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# Naturally Activated V $\gamma$ 4 $\gamma\delta$ T Cells Play a Protective Role in Tumor Immunity through Expression of Eomesodermin

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We previously demonstrated that  $\gamma\delta$  T cells played an important role in tumor immune surveillance by providing an early source of IFN- $\gamma$ . The precise role of different subsets of  $\gamma\delta$  T cells in the antitumor immune response, however, is unknown. V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells are the principal subsets of peripheral lymphoid  $\gamma\delta$  T cells and they might play distinct roles in tumor immunity. In support of this, we observed that reconstitution of TCR $\delta^{-/-}$  mice with V $\gamma$ 4, but not V $\gamma$ 1,  $\gamma\delta$  T cells restored the antitumor response. We also found that these effects were exerted by the activated (CD44<sup>high</sup>) portion of V $\gamma$ 4  $\gamma\delta$  T cells. We further determined that IFN- $\gamma$  and perforin are critical elements in the V $\gamma$ 4-mediated antitumor immune response. Indeed, CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells produced significantly more IFN- $\gamma$  and perforin on activation, and showed greater cytolytic activity than did CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells, apparently due to the high level of eomesodermin (Eomes) in these activated V $\gamma$ 4  $\gamma\delta$  T cells. Consistently, transfection of dominant-negative Eomes in V $\gamma$ 4  $\gamma\delta$  T cells diminished the level of IFN- $\gamma$  secretion, indicating a critical role of Eomes in the effector function of these  $\gamma\delta$  T cells. Our results thus reveal distinct functions of V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells in antitumor immune response, and identify a protective role of activated V $\gamma$ 4  $\gamma\delta$  T cells, with possible implications for tumor immune therapy. *The Journal of Immunology*, 2010, 185: 126–133.

There are many unique features and functions of  $\gamma\delta$  T cells (1–3). Similar to  $\alpha\beta$  T cells,  $\gamma\delta$  T cells produce an array of cytokines and possess cytolytic functions. Our previous studies have demonstrated that  $\gamma\delta$  T cells predominantly produce IFN- $\gamma$  on activation and the underlying controlling mechanisms are different from those of  $\alpha\beta$  T cells (4, 5). Moreover, we have shown that  $\gamma\delta$  T cells play an important role in tumor immune surveillance as an early source of IFN- $\gamma$  (6). However, it remained unclear whether all or only some  $\gamma\delta$  T cells contribute to host protection.

V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells are the two dominant subsets of peripheral lymphoid  $\gamma\delta$  T cells. Recently, these subsets have been demonstrated to have different functions in regulating CD4 T cell

Th1/Th2 differentiation; V $\gamma$ 1  $\gamma\delta$  T cells promote CD4 Th2 responses, whereas V $\gamma$ 4  $\gamma\delta$  T cells promote CD4 Th1 cell responses in Coxsackievirus B3 infections and airway hyperactive response (7–12). These two subsets of  $\gamma\delta$  T cells have also shown to mediate divergent functions in macrophages and play a distinctive role in autoimmune diseases models as well as infectious immunity (13–15). Although these two subsets of  $\gamma\delta$  T cells have divergent functions; however, the involvement of TCR or other receptors has not been implicated. So far, the roles of V $\gamma$ 1 versus V $\gamma$ 4  $\gamma\delta$  T cells in antitumor immune responses also remain to be investigated.

Unlike  $\alpha\beta$  T cells, most peripheral  $\gamma\delta$  T cells spontaneously activate, upregulating surface expression of the activation marker CD44 and experiencing rapid turnover (16, 17). They also expand quickly on pathogenic challenge in the first several days postinfection (18–20). In experimental models of infectious diseases,  $\gamma\delta$  T cell responses develop between 4 h and 96 h postinfection, bridging the gap between the innate immune (NK and macrophages) and adaptive immune responses (Ag-specific CD4 and CD8 T cell responses) (21, 22). Our previous studies have demonstrated that CD44<sup>high</sup>, but not CD44<sup>low</sup>,  $\gamma\delta$  T cells spontaneously express IFN- $\gamma$  and T-bet, and rapidly produce IFN- $\gamma$  on TCR activation (4, 5). Therefore, it seemed likely that CD44<sup>high</sup>  $\gamma\delta$  T cells play an important role in antitumor immune responses.

In this study, we demonstrate that V $\gamma$ 4  $\gamma\delta$  T cells are indeed protective in the immune response against the aggressive B16 melanoma, and that the CD44<sup>high</sup> fraction of this  $\gamma\delta$  T cell subset is critical. We further show that both IFN- $\gamma$  and perforin are essential for CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cell-mediated tumor protection. On activation, CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells produce higher levels of IFN- $\gamma$  and perforin than do CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells, at least in part due to the high expression level of the transcription factor eomesodermin (Eomes). Our study thus provides the first evidence for a critical role of V $\gamma$ 4  $\gamma\delta$  T cells in protective antitumor immune responses.

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Abbreviations used in this paper: DN, dominant negative; Eomes, eomesodermin; HPRT, hypoxanthine-guanine phosphoribosyltransferase; n.s., no significant difference; UD, undetected; Wt, wild-type.

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## Materials and Methods

### Mice

C57BL/6J (B6) mice were purchased from the National Cancer Institute. C57BL/6J-Tcrb<sup>tm1Mom</sup> (B6 TCR $\beta$ -deficient mice [TCR $\beta$ <sup>-/-</sup>]), C57BL/6J-Tcrd<sup>tm1Mom</sup> (B6 TCR $\delta$ <sup>-/-</sup>), C57BL/6-prf1<sup>tm1</sup> (B6 Prf1-deficient [perforin<sup>-/-</sup>]) and C57BL/6-Irfng<sup>tm1Ts</sup> (B6 IFN- $\gamma$ -deficient [IFN- $\gamma$ <sup>-/-</sup>]) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Some of experimental mice were purchased from Chinese Medical Academy of Sciences (Beijing, China). All animals were maintained under specific pathogen-free conditions and used at 6–8 wk of age.

### Reagents

Recombinant murine IL-2, IL-12 was purchased from R&D Systems (Minneapolis, MN). Anti-mouse IL-4, and Abs for phenotypic and cytokine analysis were purchased from BD Biosciences (San Jose CA).

### Tumor models

B16 F0 melanoma cells (provided by Dr. Mark Mamula, Yale School of Medicine, New Haven, CT) were injected s.c., and tumor growth was monitored and recorded daily for >3 wk, as described (6).

