

# Human Vδ2 T cells are a major source of interleukin-9

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Vδ2Vγ9 T cells are the dominant γδ T-cell subset in human peripheral blood. Vδ2 T cells recognize pyrophosphate molecules derived from microbes or tumor cells; hence, they play a role in antimicrobial and antitumor immunity. TGF-β, together with IL-15, induces a regulatory phenotype in Vδ2 T cells, characterized by forkhead box protein P3 (FoxP3) expression and suppressive activity on CD4 T-cell activation. We performed a genome-wide transcriptome analysis and found that the same conditions (TGF-β plus IL-15) strongly enhanced the expression of additional genes in Vδ2 T cells, including IKAROS family zinc finger 4 (*IKZF4*; *Eos*), integrin subunit alpha E (*ITGAE*; CD103/αEβ7), and *IL9*. This up-regulation was associated with potent IL-9 production as revealed by flow cytometry and multiplex analysis of cell culture supernatants. In contrast to CD4 and CD8 αβ T cells, γδ T cells did not require IL-4 for induction of intracellular IL-9 expression. Upon antigen restimulation of Vδ2 T cells expanded in vitro in the presence of TGF-β and IL-15, IL-9 was the most abundant among 16 analyzed cytokines and chemokines. IL-9 is a pleiotropic cytokine involved in various (patho) physiological conditions, including allergy and tumor defense, where it can promote antitumor immunity. Given the conspicuous sensitivity of many different tumors to Vδ2 T-cell-mediated killing, the conditions defined here for strong induction of IL-9 might be relevant for the development of Vδ2 T-cell-based immunotherapy.

γδ T cells | human | interleukin-9 | transforming growth factor-β

Although less well characterized than other functional T helper-cell subsets, T helper 9 (Th9) cells have been identified on the basis of their selective IL-9 production. IL-9 is a pleiotropic cytokine that promotes T-cell and mast-cell growth and mast-cell accumulation in tissue as well as IgE switching in B cells (1). In line, allergic patients have increased numbers of T cells that produce IL-9 in response to various allergens (2, 3). Moreover, Th9 cells alter intestinal epithelial cell functions (4, 5) and are key players in antiworm immunity (6). Intriguingly, Th9 cells also appear to regulate tumor immunity. Although IL-9 and Th9 cells exerted antitumor activity in some solid tumor models (7, 8), IL-9 actually promoted lymphoma development in other models (9). IL-9 is thus an important cytokine with multiple functions in the regulation of immune responses.

Similar to other functional T-cell subsets, the differentiation of Th9 cells is driven by the cytokine milieu and specific transcription factors. In general, IL-4, together with TGF-β, was found to polarize IL-9-secreting CD4 T cells (10–12), and PU.1 and interferon regulatory factor 4 (IRF4) were identified as crucially important transcription factors (11, 13). The signal strength of T-cell receptor (TCR) ligation and costimulatory signals also regulate IL-9 at the transcriptional level (14).

The γδ T cells expressing Vδ2 paired with Vγ9 (hereafter termed Vδ2 T cells) dominate in the peripheral blood of healthy adult individuals (15). Vδ2 T cells recognize via their TCR, in a CD277/butyrophilin 3A-dependent manner, microbial pyrophosphates and homologous eukaryotic pyrophosphates (isopentenyl pyrophosphate), which are produced by many tumor cells due to their dysregulated mevalonate pathway (16). Hence, Vδ2 T cells play a role in both antiinfective and antitumor immunity (17, 18). Interestingly, Vδ2 T cells exert a surprisingly large functional plasticity (17). In addition to their strong cytolytic activity (19),

Vδ2 T cells produce different cytokines, can be converted into forkhead box protein P3 (FoxP3)<sup>+</sup> regulatory cells (20, 21), and may acquire professional antigen-presenting capacity (22). Depending on priming conditions, Vδ2 T cells can secrete IFN-γ, IL-4, IL-17, and IL-22, and thus recapitulate cytokine patterns of well-defined functional Th subsets (23, 24).

Here, we identified peripheral blood Vδ2 T cells as a major source of IL-9. In the presence of TGF-β and IL-15 but the absence of IL-4, high levels of IL-9 were secreted already 4 d after initial in vitro activation. Upon antigen restimulation of Vδ2 T cells precultured for 15 d in the presence of TGF-β/IL-15, IL-9 was by far the most abundant among 16 analyzed cytokines and chemokines. Our results might help to enhance selective effector functions of human γδ T cells.

## Results

To investigate the impact of differential activation on the gene expression profile and the transcription factor and cytokine/chemokine pattern of Vδ2 T cells, the following four conditions were used for in vitro culture (note: exogenous IL-2 was always present). Purified total γδ T cells were stimulated with the Vδ2-specific phosphoantigen bromohydrin pyrophosphate (BrHPP) in the presence of irradiated peripheral blood mononuclear cells (PBMCs) and in the absence (*i*) or presence (*iii*) of TGF-β/IL-15. Alternatively, purified Vδ2 T cells were stimulated with microbeads coupled with anti-CD2/CD3/CD28 antibodies [activation/expander (A/E) beads], again in the absence (*ii*) or presence (*iv*) of TGF-β/IL-15 (21).

