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Regulatory Role of V γ 1 $\gamma\delta$ T Cells in Tumor Immunity through IL-4 Production

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It has been demonstrated that the two main subsets of peripheral $\gamma\delta$ T cells, V γ 1 and V γ 4, have divergent functions in many diseases models. Recently, we reported that V γ 4 $\gamma\delta$ T cells played a protective role in tumor immunity through eomesodermin-controlled mechanisms. However, the precise roles of V γ 1 $\gamma\delta$ T cells in tumor immunity, especially whether V γ 1 $\gamma\delta$ T cells have any interaction with V γ 4 $\gamma\delta$ T cells, remain unknown. We demonstrated in this paper that V γ 1 $\gamma\delta$ T cells suppressed V γ 4 $\gamma\delta$ T cell-mediated antitumor function both in vitro and in vivo, and this suppression was cell contact independent. Using neutralizing anti-IL-4 Ab or IL-4^{-/-} mice, we determined the suppressive factor derived from V γ 1 $\gamma\delta$ T cells was IL-4. Indeed, treatment of V γ 4 $\gamma\delta$ T cells with rIL-4 significantly reduced expression levels of NKG2D, perforin, and IFN- γ . Finally, V γ 1 $\gamma\delta$ T cells produced more IL-4 and expressed significantly higher level of GATA-3 upon Th2 priming in comparison with V γ 4 $\gamma\delta$ T cells. Therefore, to our knowledge, our results established for the first time a negative regulatory role of V γ 1 $\gamma\delta$ T cells in V γ 4 $\gamma\delta$ T cell-mediated antitumor immunity through cell contact-independent and IL-4-mediated mechanisms. Selective depletion of this suppressive subset of $\gamma\delta$ T cells may be beneficial for tumor immune therapy. *The Journal of Immunology*, 2011, 187: 4979–4986.

$\gamma\delta$ T cells have many unique features and functions (1–6). Differing from those of $\alpha\beta$ T cells, these T cells have a phenotype of spontaneous activation and release large quantities of cytokines upon activation (2, 6, 7). On the basis of these features, $\gamma\delta$ T cells are also called “innate-like lymphocytes” and can bridge the innate and adaptive immune responses (3, 6). Our earlier studies have demonstrated that $\gamma\delta$ T cells preferentially produce IFN- γ upon activation and the controlling mechanisms are quite different in comparison with those of CD4⁺ T cells (8–10). Besides many other functions, we and others (11–14) have also established that $\gamma\delta$ T cells play a critical role in tumor immunity through providing the early source of IFN- γ .

More and more evidence has been accumulated that TCR-defined subsets of $\gamma\delta$ T cells have unique functions (4, 6, 15). Most of these studies have been focused on periphery V γ 1 and V γ 4 $\gamma\delta$ T cells (16–18). It has been well documented that these

two subsets of $\gamma\delta$ T cells have divergent functions in many diseases models (4, 15). Interestingly, very often V γ 1 and V γ 4 $\gamma\delta$ T cells have opposite effects. For example, in coxsackievirus B3 infection and allergic airway hyperresponsiveness (AHR), V γ 1 $\gamma\delta$ T cells promote CD4⁺ Th2 responses, whereas V γ 4 $\gamma\delta$ T cells help CD4⁺ Th1 responses (19–24). The distinct roles of these two subsets of $\gamma\delta$ T cells have also been demonstrated in autoimmune disease models and infection immunity (25–27). Our recent studies have demonstrated a critical role of V γ 4 $\gamma\delta$ T cells in tumor immunity through eomesodermin-controlled, IFN- γ - and perforin-dependent mechanisms (28). However, the role of V γ 1 $\gamma\delta$ T cells, especially whether V γ 1 $\gamma\delta$ T cells can directly interact with V γ 4 $\gamma\delta$ T cells in tumor immunity, remains unknown.

Tumor microenvironments are composed of cancer cells, non-cancer cells, soluble factors and extracellular matrix, and cytokines are the critical soluble factors to influence antitumor immunity (29). Among these cytokines, IL-4 has been shown to have both protective as well as suppressive effect of antitumor immune responses, depending on the source of this cytokine and also the time of its production (30). Recently, it has been reported that IL-4 can downregulate the NKG2D expression on human CD8⁺ T as well as mouse NK cells, resulting in suppression of antitumor immune responses (31, 32). Although it has been determined that V γ 1 $\gamma\delta$ T cells are a major source of IL-4 (23, 33–37), the role of V γ 1 $\gamma\delta$ T cell-derived IL-4 in tumor immunity has not been investigated.

In this paper, we demonstrated a negatively regulatory role of V γ 1 $\gamma\delta$ T cells in V γ 4 $\gamma\delta$ T cell-mediated antitumor immune response. Furthermore, we determined that this regulatory effect of V γ 1 $\gamma\delta$ T cells was cell contact independent and IL-4 dependent.

Materials and Methods

Mice

C57BL/6J (B6 wild-type [Wt]), B6.129P2-Tcrd^{tm1Mom}/J (B6 TCR δ ^{-/-}), and B6.129P2-Tcrb^{tm1Mom}/J (B6 TCR β ^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P2-Il4^{tm1Cgn}/J (B6 IL-

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Abbreviations used in this article: AHR, airway hyperresponsiveness; rm, recombinant mouse; Wt, wild-type.

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$\delta^{-/-}$ mice were provided by Dr. B. Sun (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). All animals were maintained under specific pathogen-free conditions and used at 6–8 wk of age.

Reagents

Recombinant mouse (rm)IL-2 and IL-4 were purchased from R&D Systems (Minneapolis, MN). Purified IFN- γ mAb (clone XMG1.2), purified IL-4 mAb (clone 11B11), FITC-conjugated anti-mouse CD4 (clone RM4-5), and PE-conjugated anti-mouse $\gamma\delta$ TCR (clone GL3) were purchased from Tianjin Sungene (Tianjin, China). PE-conjugated anti-mouse perforin (clone OMAK-D), allophycocyanin-conjugated anti-mouse NKG2D (clone CX5), and Alexa Fluor 647-conjugated anti-mouse/human GATA-3 (clone TWAJ) were from eBioscience (San Diego, CA). Anti-mouse CD3 mAb (clone 145-2C11), anti-mouse CD28 mAb (clone PV1), hamster anti-mouse TCR V γ 1 mAb (clone 2.11), and hamster anti-mouse TCR V γ 4 mAb (clone UC3) were from Tianjin Sungene. PE-conjugated anti-mouse CD124 (IL-4R α) (clone mL4R-M1), and GolgiPlug were purchased from BD Biosciences (San Jose CA). PE-conjugated anti-mouse IFN- γ (clone XMG1.2) was from BioLegend (San Diego, CA).