### Expansion of CD44<sup>high</sup> $\gamma\delta$ T cells in vitro

CD44<sup>high</sup>  $\gamma\delta$  T cells were sorted from splenocytes of either B6 Wt (wild-type) or perforin<sup>-/-</sup> or IFN- $\gamma$ <sup>-/-</sup> mice by FACS. Sorted cells were cultured with plate-coated V $\gamma$ 4-specific Ab UC3 and V $\gamma$ 1-specific Ab 2.11, respectively (10  $\mu$ g/ml), and IL-2 (2 ng/ml) for 8 d. Expanded V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells were confirmed by FACS analysis.

### Effector function of CD44<sup>high</sup> V $\gamma$ 4 and V $\gamma$ 1 $\gamma\delta$ T cells in antitumor immune response in vivo

Expanded CD44<sup>high</sup> V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells were prepared as described previously, mixed with B16 tumor cells in a ratio of 1:4, and coinjected into B6 TCR  $\delta$ <sup>-/-</sup> mice s.c. The presence of tumors and their size was monitored and measured as described (6).

### Intracellular cytokine staining

Cells were activated as described conditions. After 3 h of culture, brefeldin A was added to the culture for additional 3 h. The cells were then collected and fixed with 2% formaldehyde in PBS, permeabilized with 0.5% saponin, and stained with fluorescently labeled cytokine-specific Abs, as described (5).

### CTL assay

The cytotoxicity of V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells was determined by JAM assay as described previously (23). Briefly, sorted CD44<sup>high</sup> V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells from B6 mice were stimulated with the V $\gamma$ 4-specific Ab UC3 and V $\gamma$ 1 specific-Ab 2.11, respectively, and cultured with IL-2 for 8 d. YAC-1 cells (target cells, 1000 cells/well) were cultured for 6 h with [<sup>3</sup>H]thymidine (5  $\mu$ Ci/ml). Various amounts of activated cells (effector cells) were added to the target cells in triplicate in a 96-well round-bottom plate. Spontaneous <sup>3</sup>H retention was determined by adding medium instead of effector cells. After 4 h of culture, cells were harvested onto filters, which were read by a scintillation counter to calculate the specific lysis using the following formula: ((spontaneous cpm – experimental cpm)  $\times$  100)/spontaneous cpm.

### Coculture of $\gamma\delta$ T cells and B16 melanoma

Expanded V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells (1.5  $\times$  10<sup>5</sup> cells/well) were mixed with B16 cells (7500 cells/well) in a total volume of 200  $\mu$ l in 96-well plate in triplicate for 24 h. Live cells were counted with trypan blue vital stain (Invitrogen, San Diego, CA) and the supernatants were collected for analysis of secreted IFN- $\gamma$ . For Ab blocking experiments, expanded V $\gamma$ 1 and V $\gamma$ 4 cells (1  $\times$  10<sup>6</sup> cells/ml) were then incubated with control Ab, anti-mouse  $\gamma\delta$  TCR (clone UC7) as TCR block or anti-mouse NKG2D (clone CX5) as NKG2D block at a concentration of 5  $\mu$ g/ml on ice for 15 min. Cells were then washed and reconstituted in RPMI 1640 supplemented with 10% FBS for the coculture experiments. The specificity of blocking NKG2D was confirmed by its effect on secondary staining with allophycocyanin-anti-mouse NKG2D as described (24). Blocking Abs and isotype control Ab were added again to the coculture system (5  $\mu$ g/ml).

### ELISA

Mouse IFN- $\gamma$  ELISA kit was purchased from BioLegend (San Diego, CA) and ELISA was performed according to the manufacturer's protocol.

Supernatants collected from the coculture system as described previously were assayed using a standard curve generated by recombinant IFN- $\gamma$ .

### Real-time PCR for gene transcription

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse-transcribed using the Strata Script First Strand Synthesis System (Stratagene, La Jolla, CA). PCR was performed on an iCycler (Bio-Rad, Hercules, CA). Cycling conditions were 12 min at 95°C, followed by 40 repeats of 95°C for 15 s and 60°C for 60 s. Analysis was performed by sequence detection software that was supplied with the instrument. Each gene transcript was analyzed concurrently on the same plate with hypoxanthine-guanine phosphoribosyltransferase (HPRT) and transcripts were normalized to HPRT using primers as described. The primers were as follows: HPRT sense, 5'-CTGGTGAAAAG GACC-TCTCG-3'; HPRT antisense, 5'-TGAAGTACTCATTATAGTCAAGG-GCA-3'; HPRT probe, VIC-5'-TGTTGGATACAGGCCAGACTTGT-TGGAT-3'-TAMRA; T-bet sense, 5'-CAACAACCCCTTTGCCAAAG-3'; T-bet antisense, 5'-TCCCCAAGCAGTTGACAGT-3'; T-bet probe, FAM-5'-CCGGGAGA ACTTTGAGTCCATGTACGC-3'-TAMRA; Eomes sense, 5'-CCTTCAC CTTCTCAGAGACACAGTT-3'; Eomes antisense, 5'-TCG-ATCTTTAG CTGGGTGATATCC-3'; Eomes probe, FAM-5'-TCGCTGT-GACGGCCT ACCAAAACA-3'-BHQ.

### Retroviral transduction

Retroviral constructs of dominant negative (DN) T-bet, DN Eomes, and empty vector were the same as used before (26). Retroviral transduction was performed as previously described (25). Naive (CD44<sup>low</sup>) V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells were sorted from the splenocytes of Wt B6 mice and were infected with viral supernatants collected from the transfected Phoenix packaging cell line under the Th1 condition (5). Cells were cultured with fresh medium with IL-2 and restimulated on day 5 for intracellular cytokine staining.

### Preparation of cell suspensions from tumor injection site

C57BL/6 mice ( $n = 10$ , aged 6 wks) were injected with 2  $\times$  10<sup>5</sup> B16 F0 melanoma cells. At day 4 postinoculation, small areas of shaved skin were resected, digested with trypsin-GNK solution (0.29% trypsin, 0.86% NaCl, 0.041% KCl, and 0.1% glucose) at 37°C for 2 h, and treated with collagenase/hyaluronidase digestion solution buffer (0.27% collagenase, 0.025% hyaluronidase, 1% DNase, 0.01% HEPES, and 0.01% sodium pyruvate in RPMI 1640) for 2 h at 37°C as described (6). The digested skin was filtered through a 40- $\mu$ m cell strainer (Becton Dickinson, San Jose, CA), and a single-cell suspension was obtained that contained resident cells and infiltrating cells. Cells were stained with anti-V $\gamma$ 1-FITC or anti-V $\gamma$ 4-FITC, fixed with 2% formaldehyde, and stained for intracellular perforin as described previously.

