>-</sup> cells. Of 20 individual T cells with Vδ5Dδ2Jδ1 *Trd* germline rearrangement, 7 had *Trg* rearrangements coding for the most frequent Vγ4 CDR3 sequence SYGLYSSGFHKV, 12 displayed other variations of the SYGXYS<sup>G</sup>GFHKV motif and one clone showed a shorter version of this CDR3, namely SYG<sub>-</sub>YSSGFHKV (Table 3). Together, these results suggest that the pool of fetal thymus-derived IL-17-producing γδ T cells of the CCR6<sup>+</sup> lineage contains a population of invariant Vγ4<sup>+</sup> cells with a TCR composed of a germline-rearranged Vδ5Dδ2Jδ1 chain and a canonical Vγ4Jγ1 chain motif.

## Discussion

This study focused on the correlation between TCR sequence and effector phenotype in Vγ4<sup>+</sup> T cells, which constitute a heterogenic population of mouse γδ T cells. It comprised high-throughput generation of hundreds of thousands of sequences of rearranged *Trg* and *Trd* genes and constitutes, to our knowledge, the first deep-sequencing report on *Trd* genes in



**Figure 6 | A frequent motif in the V $\gamma$ 4 repertoire.** (a,b) CDR3 amino-acid sequence length distribution and composition of in-frame-rearranged V $\gamma$ 4 chains in (a) V $\gamma$ 4<sup>+</sup>  $\gamma\delta$  T cells pooled from the pLN and spleen of four *TcrdH2BeGFP* or four tamoxifen-induced *Indu-Rag1*  $\times$  *TcrdH2BeGFP* mice; 10,000 V $\gamma$ 4<sup>+</sup> cells and 2,892 in-frame sequences each. (b) CCR6<sup>-</sup>CD27<sup>+</sup> or CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> T cells isolated from the pooled pLN and spleen of three *TcrdH2BeGFP* mice; 12,000 V $\gamma$ 4<sup>+</sup> cells and 6,888 in-frame sequences each. Individual in-frame-rearranged CDR3 amino-acid sequences are separated by colours. Sequences containing the SYGXYSSGFHKV motif are shown in green tones within a red box, and the sequence SYG\_YSSGFHKV (lacking X) with 11 amino acids is also shown in green. Data are representatives of three independent experiments. (c) Graphical representation of multiple sequence alignment for the frequent V $\gamma$ 4 amino-acid motif, SYGXYSSGFHKV, inspired by <http://weblogo.berkeley.edu>. (d) Percentage of the most frequent CDR3 sequence, SYGLYSSGFHKV, and the proportion of its related nucleotide sequences (in blue tone), along with percentage of other nucleotide sequences containing the SYGXYSSGFHKV motif (in light green), and the sequences for SYG\_YSSGFHKV (in dark green), within all in-frame V $\gamma$ 4 sequences of the indicated populations. Data are representative of two independent experiments that gave similar results.

**Table 2 | Most frequent V $\gamma$ 4 CDR3 nucleotide sequences that encode the V $\gamma$ 4 amino-acid sequence SYGLYSSGFHKV.**

V $\gamma$ 4 (5'-3')	N	P	J $\gamma$ 1 (5'-3')	Frequency
S Y G Stop..			..S S G F H K V	
tcctacggctaag			atagctcaggtttcacaagga	Germline
tcctacggc.....		tat	atagctcaggtttcacaagga	61-62%
tcctacggc.....		ctat	atagctcaggtttcacaagga	25-27%
tcctacgg.....	t	ctat	atagctcaggtttcacaagga	3.8-5%
tcctacgg.....	gt	tat	atagctcaggtttcacaagga	1.5-2.2%
tcctacgg.....		gctat	atagctcaggtttcacaagga	0.9-1.2%
tcctacggc.....	ctt	t	atagctcaggtttcacaagga	0.6-0.7%

The germline sequences of related gene segments are demonstrated in red. N and P refer to N- and P-nucleotides, respectively.

