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Jianlei Hao,* Siyuan Dong,* Siyuan Xia,* Weifeng He,† Hao Jia,* Song Zhang,* Jun Wei,* Rebecca L. O'Brien,‡ Willi K. Born,‡ Zhenzhou Wu,* Puyue Wang,* Jihong Han,* Zhangyong Hong,* Liqing Zhao,* and Zhinan Yin*

It has been demonstrated that the two main subsets of peripheral γδ T cells, Vγ1 and Vγ4, have divergent functions in many diseases models. Recently, we reported that Vγ4 γδ T cells played a protective role in tumor immunity through eomesodermin-controlled mechanisms. However, the precise roles of Vγ1 γδ T cells in tumor immunity, especially whether Vγ1 γδ T cells have any interaction with Vγ4 γδ T cells, remain unknown. We demonstrated in this paper that Vγ1 γδ T cells suppressed Vγ4 γδ 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 Vγ1 γδ T cells was IL-4. Indeed, treatment of Vγ4 γδ T cells with rIL-4 significantly reduced expression levels of NKG2D, perforin, and IFN-γ. Finally, Vγ1 γδ T cells produced more IL-4 and expressed significantly higher level of GATA-3 upon Th2 priming in comparison with Vγ4 γδ T cells. Therefore, to our knowledge, our results established for the first time a negative regulatory role of Vγ1 γδ T cells in Vγ4 γδ T cell-mediated antitumor immunity through cell contact-independent and IL-4–mediated mechanisms. Selective depletion of this suppressive subset of γδ T cells may be beneficial for tumor immune therapy. ***The Journal of Immunology*****, 2011, 187: 4979–4986.**

γδ T cells have many unique features and functions (1–6). Differing from those of αβ 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, γδ 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 γδ 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 γδ 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 γδ T cells have unique functions (4, 6, 15). Most of these studies have been focused on periphery Vγ1 and Vγ4 γδ T cells (16–18). It has been well documented that these two subsets of γδ T cells have divergent functions in many diseases models (4, 15). Interestingly, very often Vγ1 and Vγ4 γδ T cells have opposite effects. For example, in coxsackievirus B3 infection and allergic airway hyperresponsiveness (AHR), Vγ1 γδ T cells promote CD4$^+$ Th2 responses, whereas Vγ4 γδ T cells help CD4$^+$ Th1 responses (19–24). The distinct roles of these two subsets of γδ T cells have also been demonstrated in autoimmune disease models and infection immunity (25–27). Our recent studies have demonstrated a critical role of Vγ4 γδ T cells in tumor immunity through eomesodermin-controlled, IFN-γ– and perforin-dependent mechanisms (28). However, the role of Vγ1 γδ T cells, especially whether Vγ1 γδ T cells can directly interact with Vγ4 γδ 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 Vγ1 γδ T cells are a major source of IL-4 (23, 33–37), the role of Vγ1 γδ T cell-derived IL-4 in tumor immunity has not been investigated.

In this paper, we demonstrated a negatively regulatory role of Vγ1 γδ T cells in Vγ4 γδ T cell-mediated antitumor immune response. Furthermore, we determined that this regulatory effect of Vγ1 γδ 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-

*State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin 300071, China; †Chongqing Key Laboratory for Diseases Proteomics, State Key Laboratory of Trauma, Burns, and Combined Injury, Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China; and ‡Integrated Department of Immunology, National Jewish Health, Denver, CO 80206

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Address correspondence and reprint requests to Dr. Zhinan Yin, College of Life Sciences, Nankai University, 94 Weijin Road, Tianjin 300071, China. E-mail address: yinzn@nankai.edu.cn

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

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$4^{-/-}$) 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 γδ 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 Vγ1 mAb (clone 2.11), and hamster anti-mouse TCR Vγ4 mAb (clone UC3) were from Tianjin Sungene. PE-conjugated anti-mouse CD124 (IL-4Rα) (clone mIL4R-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).