### Statistics

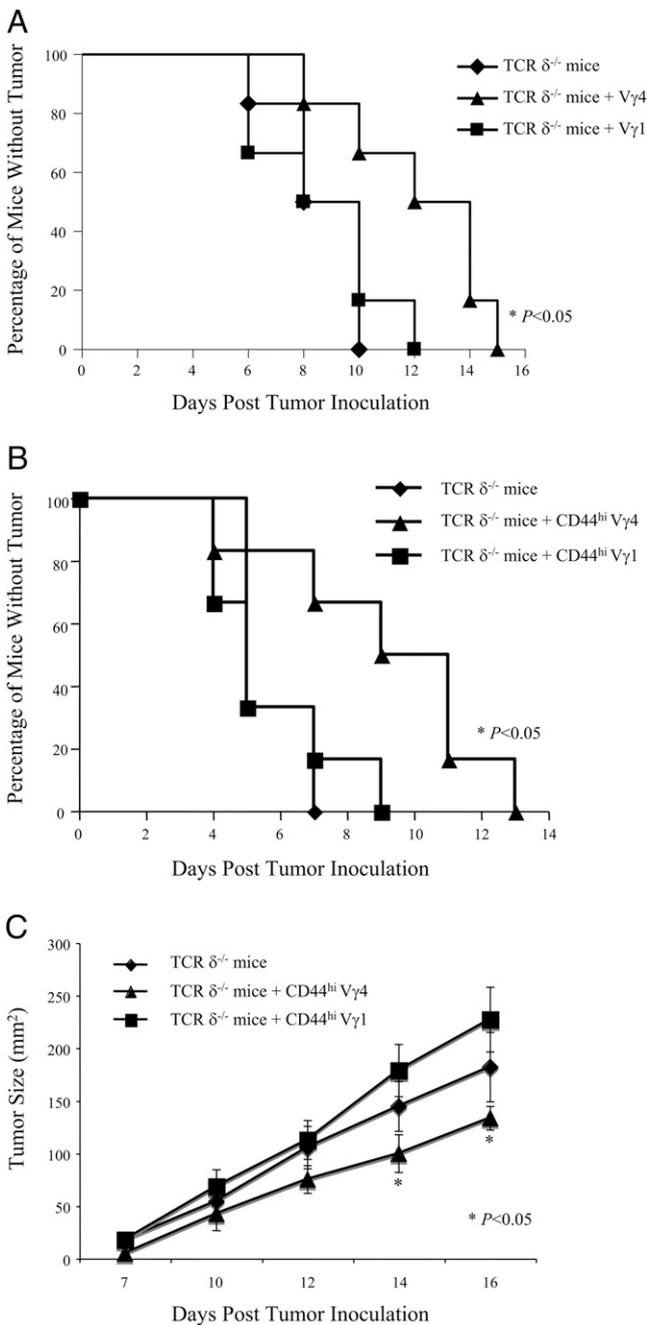
Statistical significance was evaluated by two-tailed unpaired Student  $t$  test or nonparameter analysis if SDs were significantly different between two groups using InStat version 2.03 software for Macintosh (GraphPad, San Diego, CA). The incidence of tumor development was compared and analyzed using the log-rank test, performed by GraphPad Prism version 3.0a for Macintosh (GraphPad). Throughout the text, figures, and legends, the following terminology is used to denote statistical significance: \* $p < 0.05$ ; \*\* $p < 0.01$ .

## Results

### Naturally activated (CD44<sup>high</sup>), but not naive (CD44<sup>low</sup>), V $\gamma$ 4 $\gamma\delta$ T cells protect against tumor growth

To define the role of the two subsets of  $\gamma\delta$  T cells in tumor immunity, sex- and age-matched B6 TCR $\delta$ <sup>-/-</sup> mice were transferred with small numbers of sorted V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells (1  $\times$  10<sup>5</sup>/mouse), followed by inoculation with B16 F0 cells (2  $\times$  10<sup>5</sup>/mouse,  $n = 10$ ) on the second day. Tumor growth was monitored daily. Mice reconstituted with V $\gamma$ 4  $\gamma\delta$  T cells were better protected against tumor development than those that received V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 1A,  $p < 0.05$ ). These results suggested that V $\gamma$ 4, but not V $\gamma$ 1,  $\gamma\delta$  T cells contribute to the antitumor immune response.

Our previous study demonstrated that activated (CD44<sup>high</sup>)  $\gamma\delta$  T cells express IFN- $\gamma$  and T-bet mRNA, and release IFN- $\gamma$  protein on TCR triggering (4, 5). We hypothesized that CD44<sup>high</sup> and



**FIGURE 1.** Spontaneously activated (CD44<sup>high</sup>), but not naive (CD44<sup>low</sup>), V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells play different roles in the antitumor immune response. **A**, B6 TCR  $\delta^{-/-}$  mice ( $n = 10$  per each group) were i.v. reconstituted with either V $\gamma$ 4 or V $\gamma$ 1  $\gamma\delta$  T cells sorted from B6 TCR  $\beta^{-/-}$  mice ( $1 \times 10^5$  cells/mouse). On the following day, reconstituted mice were inoculated s.c. with B16 F0 tumor cells ( $2 \times 10^5$  cells/mouse), and tumor growth was observed and recorded daily. Data represent three independent experiments. **B**, Sex- and age-matched B6 TCR $\delta^{-/-}$  mice ( $n = 10$  per each group) were reconstituted with activated (CD44<sup>high</sup>) V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells sorted from B6 TCR  $\beta^{-/-}$  mice ( $1 \times 10^5$  cells/mouse). On the following day, reconstituted mice were inoculated s.c. with B16 F0 tumor cells ( $2 \times 10^5$  cells/mouse). Recipient mice were monitored for the presence of tumors. Tumor size  $>4 \times 4$  mm<sup>2</sup> was considered positive. Data represent three independent experiments.  $*p < 0.05$ . **C**, The tumor size was measured. Each data point represents the mean size of tumors seen in 10 identically treated animals. These results are typical of three independent experiments.  $*p < 0.05$ .

CD44<sup>low</sup> V $\gamma$ 4  $\gamma\delta$  T cells have different functions in the antitumor immune response. To test our hypothesis, sex- and age-matched B6 TCR $\delta^{-/-}$  mice were reconstituted with either activated

(CD44<sup>high</sup>) or naive (CD44<sup>low</sup>) V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells ( $1 \times 10^5$ /mouse) i.v., followed with s.c. inoculation with B16 F0 cells ( $2 \times 10^5$ /mouse,  $n = 10$ ), and tumor growth was monitored and recorded daily. No significant difference was observed in tumor growth between mice reconstituted with naive V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells (data not shown). In contrast, mice that were reconstituted with activated V $\gamma$ 4  $\gamma\delta$  T cells were better protected against tumor growth than those reconstituted with activated V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 1B, 1C,  $p < 0.05$ ), suggesting that activated V $\gamma$ 4  $\gamma\delta$  T cells are the protective  $\gamma\delta$  T cell cohort in the antitumor immune response.

