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T-Bet Expression and Failure of GATA-3 Cross-Regulation Lead to Default Production of IFN-γ by $\gamma\delta$ T Cells[1]

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$\gamma\delta$ T cells predominantly produce IFN-γ upon activation. To determine the basis for default production of IFN-γ by $\gamma\delta$ T cells, we analyzed the transcription factors T-box expressed in T cells (T-bet) and GATA-3. T-bet, absent in naive $\gamma\delta$ cells, was induced upon TCR signaling, with IFN-γ production. T-bet also regulated IL-4 synthesis, as $\gamma\delta$ cells isolated from T-bet-deficient mice displayed enhanced IL-4 levels with reduced IFN-γ production. Notably, T-bet expression after TCR signaling in $\gamma\delta$ cells was not down-regulated by IL-4, in conjunction with a higher ratio of T-bet:GATA-3 expression than that found in CD4$^+$ T cells. Indeed, overexpression of GATA-3 failed to inhibit IFN-γ secretion in $\gamma\delta$ cells to the degree seen in CD4$^+$ T cells. These results indicate that T-bet enhances IFN-γ secretion and suppresses IL-4 secretion in $\gamma\delta$ cells, and that GATA-3 fails to counterbalance T-bet-mediated IFN-γ production, accounting for the default synthesis of IFN-γ by these T lymphocytes. ***The Journal of Immunology*****, 2002, 168: 1566–1571.**

Differentiation of CD4$^+$ Th1 and Th2 $\alpha\beta$ cells is tightly cross-regulated, so that development of one subset is inhibited by cytokines produced by the other (1, 2). Significant progress has been made in understanding the molecular mechanisms of this cross-regulation. T-box expressed in T cells (T-bet),[3] a newly identified Th1-specific transcription factor selectively expressed in Th1 or Tc1 cells, plays a central role in Th1 development by activating Th1 genetic programs and repressing Th2 cytokine synthesis (3). GATA-3, in contrast, is a Th2-specific transcription factor selectively expressed in Th2 cells (4, 5). It plays a major role in specifying the Th2 phenotype by promotion of Th2 cytokine secretion and inhibition of IFN-γ production through repression of IL-12 signaling (4, 6–8).

Like $\alpha\beta$ T cells, $\gamma\delta$ T cells differentiate into IFN-γ (Th1-like)- and IL-4 (Th2-like)-producing cells (9, 10); however, the molecular mechanisms underlying their polarization have yet to be defined. Recently, we described differentiation of murine splenic $\gamma\delta$ T cells in vitro using priming conditions that have been well established for $\alpha\beta$ CD4$^+$ T cells (11). In contrast to CD4$^+$ T cells, splenic $\gamma\delta$ T cells default toward type 1 cytokine production, predominantly producing IFN-γ upon activation, even in the presence of IL-4 and in the absence of IL-12. These results indicate that splenic $\gamma\delta$ T cells differ fundamentally from $\alpha\beta$ CD4$^+$ T cells in their response to exogenous cytokines and suggest the possibility that the predominant IFN-γ production by $\gamma\delta$ T cells plays an important role in protection against intracellular pathogens or tumors.

To further explore the molecular mechanisms underlying the predominant Th1-like phenotype of $\gamma\delta$ T cells, we analyzed T-bet expression in comparison with that of GATA-3 and the roles of these transcription factors in $\gamma\delta$ T cell differentiation. We demonstrate that T-bet enhances IFN-γ secretion and suppresses IL-4 secretion in $\gamma\delta$ cells, and that GATA-3 fails to counterbalance T-bet-mediated IFN-γ production, forming the molecular basis of predominant production of IFN-γ by this lineage of T lymphocytes.

Materials and Methods

Reagents

C57BL/6J (B6) and C57BL/6J-*Tcrb*^{tm1Mom} (B6 TCR β-chain-deficient (TCR β-deficient)) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6/129 T-bet-deficient mice have been described previously (20). All animals were maintained under specific pathogen-free conditions and used at 4–6 wk of age. Recombinant murine IL-2, IL-4, and IL-12 were purchased from R&D Systems (Minneapolis, MN). Anti-mouse mAb used for phenotypic and cytokine analyses were purchased from BD PharMingen (San Diego, CA). Purified anti-T-bet mAb (4B10, IgG1) and rabbit anti-T-bet serum were used as previously described (3).