Vγ1 and Vγ4 γδ T cell inactivation in vivo

For inactivation of Vγ1 or Vγ4 γδ T cells, B6 Wt mice were treated with i.v. injection of anti-Vγ1 TCR (clone 2.11, 200 μg/mouse) or anti-Vγ4 TCR (clone UC3, 200 μg/mouse) mAb on days −5 and −1 before inoculation of tumor cells. Inactivation was confirmed by analysis of TCR expression (the percentage of Vγ1 or Vγ4 γδ 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 Vγ1 and Vγ4 γδ T cells in vitro, splenic γδ T cells were sorted and expanded with anti-Vγ1 or anti-Vγ4 Abs as described previously (28). For polarization of T cells, cells were cultured with plate-coated anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/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 γδ T cells

Tumor cells (7500 cells/well) were mixed with either Vγ1 or Vγ4 cells or both in a total volume of 200 μl in a 96-well flat bottom plate. The viable tumor cells were counted using trypan blue as described previously (28). For separation of Vγ1 and Vγ4 γδ T cells, a 96-well Transwell with 0.4-μm pore size was used (Corning, NY). B16 cells (7500 cells/well) and 1.5×10^5 of Vγ1 or Vγ4 γδ T cells were placed in the lower chamber, with medium or B16 (7500 cells) plus Vγ1 (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-μm thickness, and sections were stained with biotin–anti-Vγ1 and FITC–anti-Vγ4 (20 μg/ml) at 4°C for 24 h. Slides were then washed and incubated with streptavidin-PE (2 μg/ml) (eBioscience) and Alexa Fluor 488-conjugated anti-fluorescein polyclonal Ab (10 μg/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 γδ 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 Vγ1 or Vγ4 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.

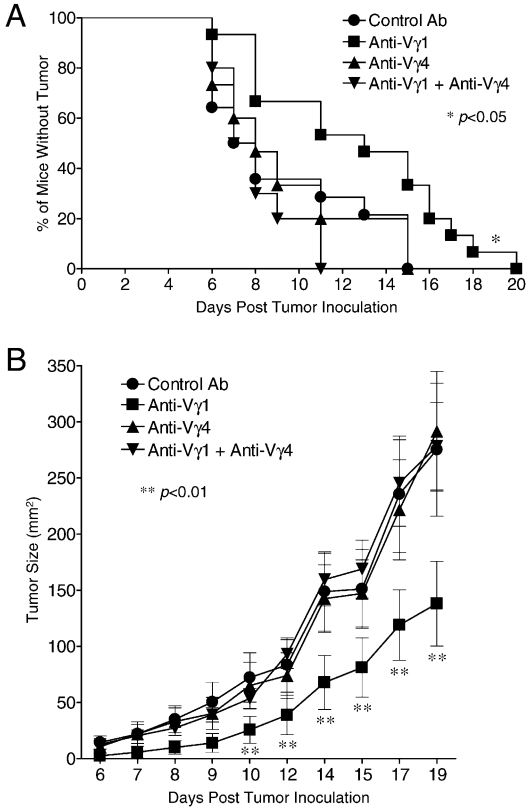

FIGURE 1. Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor immunity in vivo. Sex- and age-matched B6 Wt mice were i.v. injected with Vγ1-specific Ab 2.11 ($n = 15$), Vγ4-specific Ab UC3 ($n = 15$), or both ($n = 10$), and isotype control Ab ($n = 14$) at days −5 and −1, followed by s.c. inoculation of B16 tumor cells (2×10^5 cells/mouse) at day 0. Tumor growth was observed and recorded daily. Tumor size $> 4 \times 4$ mm^2 was considered positive. The rate of mice without tumor from one representative experiment is shown (*A*). The tumor size (mean ± 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 ($n = 10\sim15$ 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. 1*A*, 1*B*), 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. 1*A*, 1*B*), indicating a direct interaction between Vγ1 and Vγ4 γδ T cells.