V γ 1 and V γ 4 $\gamma\delta$ T cell inactivation in vivo

For inactivation of V γ 1 or V γ 4 $\gamma\delta$ T cells, B6 Wt mice were treated with i.v. injection of anti-V γ 1 TCR (clone 2.11, 200 μ g/mouse) or anti-V γ 4 TCR (clone UC3, 200 μ g/mouse) mAb on days -5 and -1 before inoculation of tumor cells. Inactivation was confirmed by analysis of TCR expression (the percentage of V γ 1 or V γ 4 $\gamma\delta$ T cells by FACS from peripheral blood cells).

Tumor models

B16-F0 melanoma cells were injected s.c., and tumor growth was monitored and recorded daily for over 3 wk, as described previously (28).

Cell preparation and activation

For expansion of V γ 1 and V γ 4 $\gamma\delta$ T cells in vitro, splenic $\gamma\delta$ T cells were sorted and expanded with anti-V γ 1 or anti-V γ 4 Abs as described previously (28). For polarization of T cells, cells were cultured with plate-coated anti-CD3 (10 μ g/ml) and soluble anti-CD28 (1 μ g/ml) and IL-2 (2 ng/ml) in neutral or Th2 (IL-4 and anti-IFN- γ) priming conditions for 2 d as described previously (8).

Coculture of tumor cells and $\gamma\delta$ T cells

Tumor cells (7500 cells/well) were mixed with either V γ 1 or V γ 4 cells or both in a total volume of 200 μ l in a 96-well flat bottom plate. The viable tumor cells were counted using trypan blue as described previously (28). For separation of V γ 1 and V γ 4 $\gamma\delta$ T cells, a 96-well Transwell with 0.4- μ m pore size was used (Corning, NY). B16 cells (7500 cells/well) and 1.5×10^5 of V γ 1 or V γ 4 $\gamma\delta$ T cells were placed in the lower chamber, with medium or B16 (7500 cells) plus V γ 1 (1.5×10^5 cells) mixture in the upper chamber. Cells were cultured for 24 h, and tumor cells in the lower chambers were counted as described previously.

Immunofluorescence histology

B16 tumor cells (2×10^5 /mouse) were s.c. injected to B6 Wt mice, and on day 4 postinoculation, tumors were excised, washed with PBS, and fixed in 4% paraformaldehyde in PBS at 4°C for 2 h. Frozen tumors were cut at 7- μ m thickness, and sections were stained with biotin-anti-V γ 1 and FITC-anti-V γ 4 (20 μ g/ml) at 4°C for 24 h. Slides were then washed and incubated with streptavidin-PE (2 μ g/ml) (eBioscience) and Alexa Fluor 488-conjugated anti-fluorescein polyclonal Ab (10 μ g/ml) (Invitrogen, San Diego, CA) at 37°C for 1 h. Finally, slides were incubated with DAPI and mounted with Fluorescent Mounting Medium (Zhongshan Goldenbridge Biotechnology, Beijing, China). Tissue sections were then examined by a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), and images were analyzed by LAS AF software (Leica Microsystems). Tumor samples without primary Ab treatment and TCR $\delta^{-/-}$ mice tumor and spleen tissues were used as negative controls. Spleen tissues of TCR $\beta^{-/-}$ mice were used as positive control, because these mice have enriched $\gamma\delta$ T cells (38).

Intracellular staining

Intracellular IFN- γ , perforin, and GATA-3 were stained using the Foxp3 Staining Buffer Set (eBioscience).

Flow cytometry

For FACS analysis, BD FACSCalibur was used. For FACS sorting, BD FACSAria was used. The software was CellQuest Pro and FACSDiva, respectively. All the machines and software were from BD Biosciences.

ELISA

Expanded V γ 1 or V γ 4 cells (1×10^6 cells/ml) were restimulated with plate-coated anti-CD3, soluble anti-CD28, and IL-2 for 12 h, supernatants were collected for ELISA. Mouse IFN- γ and IL-4 ELISA kits were purchased from BioLegend (San Diego, CA), and ELISA was performed according to the manufacturer's protocol.

Real-time PCR for gene transcription

Total RNA was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed by PrimeScript RT reagent (TaKaRa, Shiga, Japan). SYBR Premix Ex Taq (TaKaRa) and a Mastercycler ep Realplex (Eppendorf, Hamburg, Germany) were used for quantitative PCR. Cycling conditions were 30 s at 95°C, followed by 55 repeats of 95°C for 10 s and 60°C for 30 s. Hypoxanthine-guanine phosphoribosyltransferase was used as internal normalizing gene. Mouse genes were amplified by the following primers: IFN- γ , 5'-CAC AGT CAT TGA AAG CCT AGA-3' (forward) and 5'-TTG CCA GTT CCT CCA GAT AT-3' (reverse); perforin 5'-CAC AGT AGA GTG TCG CAT GTA C-3' (forward) and 5'-GGA GAT GAG CCT GTG GTA AG-3' (reverse); and hypoxanthine-guanine phosphoribosyltransferase, 5'-TCA TTA TGC CGA GGA TTT G-3' (forward) and 5'-GCC TCC CAT CTC CTT CAT-3' (reverse). Data analysis was performed using Eppendorf Realplex software.