**Gene Expression of Differentially Activated Vδ2 T Cells.** In the presence of TGF-β, the proliferative activity of freshly isolated γδ T cells as measured by <sup>3</sup>H-radiolabeled thymidine incorporation was reduced (Fig. S1A). Interestingly, however, the

## Significance

We describe in vitro cell culture conditions that induce strong secretion of IL-9 in human peripheral blood γδ T cells. IL-9 plays a role in allergy and increases the antitumor immunity of conventional CD4 and CD8 T cells. Human γδ T cells with a Vδ2 T-cell receptor kill many different tumor cells because they recognize intermediates of a metabolic pathway that is frequently dysregulated in cancer cells. Vδ2 T cells have already been used in cancer immunotherapy, as yet with limited success. Our study demonstrates that TGF-β, together with IL-15, strongly enhances IL-9 production in Vδ2 T cells. We postulate that IL-9-producing Vδ2 T cells might have enhanced therapeutic efficacy upon adoptive transfer into patients who have cancer.

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Data deposition: The data reported in this paper have been submitted to the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE85482).

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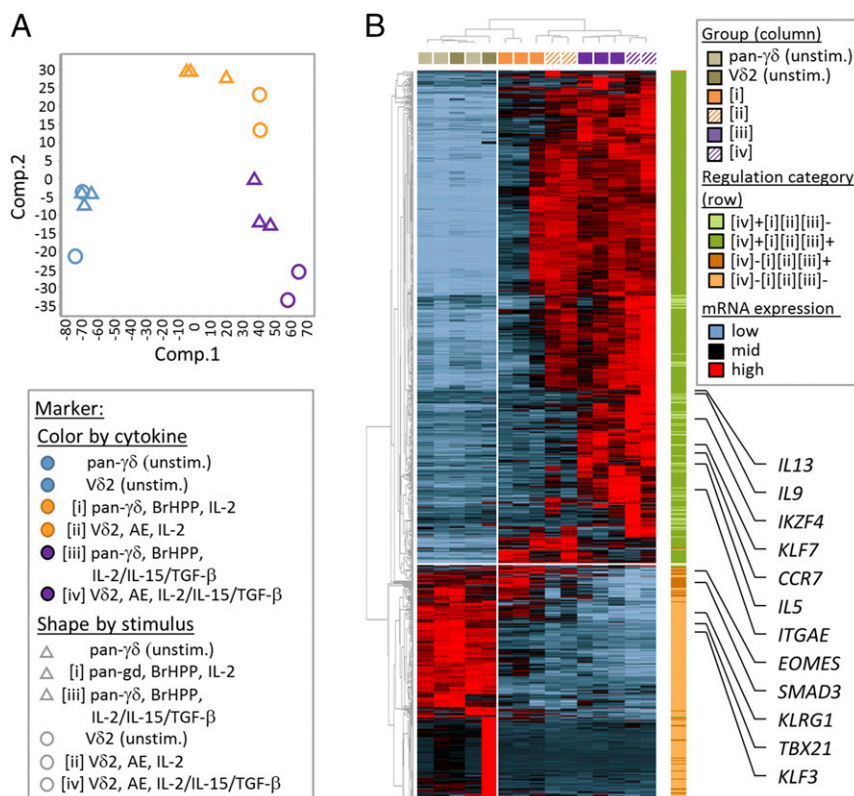
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proliferative expansion of Vδ2 T cells that had been cultured for 15 d under conditions (i) to (iv) and restimulated with BrHPP was much higher if TGF-β and IL-15 had been present during initial activation [i.e., conditions (iii) and (iv)] (Fig. S1B). We then analyzed gene expression by means of an Affymetrix whole-genome array in γδ T cells cultured for 8 d under conditions (i) to (iv). The principal component analysis depicted in Fig. 1A indicates that the overall gene expression in Vδ2 T cells cultured with TGF-β/IL-15 clustered differently from those Vδ2 T cells cultured without TGF-β/IL-15. Moreover, BrHPP stimulation [i.e., conditions (i) and (iii)] clearly differed from A/E bead stimulation [i.e., conditions (ii) and (iv)]. The heat map based on hierarchical clustering of 1,056 genes found to be regulated at least 1.5-fold in condition (iv) compared with unstimulated Vδ2 T cells is shown in Fig. 1B. Selected genes with profound modulation are identified in Fig. 1B. The median fold change of these genes induced under conditions (i) to (iv) is listed in Table S1. *IL9* was among the most strongly up-regulated genes in TGF-β/IL-15-supplemented conditions (iii) and (iv). All genes listed in Table S1 were strongly modulated (up- or down-regulated) by TGF-β/IL-15 and also were more strongly regulated by A/E bead stimulation compared with BrHPP. A Spearman's rank coefficient analysis of x-fold gene regulation revealed the highest discrepancy ( $r_s = 0.78$ ) between conditions (i) (i.e., BrHPP-activated, IL-2 only) and (iv) (i.e., A/E bead-activated with TGF-β/IL-15), which differed with regard to both the stimulus and the cytokine milieu (Fig. S24). This high discrepancy is visualized in the biplot analysis, because many genes up-regulated in condition (iv) were not up-regulated in condition (i) (e.g., *IL9*), and vice versa [e.g., Eomesodermin homolog (*Eomes*)] (Fig. S2B).

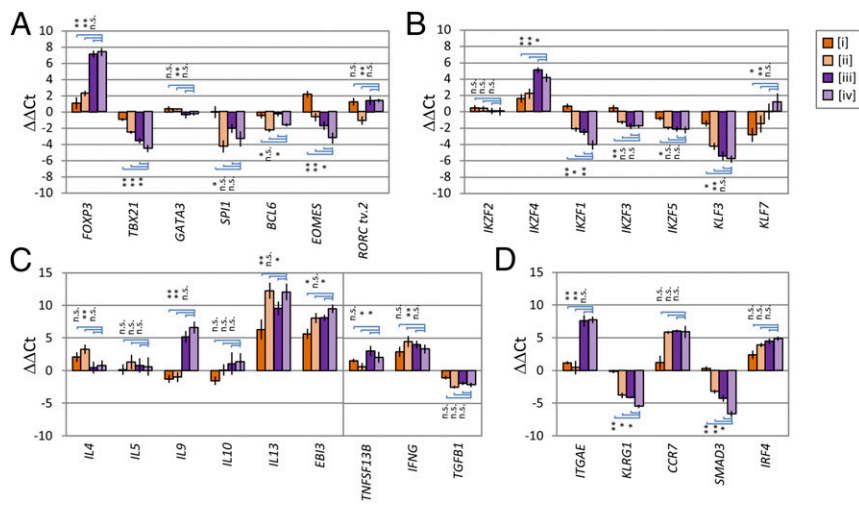
**Analysis of Gene Expression by Quantitative RT-PCR.** The regulation of 28 genes, including genes of interest from the transcriptome analysis and further lineage-specific transcription factors, cytokines, and surface receptors, was quantified by quantitative RT-PCR