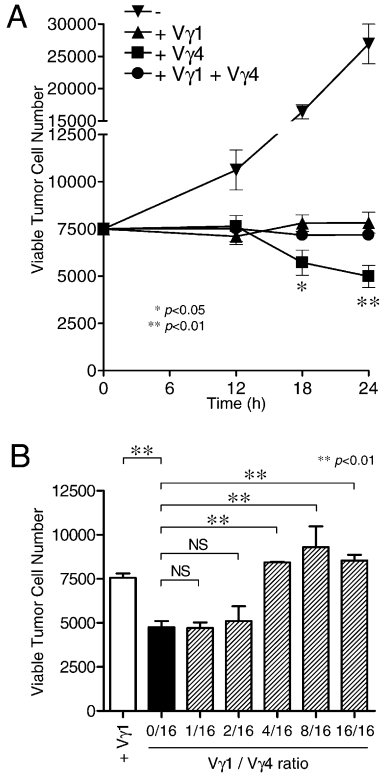

FIGURE 2. Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor immunity in vitro. *A*, B16 cells (7500 cells/well) were cocultured with 20-fold the number of Vγ1, Vγ4, or Vγ1+Vγ4 cells. At different time points postculture, viable tumor cells were counted and recorded. One representative of three 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 postculture. The viable tumor cell number (mean ± SD) is shown. Data represent three independent experiments. $**p < 0.01$.

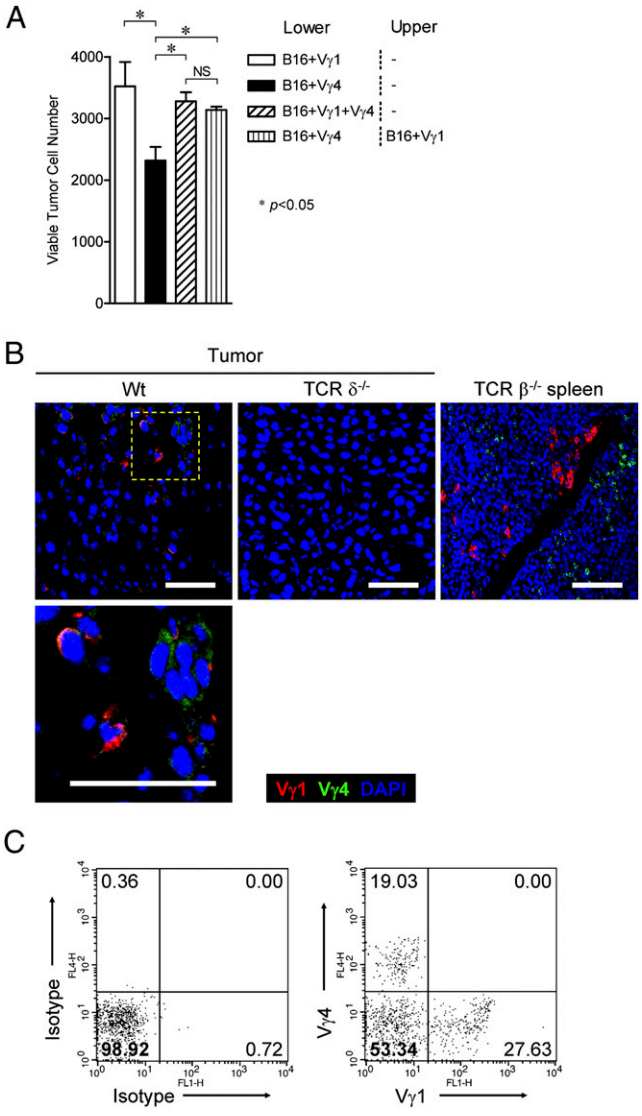

FIGURE 3. Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated antitumor effect through cell contact-independent mechanism. *A*, Vγ4 γδ T cell–B16 cocultures were set up in the lower chamber of Transwell plate (1.5×10^5 Vγ4 γδ T cells and 7500 B16 tumor cells), and Vγ1 γδ T cells (1.5×10^5 cells) were either 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 $\delta^{-/-}$ mice (negative control, *middle panel*) and spleen tissues of TCR $\beta^{-/-}$ 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), Vγ4 γδ T cells showed suppression effect on tumor cell growth with the E:T ratio of 20/1 (Fig. 2*A*). The suppression effect of Vγ4 γδ T cells was significantly abolished by the addition of Vγ1 γδ T cells (Fig. 2*A*). To test whether the suppression effect of Vγ1 γδ T cells on Vγ4 γδ T cells is dose dependent, different ratios of Vγ1/Vγ4 γδ T cells were cocultured with B16 tumor cells and the viable tumor cells were counted and recorded. Interestingly, only when the Vγ1:Vγ4 ratio reached to 1/4, Vγ1 γδ T cells significantly neutralized the suppressive effect of Vγ4 γδ T cells against tumor cell growth (Fig. 2*B*), indicating that the suppression of Vγ1 on Vγ4 γδ T cells was dose dependent.