**Table 3 | Single-cell analysis of 20 V $\gamma$ 4 chains pairing with the canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 chain.**

V $\gamma$ 4	N	P	J $\gamma$ 1 (5'-3')	AA sequence	Observed
tcctacggctaag			atagctcaggtttcacaagga	SYG..SSGFHKV	Germline
tcctacggct		tat	atagctcaggtttcacaagga	SYGLYSSGFHKV	Five times
tcctacggc.....		ctat	atagctcaggtttcacaagga	SYGLYSSGFHKV	Once
tcctacgg.....	gt	tat	atagctcaggtttcacaagga	SYGLYSSGFHKV	Once
tcctacggct...	c	at	atagctcaggtttcacaagga	SYG <sup>S</sup> YSSGFHKV	Four times
tcctacggc.....	ga	at	atagctcaggtttcacaagga	SYGEYSSGFHKV	Two times
tcctacggc.....	ac	at	atagctcaggtttcacaagga	SYGT <sup>Y</sup> SSGFHKV	Two times
tcctacggc.....	gc	at	atagctcaggtttcacaagga	SYGA <sup>Y</sup> SSGFHKV	Once
tcctacggc.....	cc	at	atagctcaggtttcacaagga	SYGP <sup>Y</sup> SSGFHKV	Once
tcctacggc.....	gtc	t	atagctcaggtttcacaagga	SYGV <sup>Y</sup> SSGFHKV	Once
tcctacggcta...	t	t	atagctcaggtttcacaagga	SYGY <sup>Y</sup> SSGFHKV	Once
tcctacggcta...			atagctcaggtttcacaagga	SYG <sub>-</sub> YSSGFHKV	Once

The germline sequences of related gene segments and the variable AA between G3 and Y5 are demonstrated in red. N and P refer to N- and P-nucleotides, respectively.

any species as well as on mouse *Trg* genes. In general, our results clearly support the view that diversity of rearranged *Trd* loci is much higher than for their *Trg* counterparts. In particular, the mouse V $\gamma$ 4 repertoire contained predominant expanded sequences that were shared between individual mice. These findings are consistent with prior deep-sequencing studies of the human *TRG* repertoire<sup>38,39</sup>, which described a limited diversity in the *TRG* repertoire. There<sup>38</sup>, the *TRG* repertoire of peripheral blood  $\gamma\delta$  T cells from three independent donors was dominated by canonical V $\gamma$ 9J $\gamma$ P sequences. In sharp contrast to the *Trg* locus, our study showed that the *Trd* repertoire was highly diverse in sorted V $\gamma$ 4<sup>+</sup> and V $\gamma$ 1<sup>+</sup> T cells from peripheral lymphoid organs. Most importantly, we revealed a novel invariant subset of innate  $\gamma\delta$  T cells, with a TCR composed of a canonical V $\gamma$ 4J $\gamma$ 1 motif paired with germline-rearranged V $\delta$ 5D $\delta$ 2J $\delta$ 1. This invariant TCR rearrangement might have evolved under selective pressure for simple and efficient modular recombination without any N- or P-nucleotides of the respective loci yielding a useful invariant TCR. Furthermore, it is conceivable that the canonical CDR3 $\delta$  loop might interact with putative TCR ligands autonomously and uncoupled from the variable CDR3 $\gamma$ . Such mode of  $\gamma\delta$  TCR-ligand interaction would be reminiscent of  $\gamma\delta$  TCR recognition of the nonclassical major histocompatibility complex class I molecules T10 and T22 (refs 40,41). While relatively short CDR3 $\delta$  chains can bind to model antigens such as phycoerythrin (PE) or Cyanine Dye Cy3 (Cy-3) haptens only in combination with a suitable CDR3 $\gamma$  partner, a relatively long CDR3 $\delta$  loop such as the canonical V $\delta$ 5D $\delta$ 2J $\delta$ 1 rearrangement (15 AA according to the international ImMunoGeneTics information system<sup>®</sup>) may be indeed be indicative of CDR3 $\gamma$ -independent ligand binding.