in Vδ2 T cells cultured for 8 d (Fig. 2) or 15 d (Fig. S3) under conditions (i) to (iv). Among the genes specifically up-regulated in the presence of TGF-β/IL-15 were *FOXP3* (Fig. 2A and Fig. S34), IKAROS family zinc finger 4 (*IKZF4*; Eos) (Fig. 2B and Fig. S3B), *IL9* (Fig. 2C), TNF superfamily member 13b (*TNFSF13B*; B cell activating factor of the TNF family, BAFF) (Fig. S3C), integrin subunit alpha E (*ITGAE*; CD103) (Fig. 2D and Fig. S3D), and *IRF4* (Fig. S3D). Interestingly, *IL9* was reduced on day 15 compared with day 8 (Fig. 2C and Fig. S3C). Other cytokine genes, including *IL13*, Epstein-Barr virus induced 3 (*EBI3*), and interferon, gamma (*IFNG*) (Fig. 2C and Fig. S3C), were up-regulated under all conditions, although *IL13* and *EBI3* were more strongly up-regulated in response to A/E bead stimulation. Kruppel-like factor 7 (*KLF7*) was up-regulated only under condition (iv) (Fig. 2B and Fig. S3B). Among the genes that were generally down-regulated were T-box 21 (*TBX21*; T-bet), *EOMES*, *SPI1* (PU.1) (Fig. 2A and Fig. S34), *IKZF1* (Ikaro), *KLF3* (Fig. 2B and Fig. S3B), *TGFB1* (TGF-β1) (Fig. 2C and Fig. S3C), killer cell lectin-like receptor G1 (*KLRG1*), and SMAD family member 3 (*SMAD3*) (Fig. 2D and Fig. S3D). Several of these genes [e.g., *SPI1*, *IKZF1*, *KLRG1*, C-C motif chemokine receptor 7 (*CCR7*)] were more potently regulated by A/E bead stimulation compared with BrHPP stimulation, pointing to an impact of the A/E bead-mediated CD28 costimulation and/or differential TCR signal strength. The down-regulation of *EOMES* was mainly observed in TGF-β/IL-15-supplemented cultures, whereas A/E bead stimulation and TGF-β/IL-15 had an additive effect on the down-regulation of *SMAD3*, *KLF3*, and *TBX21*.

**Cytokine Production of Differentially Activated Vδ2 T Cells.** Next, we analyzed the secretion of a broad panel of cytokines and chemokines in cell culture supernatants of differentially activated Vδ2 T cells by bead-based multiplex analysis. After 4 d of initial stimulation of purified γδ T cells, large amounts (>2,000 pg/mL) of IL-9 and TNF-α were detected, as well as lower concentrations (<750 pg/mL) of other cytokines and chemokines [IL-5, IL-6,



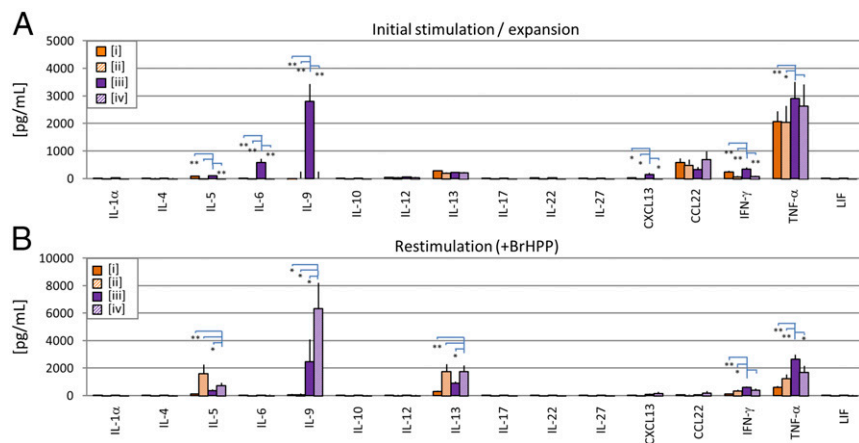
**Fig. 1.** Transcriptome analysis in differentially expanded Vδ2 T cells. Purified γδ T cells from three healthy donors were differentially stimulated as indicated. Gene expression was determined after 8 d by an Affymetrix HuGene 1.0 st v1 array analysis. (A) Principal component (Comp.) analysis (PCA) of overall gene expression in differentially expanded Vδ2 T cells. (B) Hierarchical clustering [unweighted pair group method with arithmetic mean/correlation] of the 1,056 selected genes, whose median expression was at least 1.5-fold modulated in A/E bead- and TGF-β/IL-15-expanded Vδ2 T cells [condition (iv)] compared with the corresponding freshly isolated cells, and which had a difference of modulation of at least 1.5-fold (compared with all other expansion conditions). Genes of interest are highlighted by displaying their gene symbol. For better comparability between transcripts, data were z-score-normalized before clustering. unstim., unstimulated.