The suppression of Vγ1 on Vγ4 γδ T cells is cell contact independent

On the basis of our observation that Vγ1 suppressed the antitumor effect of Vγ4 γδ 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 Vγ4-B16 cells significantly reduced the viable tumor cell numbers as described above (Fig. 3*A*). Interestingly, even in a Transwell plate, Vγ1 γδ T cells still inhibited the tumor suppression effect of Vγ4 γδ T cells (Fig. 3*A*), indicating a cell contact-independent mechanism being used by Vγ1 γδ T cells to suppress the antitumor effect of Vγ4 γδ T cells.

To visualize the tumor-infiltrating Vγ1 and Vγ4 γδ T cells, tumors were used for frozen section and stained with anti-Vγ1 or Vγ4 Abs conjugated with different fluorophores. From the sections, Vγ1 and Vγ4 γδ T cells were found to be distributed in different areas inside the tumor tissue (Fig. 3*B*), confirming a cell contact-independent communication between these two subsets of γδ T cells. To further confirm the suppressive effect of Vγ1 γδ T cells, we also found that more Vγ1 γδ T cells were infiltrated into the tumor tissues than those of Vγ4 γδ T cells through three-color FACS analysis (Fig. 3*C*). These results collectively defined a suppressive effect of Vγ1 γδ T cells in tumor immunity.

Vγ1 γδ T cells suppress Vγ4 γδ T cell-mediated tumor inhibition through IL-4 production

It has been reported that Vγ1 γδ T cells are a major source of IL-4 in thymus and periphery (23, 33–37). Our previous report also