To further confirm the role of V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells in tumor resistance, naturally activated V $\gamma$ 1 and V $\gamma$ 4 cells were expanded in vitro for 8 d, mixed with B16 cells at a 1:4 ratio and inoculated s.c. into two flanks of B6 TCR  $\delta^{-/-}$  mice, and the presence as well as growth rate of tumors in both flanks of the transplant receipts were monitored daily. As predicted, the side with inoculation of the mixture of CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells showed significantly less tumor growth than the opposite side receiving the mixture of CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 2A, 2B,  $p < 0.01$ ). This observation confirmed that activated CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells are the preeminent antitumor effectors in the in vivo response against the B16 melanoma.

#### *IFN- $\gamma$ and perforin are required for CD44<sup>high</sup> V $\gamma$ 4 $\gamma\delta$ T cell-mediated tumor protection*

Next, we tried to determine critical elements in the protective function of CD44<sup>high</sup>  $\gamma\delta$  T cells. Expanded Wt, IFN- $\gamma^{-/-}$ , or perforin<sup>-/-</sup> CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells were prepared, mixed with B16 cells at a ratio of 1:4, and inoculated into recipient B6 TCR  $\delta^{-/-}$  mice ( $n = 10$  per each group) s.c. as described previously. Tumor growth was monitored and recorded daily. Compared with mice that were injected with Wt CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells, those receiving either IFN- $\gamma^{-/-}$  or perforin<sup>-/-</sup> CD44<sup>high</sup> V $\gamma$ 4 cells were more susceptible to tumor growth, demonstrating that both IFN- $\gamma$  and perforin are required for the protective function of CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells in this model (Fig. 3). Notably, IFN- $\gamma^{-/-}$  V $\gamma$ 4  $\gamma\delta$  T cells retained some protective function; whereas, perforin<sup>-/-</sup> V $\gamma$ 4  $\gamma\delta$  T cells were no longer protective (Fig. 3). This suggests that perforin is even more critical in V $\gamma$ 4-mediated local tumor protection.

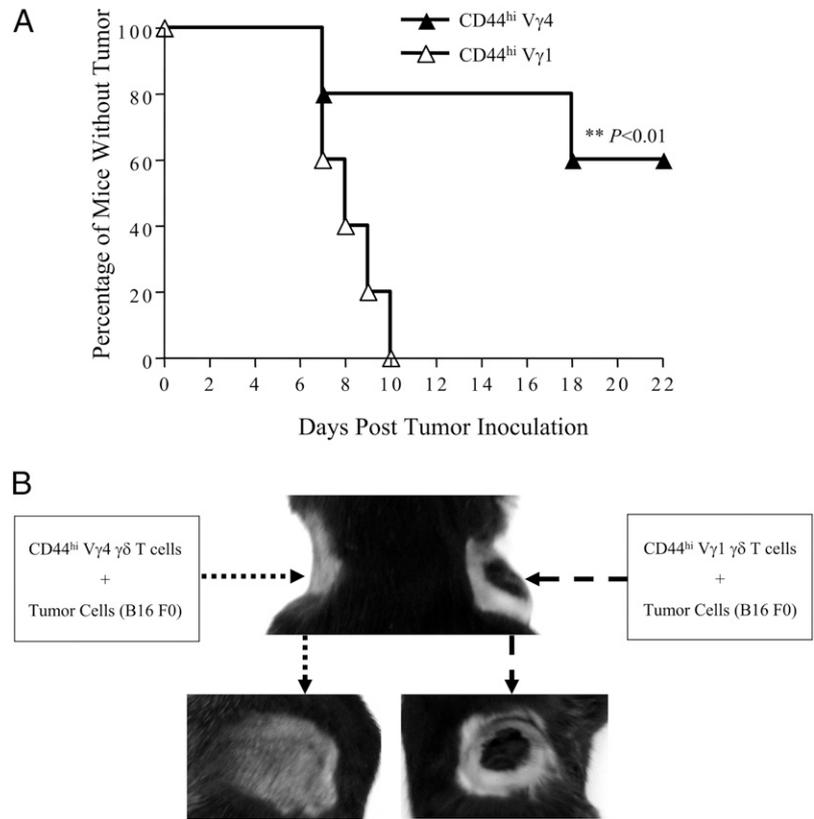
#### *Naturally activated V $\gamma$ 4 $\gamma\delta$ T cells produce more IFN- $\gamma$ than activated V $\gamma$ 1 $\gamma\delta$ T cells*

To assess the ability of spontaneously activated V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells to produce IFN- $\gamma$  on TCR stimulation, CD44<sup>high</sup> V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells were sorted from splenocytes of naive B6 mice and stimulated immediately with anti-CD3 for 6 h prior to intracellular cytokine staining. Freshly isolated CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells produced IFN- $\gamma$  at higher cell frequencies than V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 4A). Moreover, when CD44<sup>high</sup> V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells were expanded in vitro for 8 d and restimulated with anti-CD3 mAb for 6 h for intracellular staining, V $\gamma$ 4  $\gamma\delta$  T cells again produced IFN- $\gamma$  at higher cell frequencies than activated V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 4B). Conversely, activated V $\gamma$ 1  $\gamma\delta$  T cells produced IL-4 at higher cell frequency than activated V $\gamma$ 4  $\gamma\delta$  T cells (Fig. 4B).

#### *Naturally activated V $\gamma$ 4 $\gamma\delta$ T cells are more cytolytic and suppressive than V $\gamma$ 1 $\gamma\delta$ T cells due to increased IFN- $\gamma$ and perforin production*

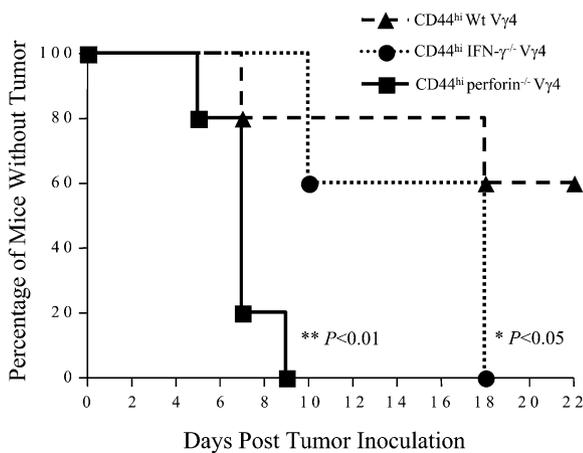
To assess the cytotoxicity of V $\gamma$ 4  $\gamma\delta$  T cells against tumor cells in vitro, sorted CD44<sup>high</sup> V $\gamma$ 4 and V $\gamma$ 1 cells were expanded and analyzed for cytolytic activity using the JAM assay, with YAC-1 tumor cells as targets. V $\gamma$ 4  $\gamma\delta$  T cells showed significantly increased CTL activity at E:T ratios of 20:1 up to 40:1 ( $p < 0.05$ ), indicating that CD44<sup>high</sup> V $\gamma$ 4 cells are more cytotoxic toward these tumor cells