In addition, the novel invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T-cell population shares several decisive features with three other prominent invariant  $\gamma\delta$  T-cell subsets in mice, which are V $\gamma$ 5 DETCs, V $\gamma$ 6<sup>+</sup>V $\delta$ 1<sup>+</sup> cells and semi-invariant V $\gamma$ 1<sup>+</sup>V $\delta$ 6<sup>+</sup> NKT cells and likewise fetal liver-derived human V $\gamma$ 9<sup>+</sup>V $\delta$ 2<sup>+</sup> cells<sup>42</sup>. First, all of these populations contain straight germline rearrangements of invariant canonical TCRs without nontemplated N-nucleotides. Second, they are of fetal or at least perinatal origin. These two features, canonical germline rearrangements and exclusive development in the fetal thymus, define such  $\gamma\delta$  T-cell populations as genuine innate T cells. Invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells were remarkably abundant in every sample of V $\gamma$ 4<sup>+</sup> T cells sorted by a CCR6<sup>+</sup>CD27<sup>-</sup> surface phenotype associated with IL-17-producing capacity and basically absent in CCR6<sup>-</sup>CD27<sup>+</sup> V $\gamma$ 4<sup>+</sup> T cells. Importantly, these results were very reproducible and consistent regardless of whether independent samples were derived from genomic DNA or cDNA and whether these were analysed by either RACE or V $\delta$ 5-specific primers. Thus, the canonical V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> TCR classifies a hitherto unrecognized conserved subset of innate and presumably IL-17-producing V $\gamma$ 4<sup>+</sup> T cells. Notably, invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells are actually abundant in peripheral lymphoid organs in the same magnitude as IL-17-producing V $\gamma$ 6<sup>+</sup>V $\delta$ 1<sup>+</sup> cells<sup>33,34</sup>. In future studies, it will be interesting to compare the common and discrete functions of these two invariant  $\gamma\delta$  T-cell subsets that are likely innate 'natural' IL-17A-producers.

Interestingly, identical V $\delta$ 5D $\delta$ 2J $\delta$ 1 germline rearrangements had 24 years ago been designated as BID, for BALB/c invariant delta<sup>43</sup>. These *Trd* germline rearrangements were described as frequent in lungs and lymph nodes of mice with a BALB/c genetic background, but are absent in mice with a C57BL/6 genetic



background<sup>43</sup>. However, BID chains were later found to be generated also in C57BL/6 fetal thymocytes at levels similar to those detected in BALB/c mice<sup>44</sup>. Hence, it was concluded that the presence of BID among resident pulmonary lymphocytes of BALB/c and of BALB/c × C57BL/6 F1 mice but not in C57BL/6 mice was because of positive selection and peripheral expansion<sup>44</sup>. Yet, in our study,  $\gamma\delta$  T cells bearing BID, that is, V $\delta$ 5D $\delta$ 2J $\delta$ 1 germline rearrangements, turned out to be an abundant innate V $\gamma$ 4<sup>+</sup> T-cell population in C57BL/6 mice. It is currently unclear, however highly relevant for future work, why the C57BL/6 mice of our study and the C57BL/6 mice of the Basel Institute used as negative controls for the original BID description in 1990 would differ in this respect. It is tempting to speculate that specific genetic variations are responsible for differential selection and peripheral expansion processes. This might provide a remarkable analogy to the seminal observation that a substrain of inbred FVB mice was uniquely depleted of invariant V $\gamma$ 5<sup>+</sup> DETCs<sup>45</sup>, which led to the discovery of the DETC-selecting determinant *Skint-1* (refs 46,47). In any case, identification of potential cognate ligands of the invariant TCRs from canonical V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells, as well as V $\gamma$ 5 DETCs, V $\gamma$ 6<sup>+</sup>V $\delta$ 1<sup>+</sup> cells and semi-invariant V $\gamma$ 1<sup>+</sup>V $\delta$ 6<sup>+</sup> NKT cells, has highest priority. Formerly suggested candidates for the selection of invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells, called BID<sup>+</sup> cells, included the murine heat-shock protein 60 (ref. 48) and a potential self-ligand that correlates with the presence of the endogenous murine leukaemia virus *Mpmv-30* (ref. 49). Furthermore, it needs to be determined in future studies whether innate invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells accumulate or expand in response to inflammatory stimuli and during bacterial infection as reported for invariant V $\gamma$ 6<sup>+</sup>V $\delta$ 1<sup>+</sup> cells<sup>12,50–52</sup>.