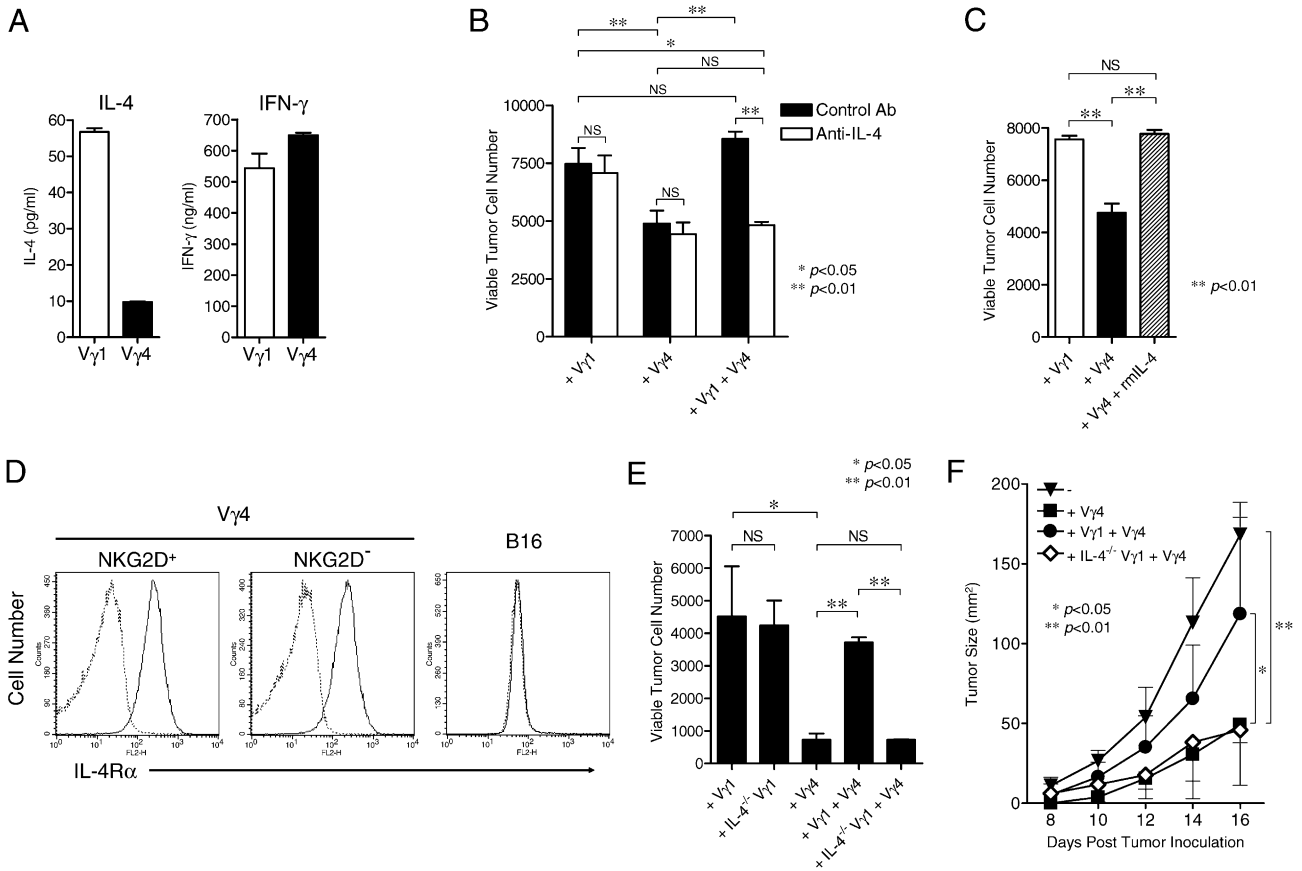

FIGURE 4. Vγ1 γδ T cells suppress Vγ4 γδ T cell antitumor function through IL-4 production. *A*, Vγ1 and Vγ4 γδ T cells were sorted from spleen, expanded, and restimulated with anti-CD3 for 12 h. The level of IFN-γ and IL-4 in the supernatant was quantified by ELISA. Data represent one of three independent experiments. *B*, B16 cells were cocultured with Vγ1, Vγ4 γδ T cells, or both as described in Fig. 2*A* in the presence of anti–IL-4 or control Ab (10 μg/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 Vγ1 or Vγ4 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*, Vγ4 γδ T cells were expanded as described previously. Vγ4 γδ T cells and B16 cells were then stained with PE-conjugated anti-mouse IL-4Rα (solid line) or PE-conjugated isotype matched control Ab (dotted line). *E*, Vγ1 γδ T cells were expanded from either Wt or IL-$4^{-/-}$ mice and cocultured with B16-Vγ4 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*, Vγ1 or Vγ4 γδ T cells were expanded from either Wt or IL-$4^{-/-}$ mice. B16 cells (1×10^5/mouse) were mixed with γδ T cells as described in the figure and injected s.c. into TCR $\delta^{-/-}$ mice. The ratios of Vγ1 or Vγ4 cell number: B16 cell number were 2:1. The tumor size (mean ± 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 Vγ1 γδ T cells than those in Vγ4 γδ 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 Vγ1 γδ T cells in suppressing Vγ4 γδ T cell functions against tumor growth. To compare the level of cytokine production from these two subsets of γδ T cells, Vγ1 and Vγ4 γδ 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, Vγ1 γδ T cells produced