**Fig. 2.** Quantification of gene expression by RT-PCR (day 8). Vδ2 T cells were activated under condition (i) BrHPP + IL-2, (ii) A/E + IL-2, (iii) BrHPP + IL-2low + IL-15 + TGF-β, or (iv) A/E + IL-2low + IL-15 + TGF-β. RT-PCR was performed after 8 d of culture. The differences between the cycle threshold values ( $\Delta C_t$ ) of the gene of interest and three different housekeeping genes (*G6PDH*, *HuPo*, and *Rpl1*) were determined.  $\Delta\Delta C_t$  values represent the difference between  $\Delta C_t$  values of freshly isolated and differentially stimulated  $\gamma\delta$  T cells at d 8. The three  $\Delta\Delta C_t$  values per gene of interest were averaged. Depicted are the mean values  $\pm$  SEM of various experiments [*FOXP3* ( $n = 7$ ), *TBX21* ( $n = 9$ ), *GATA3* ( $n = 8$ ), *SPI1* ( $n = 6$ ), *BCL6* ( $n = 5$ ), *EOMES* ( $n = 5$ ), and *RORC tv.2* ( $n = 5$ )] (A); [*IKZF2* ( $n = 9$ ), *IKZF4* ( $n = 9$ ), *IKZF1* ( $n = 7$ ), *IKZF3* ( $n = 7$ ), *IKZF5* ( $n = 7$ ), *KLF3* ( $n = 7$ ), and *KLF7* ( $n = 4$ )] (B); [*IL4* ( $n = 9$ ), *IL9* ( $n = 10$ ), *IL10* ( $n = 8$ ), *IL13* ( $n = 9$ ), *EBI3* ( $n = 5$ ), *TNFSF13B* ( $n = 5$ ), *IFNG* ( $n = 7$ ), and *TGFβ1* ( $n = 6$ )] (C); and [*ITGAE* ( $n = 5$ ), *KLRG1* ( $n = 5$ ), *CCR7* ( $n = 3$ ), *SMAD3* ( $n = 3$ ), and *IRF4* ( $n = 3$ )] (D).

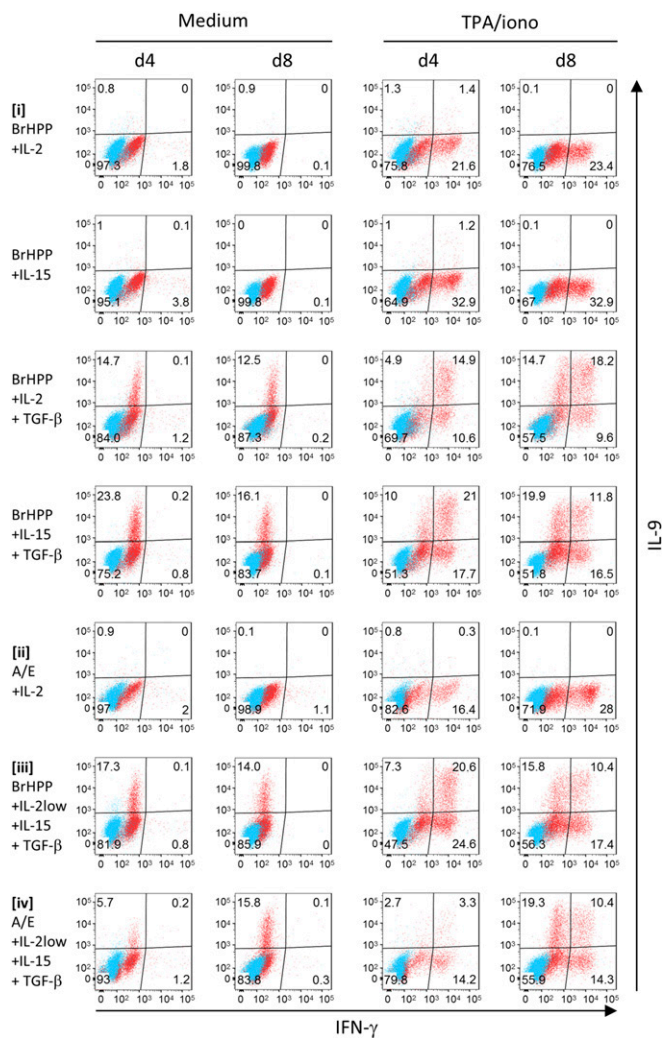
IL-13, IFN- $\gamma$ , C-C motif chemokine 22 (CCL22), and C-X-C motif chemokine 13 (CXCL13)] (Fig. 3A). Importantly, whereas TNF- $\alpha$  was induced by both BrHPP and A/E beads also in the absence of TGF- $\beta$ /IL-15 [i.e., under conditions (i) and (ii)], the high level of IL-9 secretion (as well as the much lower secretion of IL-6) was selectively induced during initial stimulation in Vδ2 T cells activated with BrHPP and TGF- $\beta$ /IL-15 [i.e., under condition (iii)] (Fig. 3A). The absence of IL-9 secretion on day 4 in Vδ2 T cells activated with A/E beads and TGF- $\beta$ /IL-15 was surprising, given that the *IL9* gene was similarly up-regulated after 8 d [condition (iv)] (Fig. 2C). Therefore, we also analyzed cytokine production at later time points. Vδ2 T cells activated and cultured for 15 d under conditions (i) to (iv) were washed and restimulated with BrHPP. Cytokines were measured in cell culture supernatants after an additional 4 d. Even larger amounts (4,000–6,000 pg/mL) of IL-9 compared with the initial activation were detected in culture supernatants of Vδ2 T cells that had been initially cultured in the presence of TGF- $\beta$ /IL-15 [(iii) and (iv)]. Interestingly, comparing both cell cultures expanded in the presence of TGF- $\beta$ /IL-15, the initially A/E bead-stimulated Vδ2 T cells [condition (iv)] secreted even higher amounts of IL-9 after BrHPP restimulation, compared with the initially BrHPP-stimulated Vδ2 T cells [condition (iii)] (Fig. 3B). In addition to IL-9, BrHPP-restimulated Vδ2 T cells secreted significant amounts (up to 2,500 pg/mL) of IL-5, IL-13, IFN- $\gamma$ , and TNF- $\alpha$ , whereas the strongest IL-5 and IL-13 secretion was observed in Vδ2 T cells [conditions (ii) and (iv)] that were initially A/E bead-stimulated (Fig. 3B). In line with cytokine measurements in cell culture