The null hypothesis, however, is that no specific antigen is required for selection and development of canonical innate V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells. This would be consistent with the hypothesis that the capacity to produce IL-17 cytokines is prewired in a subset of fetal T-cell precursors before and is independent of TCR rearrangement<sup>11</sup>. In addition, there is considerable evidence that positively selecting TCR-triggering in immature fetal  $\gamma\delta$  T-cell precursors induces differentiation towards the potential to produce IFN- $\gamma$  while suppressing the IL-17-associated factors *Sox13*, *Sox4* and *Rorc*<sup>32,53–55</sup>. Thus, if TCR-specific selection of invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells occurred during thymic development, they would, in contrast to our observations, be rather expected to adopt a CCR6<sup>-</sup>CD27<sup>+</sup> phenotype with the potential to produce IFN- $\gamma$ .

In conclusion, we establish a novel truly innate  $\gamma\delta$  T-cell subset of invariant V $\gamma$ 4<sup>+</sup>V $\delta$ 5<sup>+</sup> T cells, which is confined to presumably IL-17-producing CCR6<sup>-</sup>CD27<sup>+</sup> T cells. Future studies addressing the specific physiological functions of these cells within the pLNs and within tissues such as the lung, skin and liver will advance the understanding of innate lymphocyte and  $\gamma\delta$  T-cell biology.

## Methods

**Mice.** All mice used throughout the study were on a C57BL/6 genetic background. C57BL/6-*TcrdH2BeGFP* mice were generated at the Centre d'Immunologie de Marseille-Luminy<sup>28</sup> and C57BL/6-*Indu-Rag1* mice were a kind gift from Siggli Weiß<sup>30</sup>. *Indu-Rag1* mice were crossed to *TcrdH2BeGFP* to obtain tamoxifen-inducible *Indu-Rag1* × *TcrdH2BeGFP* mice<sup>11</sup> at the animal facilities of the Helmholtz Centre for Infection Research, Braunschweig. C57BL/6-*TcrdH2BeGFP* and C57BL/6 wild-type control mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Hannover Medical School, and used at 8–14 weeks of age (16 weeks in case of *Indu-Rag1* × *TcrdH2BeGFP* mice) if not otherwise stated in the figure legends. Animal experiments were carried out according to the institutional guidelines approved by the local government. This study was performed in accordance with the German Animal Welfare Law and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes.

**Cell isolation and cell sorting.** pLNs and spleens were mashed, filtered through 50- $\mu$ m nylon meshes and washed with PBS/3% fetal calf serum. Spleen cells were treated with erythrocyte-lysis buffer before mixing with lymph node cells as described previously<sup>56</sup>. The liver and lung were cut into small pieces and digested with 0.5 mg ml<sup>-1</sup> Collagenase D and 0.025 mg ml<sup>-1</sup> DNase-I. The digestion was stopped by adding EDTA to a final concentration of 20 mM. For the isolation of skin lymphocytes, an area of the back was shaved, the skin was removed and cut into pieces and digested with 0.5-mg ml<sup>-1</sup> Liberase and 0.025 mg ml<sup>-1</sup> DNase-I. Digestion was carried out for 45 min at 37 °C and was stopped by adding EDTA to a final concentration of 40 mM. Digested organs were meshed through a 40- $\mu$ m Cellstrainer. Lung lymphocytes were separated using Lympholyte M. Liver and skin lymphocytes were separated with density gradient centrifugation using Percoll gradients. MAbs against TCR V $\gamma$ 4 (clone UC3-10A6, PE-conjugated, 1:200) were purchased from Biologend or produced in rat hybridoma cell lines (clone 49.2-9, Cy5-conjugated, 1:100). Antibody against TCR V $\gamma$ 1 (clone 2.11, PE-conjugated, 1:100) was purchased from Biologend and antibody against TCR V $\gamma$ 7 (clone F2.67, Cy5-conjugated, 1:50) produced in rat hybridoma cell lines. Antibodies against TCR  $\beta$  (clone H57-597, PE-Cy7-conjugated, 1:200) and CD27 (clone LG.3A10, PerCP/Cy5.5-conjugated, 1:200) were obtained from Biologend. Antibodies against CD196 (CCR6; clone 140706, Alexa Fluor 647-conjugated, 1:100) were purchased from BD Biosciences. Cell suspensions were treated with FcR block (clone 2.4G2) before 20-min staining with mAbs. Antibody-labelled cell populations were sorted for high-throughput sequencing through the FACSaria IIu flow cytometer (Becton Dickinson). In experiments designed to compare two populations, we always sorted the same amount of cells as a starting population to be able to quantify TCR diversities. Likewise, single cells were sorted into 96-well plates for single-cell PCR by MoFlo or XDP flow cytometer (Beckman-Coulter). The first rows of the plates were left empty as negative controls.