**FIGURE 2.** Activated (CD44<sup>high</sup>) Vγ4, but not Vγ1, γδ T cells contribute to antitumor immune responses. *A*, CD44<sup>high</sup> γδ T cells were sorted from B6 mouse spleens and lymph nodes and stimulated with Vγ4-specific Ab UC3 and Vγ1-specific Ab 2.11, respectively, and cultured with IL-2 for 8 d. B16 F0 tumor cells ( $2 \times 10^5$  cells/mouse) were mixed with these expanded Vγ4 or Vγ1 γδ T cells ( $0.5 \times 10^5$  cells/mouse) and s.c. injected into B6 TCR δ<sup>-/-</sup> mice ( $n = 10$  per each group). Tumor growth was observed and recorded daily. Tumor size  $>4 \times 4$  mm<sup>2</sup> was considered positive. Data represent three independent experiments. \* $p < 0.05$ . *B*, Tumor growth in one representative mouse is shown.

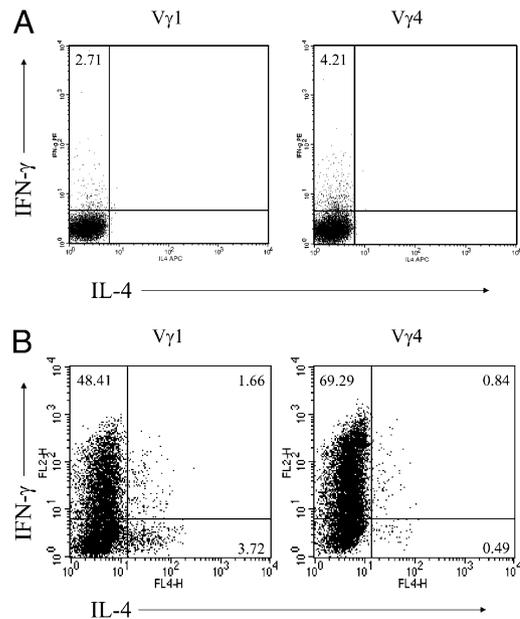


in vitro than CD44<sup>high</sup> Vγ1 cells (Fig. 5A). However, no significant cytotoxicity effect against B16 cells was observed by either Vγ4 or Vγ1 γδ T cells (data not shown). To determine whether these two γδ T cell subsets suppressed expansion of B16 cells in vitro, expanded Vγ1 or Vγ4 γδ T cells were cocultured with B16 melanoma cells (ratio of 20:1). Vγ4 γδ T cells exerted a much stronger inhibitory effect than that of Vγ1 γδ T cells (Fig. 5B). Interestingly, blocking either TCR or NKG2D partially blocked the suppressive effect of

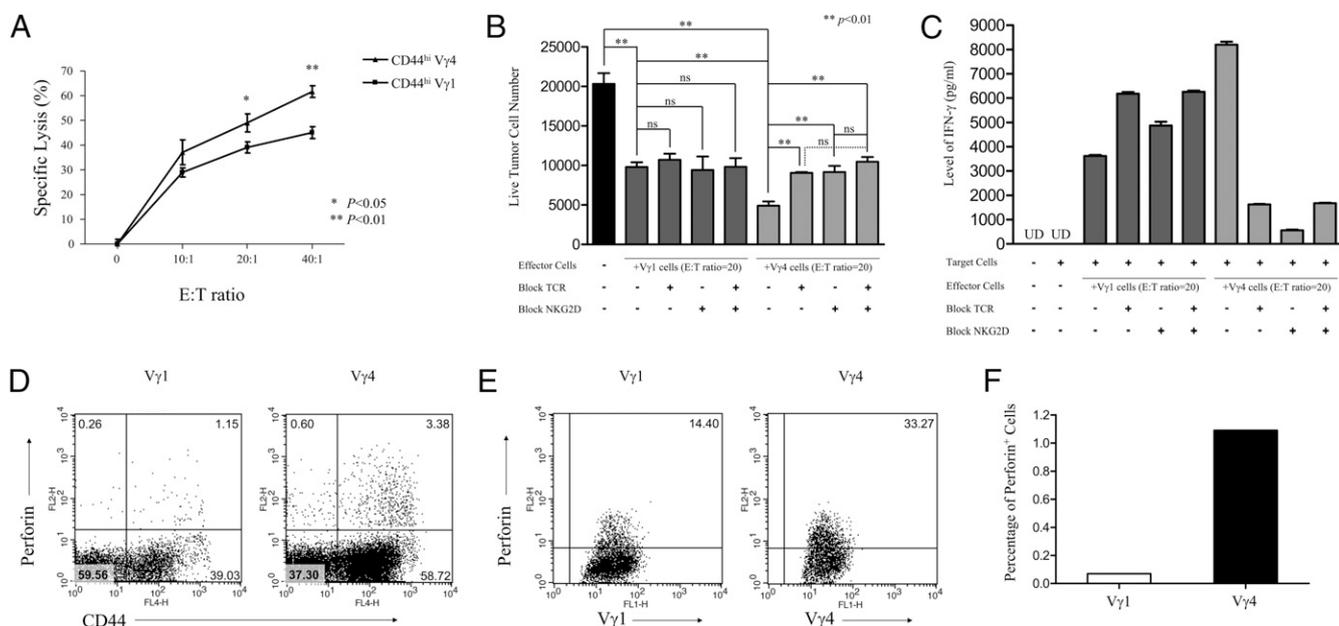
Vγ4 γδ T cells, but not Vγ1 γδ T cells (Fig. 5B), suggesting that both TCR and NKG2D are important, but not synergistic in Vγ4 γδ T cell-mediated B16 suppression. Moreover, to test the role of