supernatants, intracellular flow cytometry of IL-9 and IFN- $\gamma$  expression in Vδ2 T cells after 4 and 8 d of initial activation (without further restimulation) revealed only a few IFN- $\gamma$ -positive Vδ2 T cells but strong IL-9 expression whenever TGF- $\beta$  was present (Fig. 4, Left, Medium). To dissect the role of IL-15 versus TGF- $\beta$  in the induction of IL-9, cultures were supplemented with TGF- $\beta$  or IL-15 only (Fig. 4, Upper). We found that TGF- $\beta$ , but not IL-15, was essential for IL-9 induction, but that IL-15 increased the overall proliferation rate. Importantly, we noted significant differences in the intracellular cytokine expression pattern when  $\gamma\delta$  T cells were activated for 6 h with 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin before the cytokine staining (Fig. 4, Right), which revealed the TGF- $\beta$ -dependent appearance of IL-9 single-positive, IL-9/IFN- $\gamma$  double-positive, and IFN- $\gamma$  single-positive Vδ2 T cells. Under these conditions, IL-9 single-positive Vδ2 T cells were hardly detectable on day 4 but were more abundant on day 8 (Fig. 4, Right). Vδ2 T cells expanded for 15 d under conditions (i) to (iv) and rested afterward for 16 h in the presence of low-dose IL-2 [10 international units (IU)], showed an IL-9 and IFN- $\gamma$  expression pattern after TPA/ionomycin activation similar to day 4. Even though the *IL9* mRNA levels were no longer up-regulated on day 15 (Fig. S3C), IL-9 was produced quickly after TPA/ionomycin activation. Again, IL-9 production was only detected in Vδ2 T cells initially cultured in the presence of TGF- $\beta$ /IL-15 [conditions (iii) and (iv)]. In addition to IL-9, the production of IFN- $\gamma$  and TNF- $\alpha$  was enhanced in these cells (Fig. S4A). IL-9 and TNF- $\alpha$  were most abundant in Vδ2 T cells initially stimulated under condition (iv). Slight IL-13 production was



**Fig. 3.** Quantification of cytokines and chemokines in cell culture supernatants. Vδ2 T cells were activated under condition (i) BrHPP + IL-2, (ii) A/E + IL-2, (iii) BrHPP + IL-2low + IL-15 + TGF- $\beta$ , or (iv) A/E + IL-2low + IL-15 + TGF- $\beta$  (color-coded as indicated). Concentrations of cytokines and chemokines in cell-free supernatants were quantified by a multiplex bead array. (A) Cytokine secretion in  $\gamma\delta$  T-cell cultures after 4 d of initial stimulation. (B) Cytokine secretion in 15-d activated Vδ2 T cells, restimulated for 4 d with BrHPP. Shown are the mean values with the SEM of six (IL-1 $\alpha$ , IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-22, IL-27, CXCL13, IFN- $\gamma$ , and TNF- $\alpha$ , LIF) or four (IL-12 and CCL22) independent experiments. Statistical analysis was performed using the Student's *t* test for paired data. Asterisks refer to significant differences in  $\gamma\delta$  T cells ( $*P \leq 0.05$ ;  $**P \leq 0.01$ ).





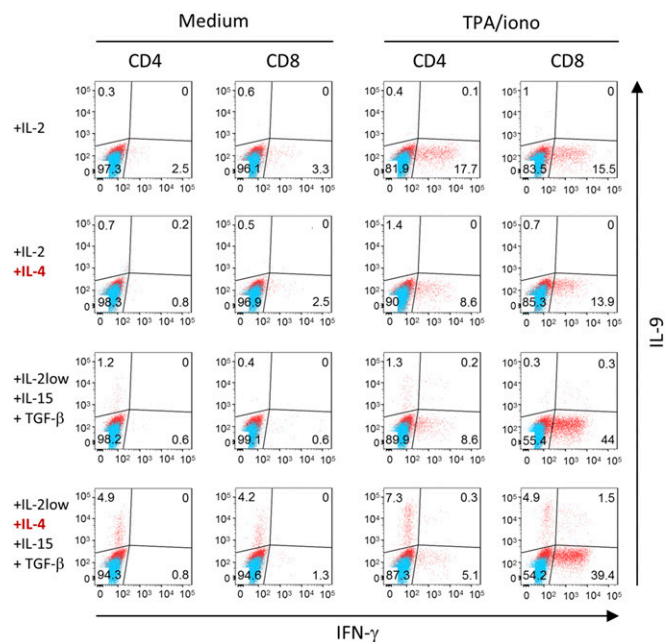
**Fig. 4.** Detection of intracellular IL-9 and IFN- $\gamma$  during primary activation of V $\delta$ 2 T cells. V $\delta$ 2 T cells were activated under conditions (i) to (iv) and under additional conditions (TGF- $\beta$  and IL-15, separately) as indicated. Intracellular IL-9 and IFN- $\gamma$  staining was performed at the indicated time points without (Left, Medium) or with 6 h of TPA/ionomycin activation (Right, TPA/iono). Monensin was added at a concentration of 3  $\mu$ M 4 h before fixation. Results of one of three independent experiments are shown. The dot plot of the specifically labeled cells is colored red, and the respective isotype control is colored blue.