**Nucleic acid isolation.** For isolation of genomic DNA, sort-purified cell fractions were resuspended in PCR lysis buffer (10 mM Tris (pH 8.4), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, 0.5% Tween-20, 400  $\mu$ g ml<sup>-1</sup> proteinase K) at 500 cells  $\mu$ l<sup>-1</sup> and incubated overnight at 50 °C. The proteinase K was inactivated at 95 °C for 10 min. This protocol was adapted from refs 56–58. Up to 20  $\mu$ l of the DNA samples were used directly for PCR. Total RNA was isolated from sorted cell populations using the RNeasy Mini Kit (QIAGEN) and was reverse-transcribed with Superscript III (Invitrogen) using Random Primers (Invitrogen).

**RACE.** To generate unbiased template libraries of rearranged CDR3 regions of the *Trd* locus, anchor sequence-containing cDNA template was synthesized using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer. RACE PCR was performed with a gene-specific primer located in the C $\delta$  gene segment (5'-CGAATTCCACAATCTTCTTG-3') and an anchor sequence-specific primer recommended in the kit.

**Gene-specific PCR.** PCR was performed to generate amplicon libraries of rearranged TCR sequences. Gene-specific primers (V $\gamma$ 4: 5'-TGCAACCCCTAC CCATATTTTCT-3' in combination with a consensus primer amplifying J $\gamma$ 1, 2 and 3: 5'-GTTCCCTCTGCAAATACCTTGTA-3' or V $\delta$ 5: 5'-AGCAGCAAGCC CAACAGAACCTT-3' in combination with J $\delta$ 1: 5'-TTGGTTCACAGTCACT TGGGTCC-3'). Either genomic DNA or cDNA were amplified using 0.375  $\mu$ M of each forward and reverse primers, 50  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub> and 2.5 U *Taq* DNA Polymerase (Invitrogen) in a 50- $\mu$ l reaction. Amplifications were performed at 94 °C for 4 min, followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 59 °C and 20 s at 72 °C, and finally a single incubation at 72 °C for 5 min.

**High-throughput sequencing.** Forward and reverse PCR primers for deep-sequencing contained at their 5' ends the respective 454 universal adaptor sequences and multiplex identifier (MID) nucleotides. PCR products were purified using gel extraction with the QIAquick Gel Extraction kit (QIAGEN) and was quantified with Qubit fluorometer (Invitrogen) using the Quant-iT dsDNA HS Assay kit (Invitrogen). Amplicons were processed with the emPCR—Lib-A SV kit (GS FLX Titanium series; Roche) according to the manufacturer to sequence on 454 Genome Sequencer FLX system (Roche). Productive rearrangements and CDR3 $\alpha$  regions were defined by comparing nucleotide sequences to the reference sequences from IMGt, the international ImMunoGeneTics information system (<http://www.imgt.org>)<sup>59</sup>. Rearrangements were analysed and CDR3 $\alpha$  regions were defined using IMGt/HighV-QUEST<sup>60</sup>.