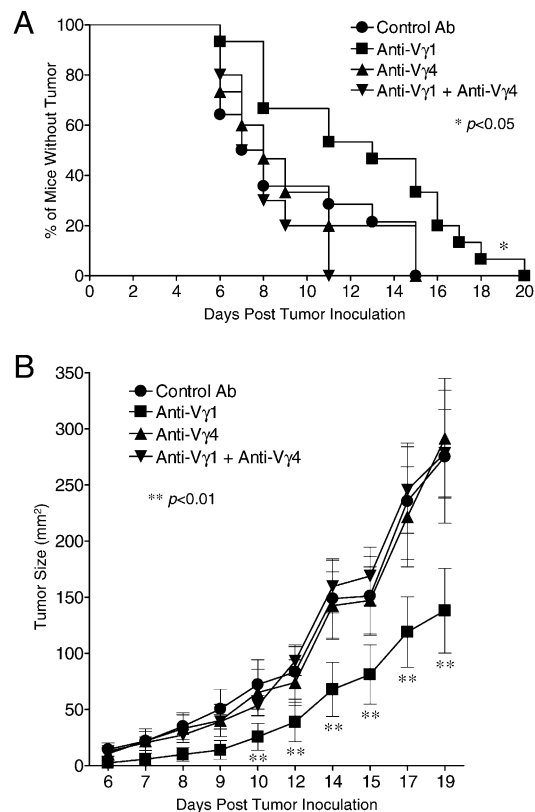


FIGURE 1. V γ 1 $\gamma\delta$ T cells suppress V γ 4 $\gamma\delta$ T cell-mediated antitumor immunity in vivo. Sex- and age-matched B6 Wt mice were i.v. injected with V γ 1-specific Ab 2.11 ($n = 15$), V γ 4-specific Ab UC3 ($n = 15$), or both ($n = 10$), and isotype control Ab ($n = 14$) at days -5 and -1, followed by s.c. inoculation of B16 tumor cells (2×10^5 cells/mouse) at day 0. Tumor growth was observed and recorded daily. Tumor size $> 4 \times 4$ mm² was considered positive. The rate of mice without tumor from one representative experiment is shown (A). The tumor size (mean \pm SD) as shown above is shown (B). Data represents one of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Statistics

Statistical significance was evaluated by two-tailed unpaired Student *t* test using InStat version 3.06 software for Windows (GraphPad, San Diego, CA). The incidence of tumor development was compared and analyzed using the log-rank test, performed by GraphPad Prism 4 for Windows (GraphPad). Throughout the text, figures, and legends, the following terminology is used to show statistical significance: **p* < 0.05, ***p* < 0.01.

Results

Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor immunity in vivo

Our previous study has demonstrated a protective role of Vγ4 γδ T cells in tumor immunity (28). To define the role of Vγ1 γδ T cells in this process, sex- and age-matched B6 Wt mice were treated with either Vγ1-inactivating Ab (2.11) or Vγ4-inactivating Ab (UC3) or both as well as the isotype control Ab (*n* = 10~15 per each group) followed by s.c. inoculation with B16-F0 tumor cells (2×10^5 /mouse). Tumor growth was monitored and recorded daily. Inactivation of Vγ1 γδ T cells resulted in significantly delay of tumor growth with significantly smaller size in comparison with those in Wt mice injected with control Ab (Fig. 1A, 1B), suggesting a negative regulatory role of Vγ1 γδ T cells in tumor immunity. Interestingly, inactivation of either Vγ4 alone or both Vγ1 and Vγ4 did not show significant impact on tumor growth (Fig. 1A, 1B), indicating a direct interaction between Vγ1 and Vγ4 γδ T cells.

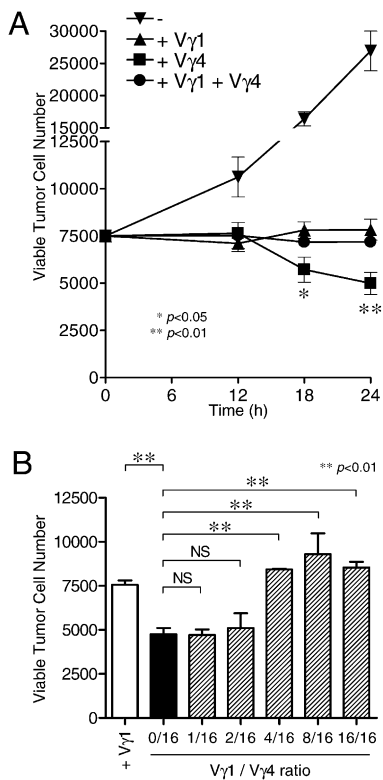


FIGURE 2. Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor immunity in vitro. *A*, B16 cells (7500 cells/well) were cocultured with 20-fold the number of Vγ1, Vγ4, or Vγ1+Vγ4 cells. At different time points postcoculture, viable tumor cells were counted and recorded. One representative of three independent experiments is shown. **p* < 0.05, ***p* < 0.01. *B*, Different number of Vγ1 γδ T cells were added into B16 cell–Vγ4 cell cocultures (Vγ4:B16 ratio = 20:1) as described above. Viable tumor cells were counted at 24 h postcoculture. The viable tumor cell number (mean ± SD) is shown. Data represent three independent experiments. ***p* < 0.01.