detected in the initially A/E bead-stimulated V $\delta$ 2 T-cell lines (ii) and (iv) (Fig. S4B). Intracellular expression of the cytotoxic mediators Perforin and Granzyme B was not differentially modulated under the various stimulation conditions (Fig. S4B). When the 15-d V $\delta$ 2 T-cell lines (iii) and (iv) were restimulated with BrHPP and cultured for an additional 4 d in the presence of IL-2 only, IL-9 production was still maintained (Fig. S4C).

**Requirements for IL-9 Production by CD4 and CD8 T Cells.** To compare the induction of IL-9 in V $\delta$ 2 T cells with CD4 and CD8 T cells, we activated purified CD4 and CD8 T cells with immobilized anti-CD3 and soluble anti-CD28 antibodies in the presence of IL-2 and absence of the presence of TGF- $\beta$ /IL-15 and IL-4 (12). Comparable to V $\delta$ 2 T cells, no intracellular IL-9 was detected in the absence of TGF- $\beta$  (Fig. 5). In contrast to V $\delta$ 2 T cells, however, very few CD4 T cells and no CD8 T cells stained positive for IL-9 in the presence of only TGF- $\beta$ /IL-15. Only in the additional presence of IL-4 was IL-9 expression detectable in CD4 and CD8 T cells (Fig. 5, Bottom). Short-term activation with TPA/ionomycin stimulated expression of

IFN- $\gamma$ , which was strongly enhanced only in CD8 T cells in the presence of TGF- $\beta$ /IL-15; however, unlike the case in V $\delta$ 2 T cells, no IL-9/IFN- $\gamma$  double-positive cells were detected (Fig. 5, Right). Analysis of the corresponding supernatants from day 8 cell cultures also revealed some IL-9 secretion in TGF- $\beta$ /IL-15-supplemented CD4 T cells (but less compared with V $\delta$ 2 T cells). In the additional presence of IL-4, IL-9 production was induced in CD8 T cells and was enhanced in CD4 T cells, respectively (Fig. S5).

**Profile of Differentially Activated V $\delta$ 2 T Cells.** Next, we attempted to correlate the gene expression profile of the differentially activated V $\delta$ 2 T cells with surface marker and transcription factor expression. A/E bead stimulation resulted in a decrease of CD27-positive but an increase of CD45RA-positive V $\delta$ 2 T cells. The presence of TGF- $\beta$ /IL-15 [conditions (iii) and (iv)] also led to fewer CD27-positive cells, but strongly induced CD103. Slight KLRG1 expression was only detected in V $\delta$ 2 T cells activated by BrHPP in the presence of IL-2 and absence of TGF- $\beta$ /IL-15 (i.e., condition (i)) (Fig. S6). The kinetics of intracellular expression of lineage-specific transcription factors was also analyzed by flow cytometry (Fig. S7). Induction of GATA-3 was stronger in V $\delta$ 2 T cells initially activated with A/E beads [i.e., conditions (ii) and (iv)]. On the other hand, induction of FoxP3 was only detected in V $\delta$ 2 T cells activated in the presence of TGF- $\beta$ /IL-15 [i.e., conditions (iii) and (iv)], and was strongest on day 8 when BrHPP was used for the initial activation [condition (iii)]. The levels of PU.1 were also slightly higher in V $\delta$ 2 T cells activated in the presence of TGF- $\beta$ /IL-15 but decreased during the 15-d culture period. T-bet expression was highest in BrHPP-only stimulated cells [condition (i)], whereas it was lowest whenever TGF- $\beta$ /IL-15 was present [conditions (iii) and (iv)]. B-cell lymphoma 6 protein (Bcl-6) was not detected at significant levels in any of the differentially activated V $\delta$ 2 T cells.



**Fig. 5.** Detection of intracellular IL-9 and IFN- $\gamma$  in differentially activated CD4 and CD8 T cells. CD4 and CD8 T cells were stimulated by immobilized anti-CD3 and soluble anti-CD28 in the presence of IL-2, IL-2low, IL-4, IL-15, and TGF- $\beta$  as indicated. Intracellular IL-9 and IFN- $\gamma$  staining was performed 8 d after initial activation without (Left, Medium) or with 6 h of TPA/ionomycin activation (Right, TPA/iono). Monensin was added at a concentration of 3  $\mu$ M 4 h before fixation. Results of one of four independent experiments are shown. The dot plot of the specifically labeled cells is colored red, and the respective isotype control is colored blue.