**FIGURE 3.** IFN-γ and perforin are required for Vγ4-mediated antitumor immune responses. CD44<sup>high</sup> Vγ4 γδ T cells were sorted from either B6 Wt mice, or perforin<sup>-/-</sup> or IFN-γ<sup>-/-</sup> mice spleens and lymph nodes, and stimulated with UC3 Ab in the presence of IL-2 for 8 d. B16 F0 tumor cells ( $2 \times 10^5$  cells/mouse) were mixed with these expanded Vγ4 γδ T cells ( $0.5 \times 10^5$  cells/mouse), injected into B6 TCR δ<sup>-/-</sup> mice ( $n = 10$  per each group) s.c. Tumor growth was observed and recorded daily. Tumor size  $>4 \times 4$  mm<sup>2</sup> was considered positive. Data represent three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**FIGURE 4.** Spontaneously activated (CD44<sup>high</sup>) Vγ4 γδ T cells produce higher levels of IFN-γ than activated Vγ1 cells. *A*, CD44<sup>high</sup> Vγ4 and Vγ1 γδ T cells were sorted from B6 spleens and lymph nodes, stimulated with anti-CD3 for 6 h, and GolgiPlug was added for the last 3 h. Cells were then fixed with 2% formaldehyde and permeabilized with 0.5% saponin for intracellular IFN-γ and IL-4 staining. Results represent one of three repeated experiments. *B*, CD44<sup>high</sup> γδ T cells were sorted from B6 spleens and lymph nodes, stimulated with UC3 and 2.11, respectively, in the presence of IL-2 for 8 d. Expanded Vγ4 and Vγ1 γδ T cells were restimulated with anti-CD3 for 6 h for intracellular cytokine staining as described previously. Results represent one of three repeated experiments.



**FIGURE 5.** Naturally activated (CD44<sup>high</sup>) V $\gamma$ 4  $\gamma\delta$  T cells are more cytolytic and suppressive than CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells. **A**, CD44<sup>high</sup>  $\gamma\delta$  T cells were sorted from B6 spleens, stimulated with UC3 and 2.11, respectively, and cultured with IL-2 for 8 d. Expanded V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells were transferred to 96-well round-bottom plates with [<sup>3</sup>H]-pulsed YAC-1 cells, and CTL activity was analyzed by JAM assay. \* $p$  < 0.05; \*\* $p$  < 0.01. **B** and **C**, V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells were expanded with UC3 and 2.11 Ab, respectively, and TCR or NKG2D were blocked with UC7 or CX5 Ab, respectively. Then V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells were mixed with B16 cells at an E:T ratio of 20:1. Cells were cocultured for 24 h. **B**, Live B16 cells were counted and recorded. **C**, IFN- $\gamma$  level in the supernatants of the coculture mediums were assessed by ELISA. **D**, Splenocytes were first stained with allophycocyanin-anti-mouse CD44, and then stimulated with anti-CD3, anti-CD28 for 3 h, and given GolgiPlug for the remaining 3 h. Cells were then fixed and permeabilized for intracellular perforin staining. One representative dot plots was shown based on the gate of V $\gamma$ 1<sup>+</sup> $\gamma\delta$  TCR<sup>+</sup> or V $\gamma$ 4<sup>+</sup> $\gamma\delta$  TCR<sup>+</sup> cells. Results represent 1 of 3 repeated experiments. **E**, Expanded V $\gamma$ 1 or V $\gamma$ 4  $\gamma\delta$  T cells were restimulated with anti-CD3 for 3 h, and given GolgiPlug for an additional 3 h. Cells were then collected, fixed and permeabilized for perforin staining. One of three independent experiments is shown. **F**, B6 Wt mice were inoculated with B16 F0 melanoma cells ( $2 \times 10^5$  cells/mouse). At day 4 after inoculation, cell suspensions were prepared from pools of 10 tissues collected from tumor injection sites, and cells were stained with anti-V $\gamma$ 1 or anti-V $\gamma$ 4 and intracellular perforin. One representative experiment is shown. ns, no significant difference; UD, undetected.

IFN- $\gamma$  in  $\gamma\delta$  T cell-mediated tumor suppression, the level of IFN- $\gamma$  in the supernatant of cocultures was determined by ELISA. The level of IFN- $\gamma$  in V $\gamma$ 4 T cell-B16 coculture medium was significantly higher than that of V $\gamma$ 1 one, and the IFN- $\gamma$  production by V $\gamma$ 4  $\gamma\delta$  T cells was significantly reduced by blockage of TCR or NKG2D (Fig. 5C).

We next determined whether perforin production also differed between V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells. B6 splenocytes or expanded V $\gamma$ 1, V $\gamma$ 4  $\gamma\delta$  T cells were stimulated with anti-CD3 for 6 h, and examined after intracellular staining. Both ex vivo isolated and expanded V $\gamma$ 4  $\gamma\delta$  T cells produced more perforin than did V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 5D, 5E), consistent with the differential cytolytic activity of these two subsets of  $\gamma\delta$  T cells.

Finally, to test whether the infiltrating V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells produce perforin at different levels in situ, B6 mice were injected with B16 melanoma cells. Subsequently, tissues from the tumor injection sites were digested, and infiltrating cells were stained directly with anti-TCR-V $\gamma$  mAbs and intracellular perforin. We found that among tumor infiltrating  $\gamma\delta$  T cells, V $\gamma$ 4  $\gamma\delta$  T cells expressed perforin at higher cell frequencies than did V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 5F), consistent with their protective role in this tumor model.

#### *Eomes is critical for IFN- $\gamma$ production in V $\gamma$ 4 $\gamma\delta$ T cells*

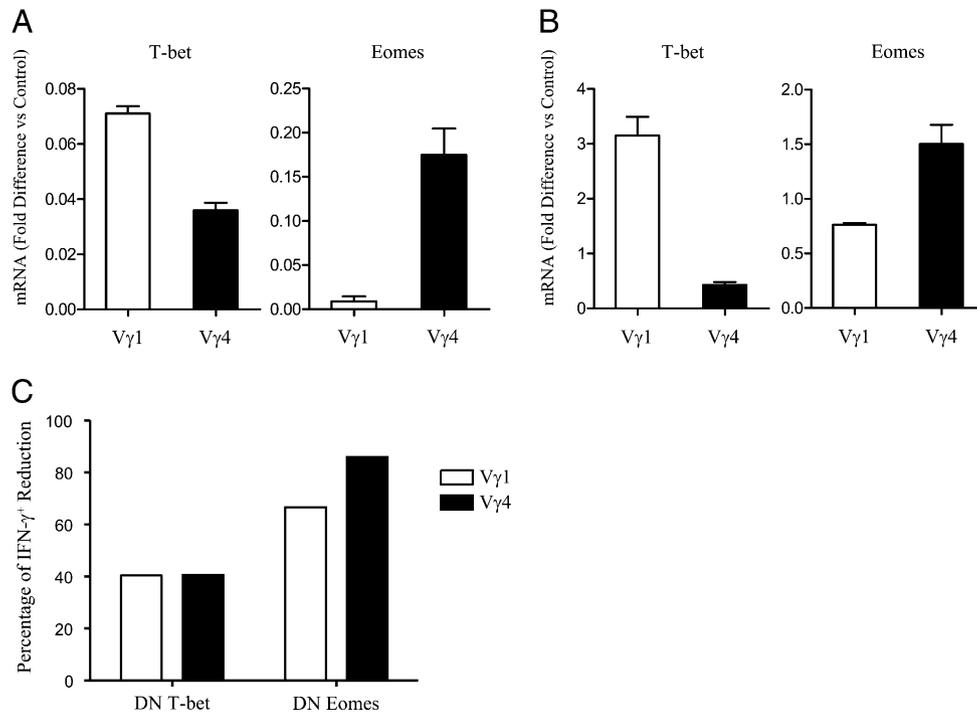
Both T-bet and Eomes are transcription factors that contribute to IFN- $\gamma$  production in  $\gamma\delta$  T cells (26). We hypothesized that differential level of IFN- $\gamma$  production between V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells might be due to the expression level of these two transcription factors. To test this hypothesis, CD44<sup>high</sup> V $\gamma$ 1 and CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells were sorted from B6 splenocytes and