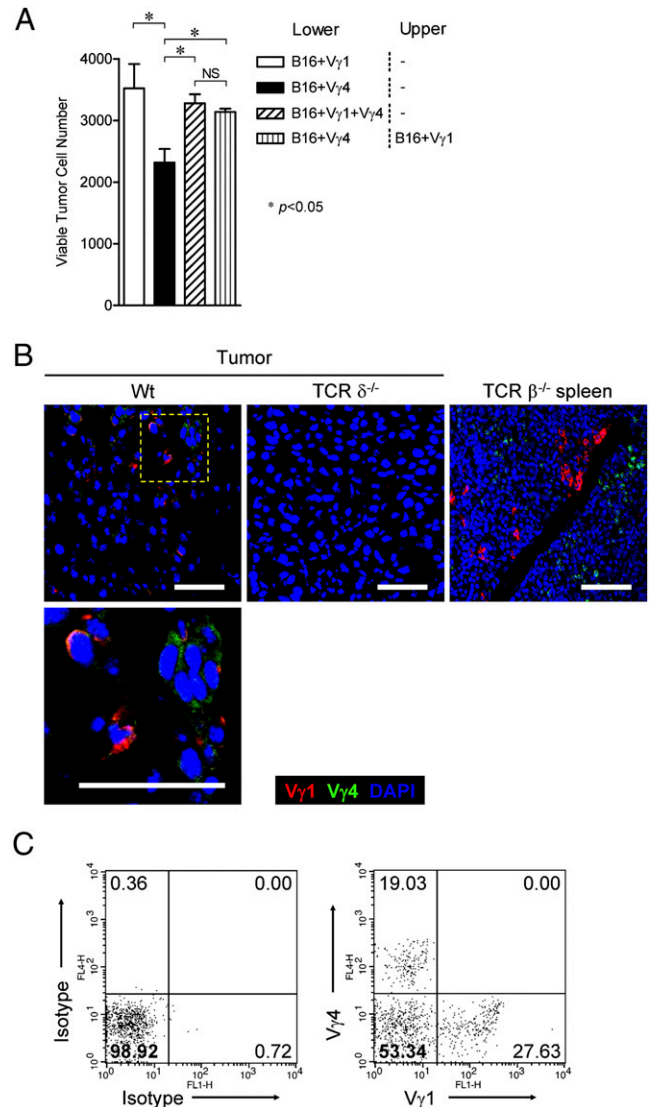


FIGURE 3. Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor effect through cell contact-independent mechanism. *A*, Vγ4 γδ T cell–B16 cocultures were set up in the lower chamber of Transwell plate (1.5×10^5 Vγ4 γδ T cells and 7500 B16 tumor cells), and Vγ1 γδ T cells (1.5×10^5 cells) were either added into the cocultures (cell contact) or in the upper chamber (without contact). The numbers of viable tumor cells in the lower chamber were counted at 24 h postculture. Data represent one of three repeated experiments. **p* < 0.05. *B*, B6 Wt mice were inoculated with B16 tumor cells (2×10^5 cells/mouse) as described previously, and at day 4 posttumor injection, tumor tissues were frozen for immunofluorescence staining of Vγ1, Vγ4 TCRs. Red represents Vγ1 TCR, green represents Vγ4 TCR, and blue represents cell nuclei stained by DAPI. One representative staining out of 15 tumor tissues is shown (left panel). Tumor tissues from TCR δ^{-/-} mice (negative control, middle panel) and spleen tissues of TCR β^{-/-} mice (positive control, right panel) were also stained with the same condition, and one representative staining is shown. The high power of the region as marked from the tumor tissues of Wt mice is shown (lower panel) (each scale bar, 50 μm). *C*, Single-cell suspensions were made from pooled tumor tissues and stained with PE–anti-γδ TCR, Alexa Fluor 488–anti-Vγ1, and Alexa Fluor 647–anti-Vγ4 (right panel). Alexa Fluor 488- and Alexa Fluor 647-isotype control Abs were also used (left panel). Both dot plots were gated on γδ TCR⁺ cells. One example of three repeatable experiments is shown.

Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated tumor suppression in vitro

To further investigate whether Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor effect, an in vitro γδ T cell–B16 cell

coculture system was adapted as described previously (28). Consistent with previous findings (28), V γ 4 $\gamma\delta$ T cells showed suppression effect on tumor cell growth with the E:T ratio of 20/1 (Fig. 2A). The suppression effect of V γ 4 $\gamma\delta$ T cells was significantly abolished by the addition of V γ 1 $\gamma\delta$ T cells (Fig. 2A). To test whether the suppression effect of V γ 1 $\gamma\delta$ T cells on V γ 4 $\gamma\delta$ T cells is dose dependent, different ratios of V γ 1/V γ 4 $\gamma\delta$ T cells were cocultured with B16 tumor cells and the viable tumor cells were counted and recorded. Interestingly, only when the V γ 1:V γ 4 ratio reached to 1/4, V γ 1 $\gamma\delta$ T cells significantly neutralized the suppressive effect of V γ 4 $\gamma\delta$ T cells against tumor cell growth (Fig. 2B), indicating that the suppression of V γ 1 on V γ 4 $\gamma\delta$ T cells was dose dependent.

The suppression of V γ 1 on V γ 4 $\gamma\delta$ T cells is cell contact independent

On the basis of our observation that V γ 1 suppressed the antitumor effect of V γ 4 $\gamma\delta$ T cells both in vitro and in vivo, next, we tested whether this suppression effect was cell contact dependent. The coculture assay as described above was set up in a Transwell plate. Similarly, coculture of V γ 4-B16 cells significantly reduced the

viable tumor cell numbers as described above (Fig. 3A). Interestingly, even in a Transwell plate, V γ 1 $\gamma\delta$ T cells still inhibited the tumor suppression effect of V γ 4 $\gamma\delta$ T cells (Fig. 3A), indicating a cell contact-independent mechanism being used by V γ 1 $\gamma\delta$ T cells to suppress the antitumor effect of V γ 4 $\gamma\delta$ T cells.

To visualize the tumor-infiltrating V γ 1 and V γ 4 $\gamma\delta$ T cells, tumors were used for frozen section and stained with anti-V γ 1 or V γ 4 Abs conjugated with different fluorophores. From the sections, V γ 1 and V γ 4 $\gamma\delta$ T cells were found to be distributed in different areas inside the tumor tissue (Fig. 3B), confirming a cell contact-independent communication between these two subsets of $\gamma\delta$ T cells. To further confirm the suppressive effect of V γ 1 $\gamma\delta$ T cells, we also found that more V γ 1 $\gamma\delta$ T cells were infiltrated into the tumor tissues than those of V γ 4 $\gamma\delta$ T cells through three-color FACS analysis (Fig. 3C). These results collectively defined a suppressive effect of V γ 1 $\gamma\delta$ T cells in tumor immunity.