## Discussion

Activation by anti-CD3/CD28 antibodies versus cognate antigen recognition differentially regulates gene transcription in CD4 T cells (14) and results in differential TCR signaling kinetics also in  $\gamma\delta$  T cells (25). This study adds a further facet to the pattern of V $\delta$ 2 T-cell gene expression, which has been described as being intermediate between  $\alpha\beta$  T cells and natural killer cells (26). The results of our present investigation extend these previous studies by demonstrating that gene expression is differentially regulated in human V $\delta$ 2 T cells by cognate TCR antigen BrHPP and A/E beads, and TGF- $\beta$ /IL-15 cytokines provide an additional layer of regulation. This additive effect is exemplified by the lowest Spearman's rank coefficient between V $\delta$ 2 T cells activated by BrHPP, IL-2 [i.e., condition (i)] and A/E, TGF- $\beta$ /IL-15 [i.e., condition (iv)]. The characteristic gene expression profiles resulting from the differential stimulation of conditions (i) to (iv) correlated with the expression of characteristic surface markers. In this regard, the expression of KLRG1, which coincided with higher expression of the transcriptional repressor KLF3 (27) in BrHPP/IL-2-activated V $\delta$ 2 T cells [condition (i)], might indicate differentiation toward a more exhausted phenotype. On the other hand, V $\delta$ 2 T cells preactivated in the presence of TGF- $\beta$  displayed a high proliferative potential upon restimulation, which was accompanied by a loss of KLRG1 and strongly induced CD103 surface expression. This observation is in line with previous findings for murine and human CD8 T cells (28). The TGF- $\beta$ /IL-15-dependent decrease of CD27 on V $\delta$ 2 T cells and the resulting shift toward an effector memory phenotype might implicate enhanced effector potential.

Various cell types have been identified as a cellular source of IL-9. The best characterized is the Th9 subset of CD4 T cells, but IL-9 production has also been found in CD8 T cells (29), innate lymphoid cells (30), and mast cells (31). So far, there is only one report of IL-9 production by  $\gamma\delta$  T cells in *Schistosoma japonicum*-infected mice (32). It is commonly accepted that TGF- $\beta$  and IL-4 are essential requirements for the induction of IL-9 in CD4 T cells in vitro (11, 12). We observed very strong IL-9 induction at the transcriptional and protein levels in human V $\delta$ 2 T cells upon in vitro activation in the presence of TGF- $\beta$ /IL-15, but an absence of exogenous IL-4. Together with our failure to detect IL-4 in any of the V $\delta$ 2 T-cell cultures, we conclude that IL-4 is not required for the strong induction of IL-9 in human V $\delta$ 2 T cells. Early studies reported that induction of IL-9 in murine CD4 T cells by TGF- $\beta$  can also occur in the absence of IL-4, but is augmented by additional IL-4 (10). In accordance, we found TGF- $\beta$ -dependent basal IL-9 production in supernatants of human CD4 T cells, which was further enhanced by IL-4, whereas the intracellular IL-9 expression on day 8 was minimal in the absence of IL-4. Therefore, the initial IL-9 production by CD4 T cells in the absence of exogenous IL-4 might be independent of IL-4 or conditioned by initial endogenous IL-4 secretion. IL-9 production in CD8 T cells always required the presence of both IL-4 and TGF- $\beta$ . Moreover, intracellular flow cytometry of V $\delta$ 2 T cells expanded in the presence of TGF- $\beta$ /IL-15 revealed that these cells mainly produced IL-9 but also had the potential to produce IFN- $\gamma$  at the same time when activated by TPA/ionomycin. This observation is striking, because IFN- $\gamma$  was found to counteract the IL-9 production in naive murine CD4 T cells (10). In line with this observation, but in contrast to our results with V $\delta$ 2 T cells, no coexpression of IL-9 and IFN- $\gamma$  was found in CD4 or CD8 T cells. We conclude that IL-9 production in V $\delta$ 2 T cells is differentially regulated compared with  $\alpha\beta$  T cells, with no requirement for IL-4 for V $\delta$ 2 T cells.

When V $\delta$ 2 T cells cultured for 15 d in the presence of TGF- $\beta$ /IL-15 were restimulated with BrHPP and were cultured for an additional 4 d, even higher levels of IL-9 were detected in cell culture supernatants than after initial stimulation. After restimulation, more IL-9 was produced by initially A/E bead-stimulated [condition (iv)] compared with initially BrHPP-stimulated [condition (iii)] V $\delta$ 2 T cells, suggesting that initial CD28 costimulation by A/E beads might induce V $\delta$ 2 T cells with a more stable IL-9-producing phenotype. Interestingly, no IL-4 or IL-10 secretion was measured in any of the culture

supernatants, whereas Th2 cytokines IL-5 and IL-13 as well as TNF- $\alpha$  and some IFN- $\gamma$  were detected. The production of IL-5 and IL-13 was not specific to TGF- $\beta$ /IL-15 conditions, but resulted from the initial CD28 costimulation (by A/E beads), in line with a previous report (33).

A multitude of signaling molecules are involved in the regulation of IL9 gene expression, of which IRF4 and PU.1 have been identified as key transcription factors (11, 13). In accordance, IRF4 and basic leucine zipper ATF-like transcription factor 3 (BATF3) gene expression was enhanced in the presence of TGF- $\beta$ /IL-15 in our study. Even though we observed the down-regulation of SPI1 (PU.1) expression by RT-PCR on days 8 and 15 after initial activation, this result does not exclude a role for PU.1 in driving IL-9 expression in V $\delta$ 2 T cells, because we found a slight increase of PU.1 on protein level in the presence of TGF- $\beta$ /IL-15. Nonetheless, TGF- $\beta$ /IL-15 had a more significant negative impact on TBX21 (T-bet) and EOMES gene expression and T-bet protein expression, which were both further down-regulated in the presence of CD28 costimulation (A/E beads). Such a negative effect of TGF- $\beta$  on T-bet (34) might foster Th9 differentiation, because it also has been shown that T-bet counteracts the development of IL-9-secreting cells (35). Moreover, in response to TGF- $\beta$ /IL-15, V $\delta$ 2 T cells up-regulated Eos and, in line with published data (23, 24), FoxP3.