significantly higher level of IL-4 than that of Vγ4 γδ T cells (Fig. 4*A*). To test whether IL-4 played any role in mediating the suppressing effect of Vγ1 γδ T cells, B16 cells were cocultured with either Vγ1 or Vγ4 γδ T cells or both in the presence of control Ab or anti–IL-4 (10 μg/ml) and the viable B16 cells (mean ± SD) were counted and recorded. Neutralizing IL-4 completely diminished the suppression effect of Vγ1 γδ T cells (Fig. 4*B*), indicating a critical role of IL-4 as the mediator of Vγ1 γδ T cells. Next, to see whether IL-4 by itself could replace the effect of Vγ1 γδ T cells, B16 tumor cells were cocultured with Vγ4 γδ T cells in the absence or presence of rmIL-4 as described above. Addition of rmIL-4 completely prevented the suppression effect of Vγ4 γδ T cells (Fig. 4*C*). To confirm the effect of IL-4 on γδ T cells, but not on B16 tumor cells, the expression level of IL-4R on both cell types was analyzed. We showed that only Vγ4 γδ 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. 4*D*). To further confirm the role of IL-4, Vγ1 γδ 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$^{-/-}$ Vγ1 γδ T cells failed to abolish the suppressive effect of Vγ4 γδ T cells (Fig. 4*E*), confirming the essential role of IL-4 in mediating the suppression effect of Vγ1 γδ T cells. Finally, to define the role of IL-4 in mediating the suppression effect of Vγ1 γδ T cells in vivo, B16 cells (1×10^5) were mixed with different types of γδ T cells (2×10^5) and coinjected s.c. into TCR δ$^{-/-}$ mice, and the size of tumors was recorded. Similarly, only Wt, but not IL-4$^{-/-}$, Vγ1 γδ T cells showed significant suppression effect on Vγ4 γδ T cell-