V γ 1 $\gamma\delta$ T cells suppress V γ 4 $\gamma\delta$ T cell-mediated tumor inhibition through IL-4 production

It has been reported that V γ 1 $\gamma\delta$ T cells are a major source of IL-4 in thymus and periphery (23, 33–37). Our previous report also

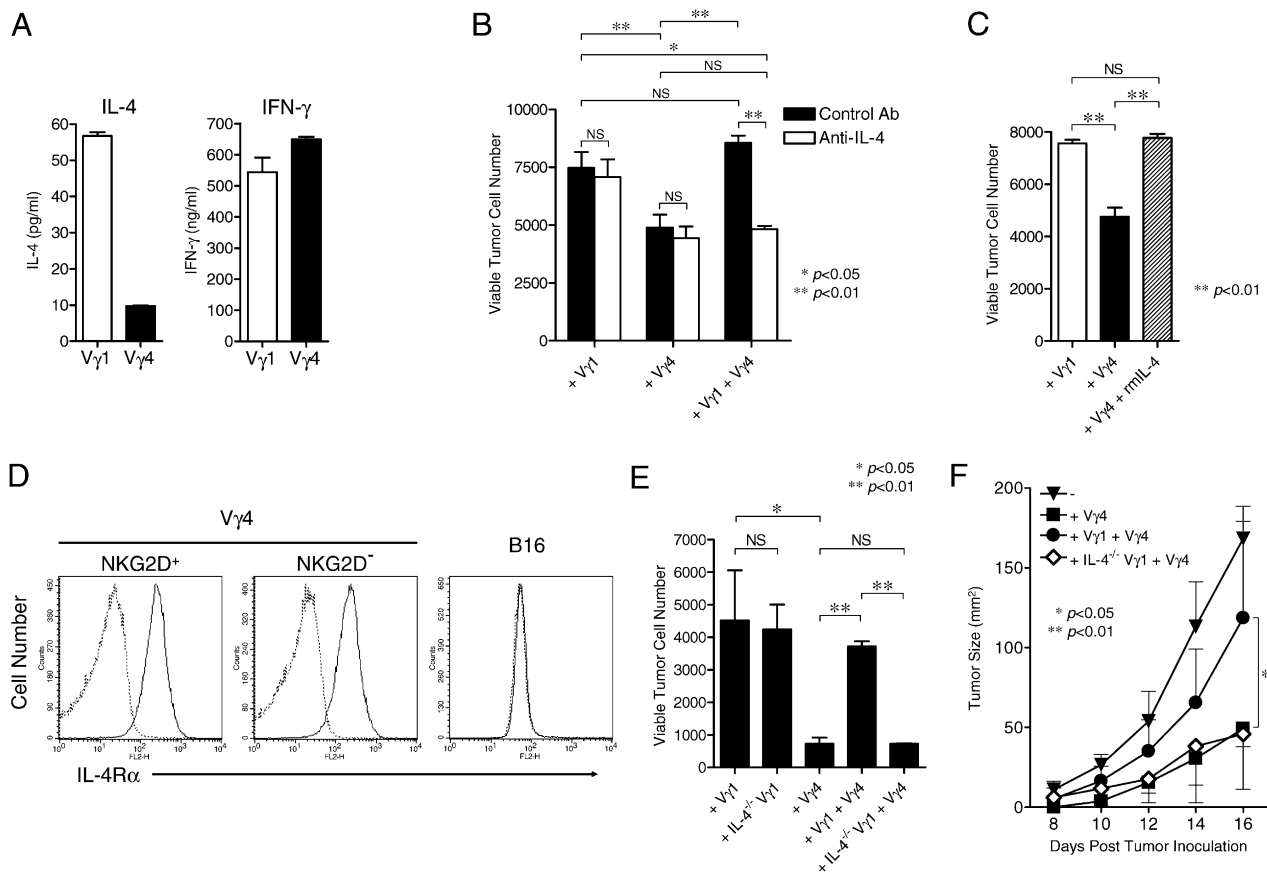
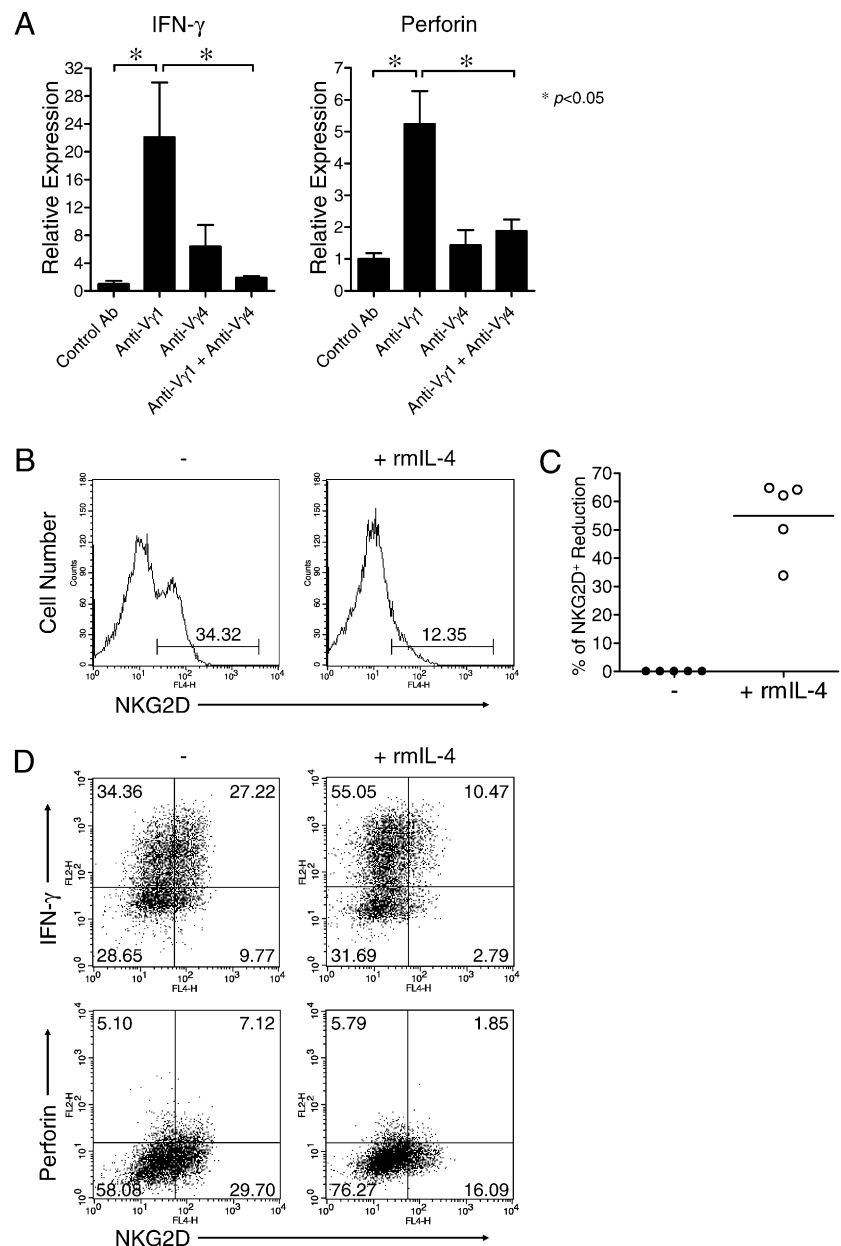