transcription of T-bet and Eomes in these different subsets of  $\gamma\delta$  T cells was examined by real-time PCR analysis. The abundance of T-bet transcripts in CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells was slightly higher than that in CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells (Fig. 6A, left panel). In sharp contrast, Eomes transcription in CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells was ~20-fold higher than that in CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells (Fig. 6A, right panel). Similar expression profiles of T-bet and Eomes were observed in the in vitro expanded  $\gamma\delta$  T cells (Fig. 6B), suggesting that Eomes plays a key role in determining the distinct function of V $\gamma$ 4  $\gamma\delta$  T cells.

To further test this hypothesis, naive V $\gamma$ 1 and V $\gamma$ 4 (CD44<sup>low</sup>)  $\gamma\delta$  T cells from Wt mice were primed in Th1 condition and transduced with DN T-bet, DN-Eomes, or control retrovirus. Five days later, cells were restimulated with anti-CD3 for 6 h for intracellular cytokine staining as described previously. It has to be mentioned that most of these cultured V $\gamma$ 1 and V $\gamma$ 4  $\gamma\delta$  T cells were CD44<sup>high</sup> (data not shown). Interestingly, transduction of DN T-bet reduced frequencies of IFN- $\gamma$  producing V $\gamma$ 4 and V $\gamma$ 1  $\gamma\delta$  T cells partially, and to a similar degree, consistent with the similar levels of T-bet expression in the two types of  $\gamma\delta$  T cells. Transduction of DN-Eomes reduced frequencies of IFN- $\gamma$  producing  $\gamma\delta$  T cells even more drastically, especially among V $\gamma$ 4  $\gamma\delta$  T cells where IFN- $\gamma$  producing cells disappeared almost entirely (Fig. 6C). This result emphasizes the critical role of the transcription factor Eomes in the functional differentiation of tumoricidal V $\gamma$ 4  $\gamma\delta$  T cells.

## Discussion

$\gamma\delta$  T cells have many unique features and functions. Our earlier studies established that splenic  $\gamma\delta$  T cells predominantly produce IFN- $\gamma$  on activation and play a critical role in tumor immune



**FIGURE 6.** Eomes is critical for V $\gamma$ 4  $\gamma$  $\delta$  T cell IFN- $\gamma$  production. *A*, CD44<sup>high</sup> V $\gamma$ 4 and V $\gamma$ 1  $\gamma$  $\delta$  T cells were sorted from B6 mice for real-time PCR analysis. T-bet and Eomes transcripts were normalized against HPRT. One example of three repeated experiments is shown. *B*, V $\gamma$ 1 or V $\gamma$ 4 cells were expanded as described previously and the transcription of T-bet and Eomes was determined by real-time PCR. *C*, Naive (CD44<sup>low</sup>) V $\gamma$ 4 and V $\gamma$ 1  $\gamma$  $\delta$  T cells were sorted from Wt mice and activated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL-4. Cells were then infected with either control, DN T-bet, or DN Eomes retrovirus-GFP as described in *Materials and Methods*. After 48 h, cells were cultured in the presence of IL-2 for additional 3 d, restimulated with anti-CD3 and anti-CD28 for 6 h for intracellular cytokine staining. One representative experiment for the percentage of IFN- $\gamma$ <sup>+</sup> reduction [(% of IFN- $\gamma$ <sup>+</sup> cells from control retrovirus infected – % of IFN- $\gamma$ <sup>+</sup> cells from DN retrovirus infected)/% of IFN- $\gamma$ <sup>+</sup> cells from control retrovirus infected] is shown.

surveillance through providing an early source of IFN- $\gamma$  (4–6). However, the role of TCR-defined different subsets of  $\gamma$  $\delta$  T cells in this capacity has not been identified. In the current study, we provide evidence that spontaneously activated CD44<sup>high</sup> V $\gamma$ 4  $\gamma$  $\delta$  T cells play a critical role in tumor immunity through production of perforin and IFN- $\gamma$ , which in turn depends on Eomes expression.

It has been shown in many experimental systems that TCR-defined subsets of  $\gamma$  $\delta$  T cells have distinctive functions. This is also true for V $\gamma$ 1 and V $\gamma$ 4  $\gamma$  $\delta$  T cells, the two main subsets in peripheral lymphoid tissues. V $\gamma$ 4  $\gamma$  $\delta$  T cells tend to produce more IFN- $\gamma$  and enhance CD4<sup>+</sup> Th1 responses, whereas, V $\gamma$ 1  $\gamma$  $\delta$  T cells seem to produce more IL-4 and promote airway hyperactivity and type 2 inflammation (9–11). However, the role of these two subsets of  $\gamma$  $\delta$  T cells in tumor immunity has not yet been defined. Based on our previous studies that  $\gamma$  $\delta$  T cells provide an early source of IFN- $\gamma$  in tumor surveillance (6), we hypothesized that the Th1-like V $\gamma$ 4  $\gamma$  $\delta$  T cells might be more critical in comparison with V $\gamma$ 1  $\gamma$  $\delta$  T cells in immune response against tumor growth. We first demonstrated in this study that reconstitution of mice deficient in all  $\gamma$  $\delta$  T cells with CD44<sup>high</sup> V $\gamma$ 4  $\gamma$  $\delta$  T cells, but not V $\gamma$ 1  $\gamma$  $\delta$  T cells rendered them better protected against tumor growth (Fig. 1). The importance of our results not only establishes a critical role of V $\gamma$ 4  $\gamma$  $\delta$  T cells in tumor immunity, but also highlights a physiological function of spontaneously activated V $\gamma$ 4  $\gamma$  $\delta$  T cells in vivo. It has been demonstrated from our previous studies that CD44<sup>high</sup>  $\gamma$  $\delta$  T cells express IFN- $\gamma$  and T-bet mRNA (4), but the physiological function of this subpopulation was unclear. Although it is uncertain how  $\gamma$  $\delta$  T cells obtain the spontaneous activation phenotype in vivo, our work thus provides

clear evidence that these activated V $\gamma$ 4  $\gamma$  $\delta$  T cells join the defenses and play an important role in tumor immunity.