The (patho)physiological significance of IL-9 producing V $\delta$ 2 T cells is currently unknown, but the strongly enhanced CD103 (ITGAE) surface expression might point to a role in the mucosal or epithelial environment. Increased proportions of IL-5- and IL-13-producing  $\gamma\delta$  T cells are present in the bronchoalveolar lavage of patients with asthma (36). Accordingly, TGF- $\beta$ , which is produced in response to allergen challenge (37), might drive the differentiation of local V $\delta$ 2 T cells into IL-9 producers. Furthermore, we found IL-9 production in V $\delta$ 2 T cells accompanied by elevated levels of CXCL13 and TNFSF13B (BAFF). CD4 Th9 cells can coexpress TNFSF13B (38), and CXCL13 can be induced in response to TGF- $\beta$  (39). Both mediators might be relevant for the interaction with B cells. Because IL-9 promotes IgE production in B cells (40), V $\delta$ 2 T cells might thus contribute to the enhanced IgE production (e.g., by coproducing CXCL13, TNFSF13B, and IL-9) in asthma.

A second scenario where IL-9-producing  $\gamma\delta$  T cells might be highly relevant is tumor defense. Th9 cells themselves can exert enhanced antitumor activity (41), but IL-9 might also act indirectly via mast cells (8) or recruitment of dendritic cells to the tumor site (7). Significant numbers of  $\gamma\delta$  T cells are present among tumor-infiltrating lymphocytes (TILs) in many cancer types (42), and, in fact, the proportion of  $\gamma\delta$  T cells among TILs is the best predictive parameter across 25 human tumor entities (43). Many tumors secrete TGF- $\beta$ , which could induce IL-9 production in tumor-infiltrating  $\gamma\delta$  T cells. In murine models, IL-9-producing CD8 cytotoxic T lymphocytes are superior effector cells in adoptive cancer immunotherapy, due to their extended lifespan (44, 45). Human V $\delta$ 2 T cells have raised great attention as effector cells in immunotherapy because of their broad reactivity toward many different tumors (46). Adoptive transfer of in vitro expanded V $\delta$ 2 T cells has already been performed, with promising results in some studies (47). We hypothesize that induction of potent IL-9 production upon in vitro expansion in the presence of TGF- $\beta$ /IL-15 might also greatly improve the therapeutic efficacy of adoptively transferred V $\delta$ 2 T cells.

## Materials and Methods

**Blood Donors.** Leukocyte concentrates from 30 healthy adult donors (25 male and 5 female) with a mean age of 34.6 y were provided by the Institute of Transfusion Medicine, University Hospital Schleswig-Holstein. Informed consent was obtained from all blood donors. This study was approved by the Ethics Committee of Kiel University Medical Faculty (D402/14).

**Cell Culture.** Pan- $\gamma\delta$  T cells and V $\delta$ 2 T cells were positively isolated and CD4 and CD8 T cells were negatively isolated by magnetic sorting (Miltenyi Biotec). Cell culture was performed in serum-free X-VIVO 15 medium (Lonza) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. A total of 40 × 10<sup>3</sup> pan- $\gamma\delta$  T cells were stimulated in 96-well, round-bottomed plates in the presence of 50 × 10<sup>3</sup> irradiated (40 Gy) PBMC



feeder cells with 300 nmol/L BrHPP (kindly provided by Innate Pharma). A total of  $40 \times 10^3$  V $\delta$ 2 T cells were stimulated with A/E beads (Miltenyi Biotec), and CD4 and CD8 T cells were stimulated by plate-bound anti-CD3 and soluble anti-CD28, all in the absence of additional feeder cells. Cell cultures were supplemented with 50 IU/mL IL-2 or 10 IU/mL IL-2 (referred to as IL-2low) (Novartis), IL-15 (10 ng/mL), and TGF- $\beta$ 1 (1.7 ng/mL) (R&D Systems/Biotechne) as indicated. Where indicated, CD4 and CD8 T-cell cultures were additionally supplemented with 20 ng/mL IL-4. Fresh cytokines were also added on days 4 and 12, and on day 8, the cells were transferred to a 24-well plate into fresh medium supplemented with the respective cytokines. Additional information is provided in *SI Materials and Methods*.

**Flow Cytometry.** Antibodies and techniques used for cell surface and intracellular staining are listed in *SI Materials and Methods*.

**Measurement of Cytokine Secretion.** Cell culture supernatants were collected at day 8, and up to 16 analytes were measured using a Magnetic Luminex Screening Assay (R&D Systems/Biotechne) (additional information is provided in *SI Materials and Methods*).

**mRNA Expression Analysis.** RNA was isolated using the RNeasy Mini-Kit (Qiagen). Total RNA was hybridized to an Affymetrix Human Gene 1.0 st v1 Array according to the manufacturer's guidelines. Raw data were normalized

using RMA (R; Bioconductor). Expression of selected genes was validated by quantitative RT-PCR. Experimental details and a list of used primers are provided in *SI Materials and Methods*.

**Statistical Analysis.** For statistical analysis, Microsoft Excel 2007 and GraphPad Prism version 5.0 software were used.

**Supporting Material.** Table S1 lists the x-fold change in gene expression of 12 genes in V $\delta$ 2 T cells activated under conditions (i) to (iv). Table S2 describes the PCR primers. Fig. S1 shows the modulation of V $\delta$ 2 T-cell expansion by TGF- $\beta$ . Fig. S2 compares the gene regulation patterns of differentially expanded V $\delta$ 2 T cells. Fig. S3 depicts the gene expression measured by RT-PCR on day 15. Fig. S4 shows different intracellular mediators analyzed in expanded V $\delta$ 2 T cells. Fig. S5 shows the concentration of IL-9 detected in the supernatants of activated CD4 and CD8 T cells. Fig. S6 illustrates surface marker expression on differentially activated V $\delta$ 2 T cells. Fig. S7 shows the kinetics of transcription factor expression in differentially activated V $\delta$ 2 T cells.

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