FIGURE 4. V γ 1 $\gamma\delta$ T cells suppress V γ 4 $\gamma\delta$ T cell antitumor function through IL-4 production. *A*, V γ 1 and V γ 4 $\gamma\delta$ T cells were sorted from spleen, expanded, and restimulated with anti-CD3 for 12 h. The level of IFN- γ and IL-4 in the supernatant was quantified by ELISA. Data represent one of three independent experiments. *B*, B16 cells were cocultured with V γ 1, V γ 4 $\gamma\delta$ T cells, or both as described in Fig. 2A in the presence of anti-IL-4 or control Ab (10 μ g/ml) for 24 h, and the viable B16 cells were counted. One out of three repeatable experiments is shown. * p < 0.05, ** p < 0.01. *C*, B16 cells were cocultured with either V γ 1 or V γ 4 cells in the presence or absence of rmIL-4 (1 ng/ml), and the viable tumor cells were counted at 24 h postcoculture. One representative experiment is shown. ** p < 0.01. *D*, V γ 4 $\gamma\delta$ T cells were expanded as described previously. V γ 4 $\gamma\delta$ T cells and B16 cells were then stained with PE-conjugated anti-mouse IL-4R α (solid line) or PE-conjugated isotype matched control Ab (dotted line). *E*, V γ 1 $\gamma\delta$ T cells were expanded from either Wt or IL-4^{-/-} mice and cocultured with B16-V γ 4 cells as described previously. The viable tumor cells were counted at 24 h postcoculture. Data represent one out of three repeatable experiments. * p < 0.05, ** p < 0.01. *F*, V γ 1 or V γ 4 $\gamma\delta$ T cells were expanded from either Wt or IL-4^{-/-} mice. B16 cells (1 \times 10⁵/mouse) were mixed with $\gamma\delta$ T cells as described in the figure and injected s.c. into TCR δ ^{-/-} mice. The ratios of V γ 1 or V γ 4 cell number: B16 cell number were 2:1. The tumor size (mean \pm SD) was recorded and shown (n = 6 mice/group). Data represent one of three independent experiments. * p < 0.05, ** p < 0.01.

demonstrated a higher percentage of IL-4 in V γ 1 $\gamma\delta$ T cells than those in V γ 4 $\gamma\delta$ T cells (28). On the basis of the fact that IL-4 suppresses antitumor immune responses (30), we hypothesized that IL-4 might be the mediator of V γ 1 $\gamma\delta$ T cells in suppressing V γ 4 $\gamma\delta$ T cell functions against tumor growth. To compare the level of cytokine production from these two subsets of $\gamma\delta$ T cells, V γ 1 and V γ 4 $\gamma\delta$ T cells were expanded and restimulated for 12 h as described in *Materials and Methods*. The amount of IFN- γ and IL-4 in culture supernatant was quantified by ELISA. Consistently, V γ 1 $\gamma\delta$ T cells produced significantly higher level of IL-4 than that of V γ 4 $\gamma\delta$ T cells (Fig. 4A). To test whether IL-4 played any role in mediating the suppressing effect of V γ 1 $\gamma\delta$ T cells, B16 cells were cocultured with either V γ 1 or V γ 4 $\gamma\delta$ T cells or both in the presence of control Ab or anti-IL-4 (10 μ g/ml) and the viable B16 cells (mean \pm SD) were counted and recorded. Neutralizing IL-4 completely diminished the suppression effect of V γ 1 $\gamma\delta$ T cells (Fig. 4B), indicating a critical role of IL-4 as the mediator of V γ 1 $\gamma\delta$ T cells. Next, to see whether IL-4 by itself could replace the effect of V γ 1 $\gamma\delta$ T cells, B16 tumor cells were

cocultured with V γ 4 $\gamma\delta$ T cells in the absence or presence of rmIL-4 as described above. Addition of rmIL-4 completely prevented the suppression effect of V γ 4 $\gamma\delta$ T cells (Fig. 4C). To confirm the effect of IL-4 on $\gamma\delta$ T cells, but not on B16 tumor cells, the expression level of IL-4R on both cell types was analyzed. We showed that only V γ 4 $\gamma\delta$ T cells, regardless of their NKG2D expression level, highly expressed the IL-4R. In contrast, no detectable level of IL-4R was found on B16 cells (Fig. 4D). To further confirm the role of IL-4, V γ 1 $\gamma\delta$ T cells from either Wt or IL-4^{-/-} mice were expanded and cocultured with B16 cells as above. In contrast to those from Wt mice, IL-4^{-/-} V γ 1 $\gamma\delta$ T cells failed to abolish the suppressive effect of V γ 4 $\gamma\delta$ T cells (Fig. 4E), confirming the essential role of IL-4 in mediating the suppression effect of V γ 1 $\gamma\delta$ T cells. Finally, to define the role of IL-4 in mediating the suppression effect of V γ 1 $\gamma\delta$ T cells in vivo, B16 cells (1 \times 10⁵) were mixed with different types of $\gamma\delta$ T cells (2 \times 10⁵) and coinjected s.c. into TCR δ ^{-/-} mice, and the size of tumors was recorded. Similarly, only Wt, but not IL-4^{-/-}, V γ 1 $\gamma\delta$ T cells showed significant suppression effect on V γ 4 $\gamma\delta$ T cell-