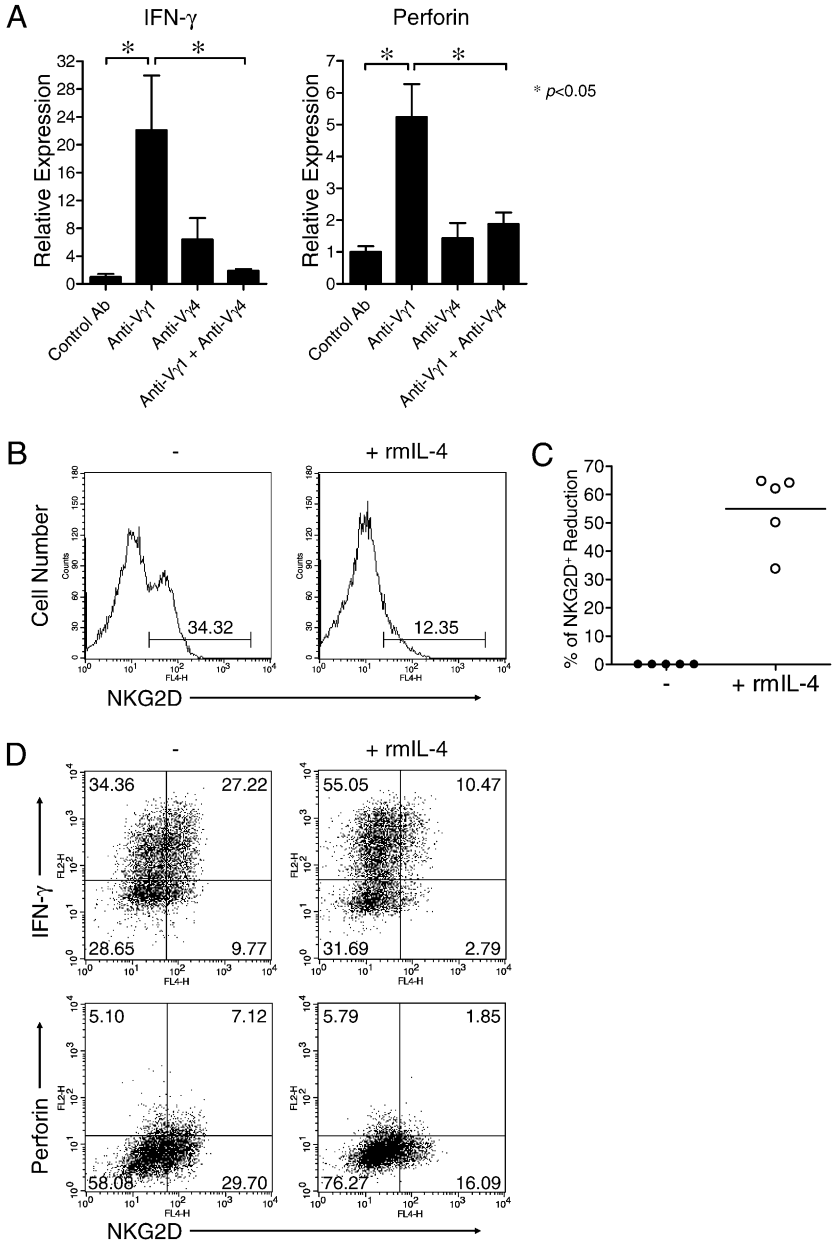

FIGURE 5. Vγ1 γδ T cell-derived IL-4 suppresses Vγ4 γδ T cell function through mechanisms involving NKG2D, IFN-γ, and perforin. *A*, B6 Wt mice were treated with different inactivation Abs as indicated, followed by tumor inoculation (2×10^5 B16 tumor cells/mouse) as in Fig. 1. At day 7 postinoculation, 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 Vγ4 γδ T cells were isolated and expanded as described previously. These expanded Vγ4 γδ 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 Vγ4 γδ 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. 4*F*). With these results taken together, we firmly defined a critical role of Vγ1 γδ T cell-derived IL-4 in suppressing the Vγ4 γδ T cell-mediated antitumor immune responses.

Vγ1 γδ T cell-derived IL-4 suppresses Vγ4 γδ 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 Vγ4 γδ T cell-mediated antitumor immunity (28). We hypothesized that Vγ1 γδ T cells might affect the expression level of these key factors from Vγ4 γδ T cells. To test our hypothesis, B6 Wt mice were treated with inactivation Abs of either Vγ1 or Vγ4 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 Vγ1 γδ T cells significantly increased the expression level of IFN-γ and perforin (Fig. 5*A*), whereas inactivation of Vγ4 γδ T cells or both subsets showed significantly lower level of these key factors (Fig. 5*A*), indicating that Vγ1 γδ T cells acted on Vγ4 γδ T cells to downregulate the expression level of IFN-γ and perforin. Because both NKG2D and TCR are involved in Vγ4 γδ T cell-mediated antitumor immune responses (28), we next tested whether Vγ1-derived IL-4 had any effect on NKG2D expression of Vγ4 γδ T cells. Vγ4 γδ T cells were expanded from B6 spleens and treated with IL-4 for 24 h. IL-4 treatment significantly reduced NKG2D expression in Vγ4 γδ T cells (Fig. 5*B*, 5*C*). Moreover, addition of IL-4 significantly reduced the percentage of IFN-γ–producing NKG2D$^+$ cells and even more, the percentage of perforin-expressing NKG2D$^+$ Vγ4 γδ T cells (Fig. 5*D*). Our results thus confirmed a negative regulatory effect of Vγ1 γδ T cell-derived IL-4 on the key elements of Vγ4 γδ T cells.

Vγ1 γδ T cells express significantly higher level of GATA-3 upon Th2 priming

It has been reported that Vγ1 γδ 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 γδ 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 γδ T cells, naive Vγ1 and Vγ4 γδ 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, Vγ1 γδ T cells expressed significantly higher level of GATA-3 than that of Vγ4 γδ T cells (Fig. 6), indicating a possible role of GATA-3 in controlling IL-4 production of Vγ1 γδ T cells.

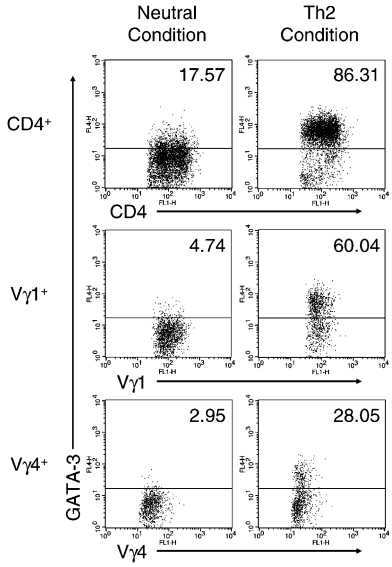

FIGURE 6. Vγ1 γδ T cells express higher level of GATA-3 upon Th2-priming. Naive Vγ1, Vγ4, and CD4$^+$ T cells were sorted and cultured as indicated culture conditions for 48 h. Cells were then collected and stained with anti-Vγ1, or anti-Vγ4, or anti-CD4 followed with intracellular staining of GATA-3. One out of three independent experiments is shown.