**Single-cell PCR.** For single-cell PCR, CCR6<sup>+</sup>CD27<sup>-</sup> V $\gamma$ 4<sup>+</sup> single cells were sorted directly into 96 wells. We reverse-transcribed RNA from each sort-purified cell to cDNA, and used it as a template to amplify corresponding  $\gamma$  and  $\delta$  TCR chains. Single cells sorted in 6- $\mu$ l PBS were immediately frozen on dry ice and transferred to -80 °C. Frozen cells were lysed by heating to 65 °C for 2 min and were cooled to 4 °C. RNA transcription and a first round of PCR were sequentially performed in one reaction using the OneStep RT-PCR kit (QIAGEN) according to the manufacturer with some modifications. A combination of specific 5'- primer pair for V $\gamma$ 4 chain (V $\gamma$ 4 outer: 5'-ASCAAGAGATGAGACTGCACAAAT-3' in

combination with J $\gamma$ 1, 2 and 3: 5'-GTTCTCTGCAAATACCTTGTGA-3') and V $\delta$ 5 chain (V $\delta$ 5 outer: 5'-TGCGGATTCCTCCAAACCCAGATTTA-3' in combination with J $\delta$ 1: 5'-TTGGTTCCACAGTCACCTTGGGTTCC-3') was used for this multiplex reaction. For a 25- $\mu$ l reaction, the components were as following: 1  $\times$  buffer, 400  $\mu$ M of each dNTP, 0.4  $\mu$ M of each primer and 1- $\mu$ l OneStep RT-PCR Enzyme mix. Reverse transcription was performed for 30 min at 50  $^{\circ}$ C and was directly followed by amplification. PCR activation was initiated at 95  $^{\circ}$ C for 15 min, before 30 cycles, consisting of 30 s at 94  $^{\circ}$ C, 30 s at 65  $^{\circ}$ C and 60 s at 72  $^{\circ}$ C, and finally a single incubation at 72  $^{\circ}$ C for 10 min. Next, second rounds of PCR with seminested primers (V $\gamma$ 4: 5'-TGCAACCCCTACCCATATTTTCT-3' and V $\delta$ 5 inner: 5'-TAGGGACGACACTAGTCCCATGAT-3') were separately performed for V $\gamma$ 4 and V $\delta$ 5 chains. For this, 0.2  $\mu$ l from the first PCR products were used as template in a 20- $\mu$ l reaction. The PCR fragments were amplified using 0.5 unit AmpliTaq Gold DNA polymerase (Applied Biosystems) in combination with GeneAmp 1  $\times$  PCR puffer II, 2 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP and 0.3  $\mu$ M of each primer. After 10 cycles, samples positive for V $\gamma$ 4 and V $\delta$ 5 were detected using agarose gel electrophoresis. After additional 20 cycles of PCR, gel-extracted (QIAGEN) PCR products of V $\gamma$ 4 and V $\delta$ 5 chains from each cell were separately cloned in pCR4-TOPO vector through the TOPO TA Cloning Kit for sequencing (Invitrogen). These plasmid vectors were isolated via the QIAprep kit (QIAGEN) and sequenced by GATC Biotech (Germany).

**Sequence analysis.** First, *fna* files generated by 454 high-throughput sequencing were converted and partitioned into separate FASTA files using MIDs and gene-specific primer sequence identifiers. Next, these files were uploaded to HighV-QUEST, an online tool available on the IMGT website<sup>59</sup>, and compared with the IMGT data base. Analysed txt files returned from IMGT were further processed with Excel to segregate productive and unproductive TCR rearrangements to quantify and merge similar sequences and for further statistical analysis. Via specifically selecting only in-frame productive TCR rearrangements, sequences that contained insertions and deletions from the 454 platform were routinely excluded. Shannon indices were calculated using Vegan R package (2.14.0). Venn diagrams were produced with VennMaster (0.37.5; ref. 61). All raw sequence data were uploaded to the NCBI Sequence Read Archive under the SRP accession number SRP050364.

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## Author contributions

E.K., L.F., S.R. and I.P. designed the studies, performed the experiments and analysed the data. E.K., L.F., I.S. and L.O. performed cellular and molecular experiments. E.K. and L.F. performed bioinformatics analyses. S.S. contributed to deep-sequence experiments. S.W. provided vital reagents. S.W., C.K. and S.S. contributed to study design. I.P. and E.K. co-wrote the manuscript.

## Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>.

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