FIGURE 5. V γ 1 $\gamma\delta$ T cell-derived IL-4 suppresses V γ 4 $\gamma\delta$ T cell function through mechanisms involving NKG2D, IFN- γ , and perforin. *A*, B6 Wt mice were treated with different inactivation Abs as indicated, followed by tumor inoculation (2 \times 10⁵ B16 tumor cells/mouse) as in Fig. 1. At day 7 postinoculation, small tumors were recovered and used for analysis of gene expression by real-time PCR. The relative expression level of IFN- γ and perforin is shown (n = 3 mice/group). One of three independent experiments is shown. * p < 0.05. *B*, Splenic V γ 4 $\gamma\delta$ T cells were isolated and expanded as described previously. These expanded V γ 4 $\gamma\delta$ T cells were treated in the presence or absence of IL-4 (1 ng/ml) for 24 h. Cells were then stained with allophycocyanin-anti-NKG2D. The percentage of NKG2D⁺ cell reduction ([% of NKG2D⁺ of untreated cells - percentage of NKG2D⁺ of IL-4-treated cells]/percentage of NKG2D⁺ of untreated cells) was calculated from five independent experiments. One representative staining is shown (*B*). Pooled is shown (*C*). *D*, Expanded V γ 4 $\gamma\delta$ T cells were treated with or without IL-4 as described in *B* and were then restimulated with anti-CD3 and anti-CD28 for 6 h, and GolgiPlug was added at last 3 h during restimulation. Cells were then collected for NKG2D staining followed with intracellular IFN- γ and perforin staining. One representative staining is shown.



mediated tumor inhibition (Fig. 4F). With these results taken together, we firmly defined a critical role of V γ 1 $\gamma\delta$ T cell-derived IL-4 in suppressing the V γ 4 $\gamma\delta$ T cell-mediated antitumor immune responses.

V γ 1 $\gamma\delta$ T cell-derived IL-4 suppresses V γ 4 $\gamma\delta$ T cell function through mechanisms involving NKG2D, IFN- γ , and perforin

In our previous study, we have demonstrated that IFN- γ and perforin are critical factors in V γ 4 $\gamma\delta$ T cell-mediated antitumor immunity (28). We hypothesized that V γ 1 $\gamma\delta$ T cells might affect the expression level of these key factors from V γ 4 $\gamma\delta$ T cells. To test our hypothesis, B6 Wt mice were treated with inactivation Abs of either V γ 1 or V γ 4 or both, or left untreated, followed by B16 tumor cell inoculation. On day 7 posttumor injection, local tumor tissues were used for analysis of the expression level of IFN- γ and perforin. Interestingly, inactivation of V γ 1 $\gamma\delta$ T cells significantly increased the expression level of IFN- γ and perforin (Fig. 5A), whereas inactivation of V γ 4 $\gamma\delta$ T cells or both subsets showed significantly lower level of these key factors (Fig. 5A), indicating that V γ 1 $\gamma\delta$ T cells acted on V γ 4 $\gamma\delta$ T cells to downregulate the expression level of IFN- γ and perforin. Because both NKG2D and TCR are involved in V γ 4 $\gamma\delta$ T cell-mediated antitumor immune responses (28), we next tested whether V γ 1-derived IL-4 had any effect on NKG2D expression of V γ 4 $\gamma\delta$ T cells. V γ 4 $\gamma\delta$ T cells were expanded from B6 spleens and treated with IL-4 for 24 h. IL-4 treatment significantly reduced NKG2D expression in V γ 4 $\gamma\delta$ T cells (Fig. 5B, 5C). Moreover, addition of IL-4 significantly reduced the percentage of IFN- γ -producing NKG2D⁺ cells and even more, the percentage of perforin-expressing NKG2D⁺ V γ 4 $\gamma\delta$ T cells (Fig. 5D). Our results thus confirmed a negative regulatory effect of V γ 1 $\gamma\delta$ T cell-derived IL-4 on the key elements of V γ 4 $\gamma\delta$ T cells.

V γ 1 $\gamma\delta$ T cells express significantly higher level of GATA-3 upon Th2 priming

It has been reported that V γ 1 $\gamma\delta$ T cells are a major source of IL-4 (23, 33–37). However, the underlying transcriptional control mechanisms are unknown. From our early studies, overexpression of GATA-3 significantly induced IL-4 production from total splenic $\gamma\delta$ T cells, although it failed to downregulate IFN- γ production by these T cells (9). To investigate whether GATA-3 was differentially expressed between these two subsets of $\gamma\delta$ T cells, naive V γ 1 and V γ 4 $\gamma\delta$ T cells were sorted and primed under neutral or Th2-priming conditions as described previously (8), and naive CD4⁺ T cells were used as control. Upon Th2-priming, V γ 1 $\gamma\delta$ T cells expressed significantly higher level of GATA-3 than that of V γ 4 $\gamma\delta$ T cells (Fig. 6), indicating a possible role of GATA-3 in controlling IL-4 production of V γ 1 $\gamma\delta$ T cells.

Discussion

More and more evidence has been accumulated that TCR-determined different subsets of peripheral $\gamma\delta$ T cells, especially V γ 1 and V γ 4 $\gamma\delta$ T cells, have divergent functions (4, 6, 15). On the basis of our previous findings, V γ 4 $\gamma\delta$ T cells play a protective role in tumor immunity through both TCR and NKG2D recognition and comesodermin-controlled IFN- γ and perforin production (28). In this study, we demonstrated that V γ 1 $\gamma\delta$ T cells had a negative regulatory role in V γ 4-mediated tumor immunity through cell contact-independent and IL-4-dependent mechanisms.

One of the most striking findings in this study was the demonstration that V γ 1 $\gamma\delta$ T cells were the negative regulator of V γ 4 $\gamma\delta$ T cells. It has been emphasized again and again by many studies that different kinds of regulatory T cells play an extremely

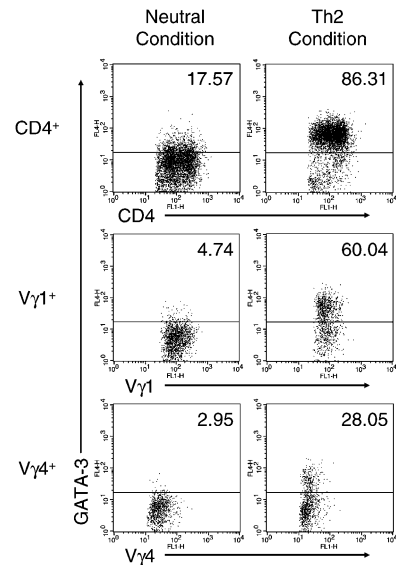


FIGURE 6. V γ 1 $\gamma\delta$ T cells express higher level of GATA-3 upon Th2-priming. Naive V γ 1, V γ 4, and CD4⁺ T cells were sorted and cultured as indicated culture conditions for 48 h. Cells were then collected and stained with anti-V γ 1, or anti-V γ 4, or anti-CD4 followed with intracellular staining of GATA-3. One out of three independent experiments is shown.