What are the molecules derived from activated V $\gamma$ 4  $\gamma$  $\delta$  T cells that mediate the protective effect in tumor immunity? It is well established that certain key components of the host immune response, such as NK, NKT cells, IFN- $\gamma$ , and perforin, are essential for immune surveillance (27–31). In support of this, both IFN- $\gamma$ <sup>-/-</sup> or perforin<sup>-/-</sup> mice are highly susceptible to tumor growth. Likewise, humans with IFN- $\gamma$  signaling mutations are also more susceptible to mycobacterial infections (32, 33). Consistently, we defined in this study IFN- $\gamma$  and perforin as the critical factors produced by activated V $\gamma$ 4  $\gamma$  $\delta$  T cells in tumor immune surveillance. Indeed, in comparison with V $\gamma$ 1  $\gamma$  $\delta$  T cells, V $\gamma$ 4  $\gamma$  $\delta$  T cells were more likely to produce IFN- $\gamma$  and perforin on activation ex vivo and after culture in vitro (Figs. 4, 5), correlated with significantly enhanced cytolytic and the in vitro tumor suppressive functions (Fig. 5A, 5B). Interestingly, both TCR and NKG2D were involved in V $\gamma$ 4-mediated tumor suppression, probably through affecting IFN- $\gamma$  secretion by this subset of  $\gamma$  $\delta$  T cells (Fig. 5B, 5C). Although both expanded V $\gamma$ 1 and V $\gamma$ 4  $\gamma$  $\delta$  T cells upregulated the expression of NKG2D (data not shown), it only involved in V $\gamma$ 4-mediated antitumor effect. Our results thus provide additional evidence that TCR-defined subsets of  $\gamma$  $\delta$  T cells have divergent functional and recognition properties. Reconstitution of TCR  $\delta$ <sup>-/-</sup> mice with IFN- $\gamma$ <sup>-/-</sup> or perforin<sup>-/-</sup> CD44<sup>high</sup> V $\gamma$ 4  $\gamma$  $\delta$  T cells showed that these  $\gamma$  $\delta$  T cells were functionally impaired and no longer protective in vivo (Fig. 3), further supporting a critical role of IFN- $\gamma$  and perforin in mediating the protective effect of V $\gamma$ 4  $\gamma$  $\delta$  T cells in tumor immunity. In contrast, although Granzyme B was found to play an important role in tumor immunity as well (34), we

did not observe differences in its expression level between these two subsets of  $\gamma\delta$  T cells (data not shown). Additional studies will be needed to address the role of Granzyme B in  $\gamma\delta$  T cell-mediated tumor immunity.

What are the mechanisms that might determine the functional differences between TCR-defined  $\gamma\delta$  T cells? It has been shown from our early studies that both T-bet and Eomes contribute to the IFN- $\gamma$  production by  $\gamma\delta$  T cells (26). A striking finding in this study was that the expression level of Eomes in CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells was so much higher than those in CD44<sup>high</sup> V $\gamma$ 1  $\gamma\delta$  T cells (~20-fold), in contrast to the expression of T-bet (Fig. 6A). Expanded V $\gamma$ 4  $\gamma\delta$  T cells also have a higher Eomes level (Fig. 6B). Moreover, forced reduction of Eomes by retroviral infection significantly reduced IFN- $\gamma$  expression in V $\gamma$ 4  $\gamma\delta$  T cells (Fig. 6C), emphasizing the critical role of Eomes in the protective effector function of V $\gamma$ 4  $\gamma\delta$  T cells. Indeed, Eomes knockdown also strongly abrogated V $\gamma$ 1  $\gamma\delta$  T cell IFN- $\gamma$  production (Fig. 6C), most probably due to its effect on T-bet, which was highly expressed in expanded V $\gamma$ 1  $\gamma\delta$  T cells. Consistently, it has been previously reported that the DN Eomes can inhibit T-box factors, including T-bet (25). However, it is unclear what causes the differential expression level of Eomes in V $\gamma$ 4 versus V $\gamma$ 1  $\gamma\delta$  T cells. One possible mechanism would be that different TCR (V $\gamma$ 4 versus V $\gamma$ 1) signals differently on TCR-ligation (35). Additional studies to define the signaling via the TCR in these two subsets of  $\gamma\delta$  T cells are therefore needed.

What is the significance of defining the function of TCR-defined  $\gamma\delta$  T cell subsets in tumor immunity?  $\gamma\delta$  T cells might be better objects for tumor immunotherapy when compared with conventional T cells. They can recognize tumor-associated Ags directly, even when the function of traditional Ag-presenting dendritic cells is impaired or suppressed in tumor microenvironment (3, 36). They also have direct cytolytic functions against tumor cells, which, in sharp contrast to CD8<sup>+</sup> T cells, are not affected by reduced expression of MHC class I molecules in tumor tissues (37). Furthermore, the  $\gamma\delta$  T cells themselves can also serve as APCs, and thus support responses of Ag-specific  $\alpha\beta$  T cells (38). Because different TCR-defined subsets of  $\gamma\delta$  T cells have divergent functions in many disease models, including models of tumor immunity, and are highly effective in small cell numbers, intervention with mAbs against the  $\gamma\delta$  TCR or via transfer of purified  $\gamma\delta$  T cells may provide a novel therapeutic approach against tumors, without significantly affecting general immune competence.

Given the limited number of  $\gamma\delta$  T cells *ex vivo*, we used *in vitro* expanded  $\gamma\delta$  T cells in some of our studies. It has to be emphasized that although we cannot rule out quantitative changes in functional efficiency, expanded  $\gamma\delta$  T cells showed very similar functions as those of *ex vivo*  $\gamma\delta$  T cells, such as IFN- $\gamma$  production (Fig. 4A, 4B), perforin expression (Fig. 5D, 5E), and T-bet/Eomes profiles (Fig. 6A, 6B).

In summary, we have presented novel evidence that CD44<sup>high</sup> V $\gamma$ 4  $\gamma\delta$  T cells play a critical role in preventing tumor formation and tumor growth, and that these cells in particular express high level of Eomes, resulting in their enhanced ability to produce more IFN- $\gamma$  and perforin as well as an increased cytolytic function. These findings not only shed light on the molecular mechanisms of tumor immune surveillance, but also might lead to the development of new strategies for tumor immunotherapy.

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## Disclosures

The authors have no financial conflicts of interest.

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