Discussion

More and more evidence has been accumulated that TCR-determined different subsets of peripheral γδ T cells, especially Vγ1 and Vγ4 γδ T cells, have divergent functions (4, 6, 15). On the basis of our previous findings, Vγ4 γδ T cells play a protective role in tumor immunity through both TCR and NKG2D recognition and eomesodermin-controlled IFN-γ and perforin production (28). In this study, we demonstrated that Vγ1 γδ T cells had a negative regulatory role in Vγ4-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 Vγ1 γδ T cells were the negative regulator of Vγ4 γδ T cells. It has been emphasized again and again by many studies that different kinds of regulatory T cells play an extremely 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 γδ T cells, Vγ1 γδ T cells served as a subset of regulatory γδ T cells that controlled or inhibited Vγ4 γδ T cell-mediated antitumor immunity. It would be interesting to know whether Vγ1 γδ T cells also play a similar role in other Vγ4 γδ T cell-mediated immune responses. Given the fact that Vγ1 γδ 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 Vγ1 γδ T cells, which may provide better target for immune intervention.

We next defined the mechanisms underlying this negative regulatory function of Vγ1 γδ T cells. We showed that Vγ1 γδ T cells interacted with Vγ4 γδ T cells through cell contact-independent mechanisms. Although both Vγ1 and Vγ4 γδ 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. 3*B*). Our in vitro coculture assay in the Transwell plate also confirmed this conclusion (Fig. 3*A*). 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 γδ T cells with other types of immune cells.

More interestingly, we determined a critical role of IL-4 as the key mediator of Vγ1 γδ T cells. Vγ1 γδ 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 Vγ1 γδ T cells is found in our previous studies (28). We further confirmed that Vγ1 γδ T cells produced significantly higher amount of IL-4 than those of Vγ4 γδ T cells (Fig. 4*A*). 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 Vγ1 γδ T cells suppressed the Vγ4 γδ T cell-mediated antitumor function through their IL-4 production. Our results thus provided additional information about the role of IL-4–producing Vγ1 γδ T cells in tumor immunity.

What was the target of Vγ1 γδ T cell-derived IL-4? Our previous study has demonstrated that both TCR and NKG2D are involved in tumor recognition of Vγ4 γδ 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 Vγ1 γδ T cell-derived IL-4 inhibited expression level of NKG2D on Vγ4 γδ T cells (Fig. 5*B*, 5*C*), which might indirectly inhibited the cytolytic function of this subset of γδ T cells. On the basis of the expression level of IL-4R, the effect of rmIL-4 was only through targeting Vγ4 γδ 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^+ Vγ4 γδ T cells, rather it significantly reduced the percentage of NKG2D$^+$IFN-γ^+ cells (Fig. 5*D*). Moreover, IL-4 directly inhibited the expression level of perforin as well as NKG2D$^+$perforin$^+$ Vγ4 γδ T cells (Fig. 5*D*). 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 γδ 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, Vγ1 γδ T cells expressed significantly higher level of GATA-3 than those of Vγ4 γδ 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 Vγ1 γδ 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 Vγ1 γδ T cells.

It has to be noted that TCR-defined subsets of γδ T cells are still heterogeneous populations and they may contain many different subgroups of cells with diversified functions. For example, thymus-derived "Ag-experienced" γδ T cells tend to produce IFN-γ, whereas "Ag-unexperienced" γδ 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 γδ T cells. It would be interesting to explore the differences between the effector Vγ1 γδ T cells in coxsackievirus B3 infection and AHR model as demonstrated in previous studies (19–24) and the regulatory function of Vγ1 γδ T cells as described in this paper.

In summary, our study demonstrated a critical negative regulatory role of Vγ1 γδ T cells in Vγ4 γδ T cell-mediated antitumor immunity through cell contact-independent and IL-4–dependent mechanisms. These results suggested the existence of regulatory γδ T cell subset and thus added novel information to our understanding of γδ T cell biology. Depletion of this regulatory γδ 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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