important role in maintaining the peripheral tolerance. In the absence of regulatory T cells, severe autoimmune diseases would occur (39, 40). To our knowledge, our results reported for the first time that among $\gamma\delta$ T cells, V γ 1 $\gamma\delta$ T cells served as a subset of regulatory $\gamma\delta$ T cells that controlled or inhibited V γ 4 $\gamma\delta$ T cell-mediated antitumor immunity. It would be interesting to know whether V γ 1 $\gamma\delta$ T cells also play a similar role in other V γ 4 $\gamma\delta$ T cell-mediated immune responses. Given the fact that V γ 1 $\gamma\delta$ T cells also mediate other effector functions (41–44), such as enhancing allergic AHR and IgE responses (21–24), it would be important to further characterize these different subgroups among V γ 1 $\gamma\delta$ T cells, which may provide better target for immune intervention.

We next defined the mechanisms underlying this negative regulatory function of V γ 1 $\gamma\delta$ T cells. We showed that V γ 1 $\gamma\delta$ T cells interacted with V γ 4 $\gamma\delta$ T cells through cell contact-independent mechanisms. Although both V γ 1 and V γ 4 $\gamma\delta$ T cells were recruited from peripheral lymphoid tissues into the tumor tissues as demonstrated from our previous studies (13) as well as our current study, they were not colocalized (Fig. 3B). Our *in vitro* coculture assay in the Transwell plate also confirmed this conclusion (Fig. 3A). However, it is unclear at the present time what factors determine their distribution inside the tumor tissue and also whether they colocalize with other tumor-infiltrating immune cells. Further studies are needed to explore the interactions of these two subsets of $\gamma\delta$ T cells with other types of immune cells.

More interestingly, we determined a critical role of IL-4 as the key mediator of V γ 1 $\gamma\delta$ T cells. V γ 1 $\gamma\delta$ T cells has been reported as the primary source of IL-4 (23, 33–37), and indeed, a higher percentage of IL-4-producing cells in activated V γ 1 $\gamma\delta$ T cells is found in our previous studies (28). We further confirmed that V γ 1 $\gamma\delta$ T cells produced significantly higher amount of IL-4 than those of V γ 4 $\gamma\delta$ T cells (Fig. 4A). Using several approaches, including neutralizing IL-4 with Abs, addition of rIL-4, as well as IL-4^{-/-} mice (Fig. 4), we collectively demonstrated that V γ 1 $\gamma\delta$ T cells suppressed the V γ 4 $\gamma\delta$ T cell-mediated antitumor function through their IL-4 production. Our results thus provided additional information about the role of IL-4-producing V γ 1 $\gamma\delta$ T cells in tumor immunity.

What was the target of V γ 1 $\gamma\delta$ T cell-derived IL-4? Our previous study has demonstrated that both TCR and NKG2D are involved in tumor recognition of V γ 4 $\gamma\delta$ T cells, and both IFN- γ and perforin are the critical mediators in the protective immune responses (28). In this study, we extended our previous findings and determined that V γ 1 $\gamma\delta$ T cell-derived IL-4 inhibited expression level of NKG2D on V γ 4 $\gamma\delta$ T cells (Fig. 5B, 5C), which might indirectly inhibited the cytolytic function of this subset of $\gamma\delta$ T cells. On the basis of the expression level of IL-4R, the effect of rmIL-4 was only through targeting V γ 4 $\gamma\delta$ T cells but not on B16 tumor cells. Indeed, addition of IL-4 did not significantly alter the proliferation of B16 tumor cells (data not shown). Interestingly, rmIL-4 did not change the total percentage of IFN- γ ⁺ V γ 4 $\gamma\delta$ T cells, rather it significantly reduced the percentage of NKG2D⁺IFN- γ ⁺ cells (Fig. 5D). Moreover, IL-4 directly inhibited the expression level of perforin as well as NKG2D⁺perforin⁺ V γ 4 $\gamma\delta$ T cells (Fig. 5D). At present time, we do not understand the molecular mechanisms underlying the downregulation of NKG2D by IL-4, and further studies are therefore needed to shed light on the role of IL-4 in protective antitumor immune responses.

GATA-3 is a critical factor for IL-4 gene transcription and production in CD4⁺ T cells (45–47). However, the role of GATA-3 in controlling IL-4 production of $\gamma\delta$ T cells is unknown. Our previous study has demonstrated that overexpression of GATA-3 results in increased level of IL-4 production, even though it fails to suppress IFN- γ production (9). Interestingly, V γ 1 $\gamma\delta$ T cells expressed significantly higher level of GATA-3 than those of V γ 4 $\gamma\delta$ T cells upon Th2 priming (Fig. 6), indicating that GATA-3 might play a role in determining the relatively higher level of IL-4 in V γ 1 $\gamma\delta$ T cells. It has to be emphasized that further studies are needed to determine whether other transcriptional factors or epigenetic programs are involved in IL-4 production in V γ 1 $\gamma\delta$ T cells.

It has to be noted that TCR-defined subsets of $\gamma\delta$ T cells are still heterogeneous populations and they may contain many different subgroups of cells with diversified functions. For example, thymus-derived “Ag-experienced” $\gamma\delta$ T cells tend to produce IFN- γ , whereas “Ag-unexperienced” $\gamma\delta$ T cells produce IL-17 upon activation (48). What we defined in this report may add new information on the functional complexity of TCR-defined subsets of $\gamma\delta$ T cells. It would be interesting to explore the differences between the effector V γ 1 $\gamma\delta$ T cells in coxsackievirus B3 infection and AHR model as demonstrated in previous studies (19–24) and the regulatory function of V γ 1 $\gamma\delta$ T cells as described in this paper.

In summary, our study demonstrated a critical negative regulatory role of V γ 1 $\gamma\delta$ T cells in V γ 4 $\gamma\delta$ T cell-mediated antitumor immunity through cell contact-independent and IL-4-dependent mechanisms. These results suggested the existence of regulatory $\gamma\delta$ T cell subset and thus added novel information to our understanding of $\gamma\delta$ T cell biology. Depletion of this regulatory $\gamma\delta$ T cell subset may be beneficial for tumor immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

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