Polarization and flow cytometry of $\gamma\delta$ and CD4$^+$ T cells

$\gamma\delta$ and CD4$^+$ T cells were purified from splenocytes of B6 TCR β-deficient and B6 wild-type mice, respectively, using a MACS (Miltenyi Biotec, Auburn, CA) as described previously (11) and were sorted into naive (CD44^{low}CD62 ligand^{high}) and activated (CD44^{high}) populations by flow cytometry (Vantage; BD Biosciences, San Jose, CA) (12, 13). Naive $\gamma\delta$ T cells were also sorted from B6/129 T-bet-deficient mice and their wild-type littermates using the same surface markers. After sorting, cell purity was >99%, as determined by cell surface markers. T cells were cultured in complete Click's medium with plate-coated anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of IL-2 (neutral condition), IL-12 (5 ng/ml), and anti-IL-4 (10 μg/ml; Th1-priming condition), or IL-4 (20 ng/ml) and anti-IFN-γ (10 μg/ml; Th2-priming condition). IL-2 was added to the culture medium on day 3. Cells were collected after 3 days of culture and were analyzed for T-bet and GATA-3 protein and mRNA detection by immunoblots and Northern analysis, respectively. Primed cells were restimulated on day 6 with coated anti-CD3 and soluble anti-CD28 in the

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[3] Abbreviations used in this paper: T-bet, T-box expressed in T cells; GFP, green fluorescent protein; TCR β deficient, TCR β-chain deficient.

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presence of brefeldin A (1 μM; BD PharMingen) for 6 h and used for intracellular cytokine and T-bet staining as previously described (11), the latter using an anti-T-bet mAb (3), followed by FITC-anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). IFN-γ- and T-bet-stained cells were also identified by confocal microscopy. T-bet was stained in green (FITC) and was located in the nucleus, whereas IFN-γ was stained in red (PE) and was retained in the cytoplasm due to culture with brefeldin A.

Immunoblots and Northern analysis

Nuclear extracts from sorted (naive and activated) or cultured $\gamma\delta$ and $CD4^+$ T cells were prepared, and their protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, CA). The same amount of protein was loaded in each lane, and immunoblots were performed as previously described (14) using rabbit anti-T-bet (3). The same blots were then stripped and probed with mouse anti-GATA-3 mAbs (Santa Cruz Biotechnology, Santa Cruz, CA). Total cellular RNA was prepared, and Northern analysis was performed as previously described (15) using as probes an ~2500-bp fragment of murine T-bet cDNA and an ~360-bp fragment of murine GATA-3 cDNA (*Cla*I and *Bgl*II) (3, 11).

Retroviral transfection of $\gamma\delta$ T cells with GATA-3

The bicistronic retrovirus coexpressing green fluorescent protein (GFP) and GATA-3 or a control vector (retrovirus containing only GFP) was provided by Dr. A. O'Garra from DNAX (Palo Alto, CA) (7). $CD4^+$ T cells or $\gamma\delta$ T cells, sorted by flow cytometry, were activated with anti-CD3 and anti-CD28 in the presence of IL-2, IL-12 plus anti-IL-4, or IL-4 plus anti-IFN-γ for 24 h. Cells were then infected with viral supernatant collected from the Phoenix-Eco packaging cell line (gift from Drs. B. Lu and R. Flavell, Yale University (New Haven, CT), originally obtained from Dr. G. Nolan, Stanford University (Stanford, CA)) supplemented with the same cytokine conditions as on day 1 (described above), according to the previously described protocol (6, 7). Cells were cultured with fresh medium with IL-2 on day 3, and GFP-positive cells were sorted on day 7. Sorted GFP cells were further activated with anti-CD3 and anti-CD28 in the presence of brefeldin A for IFN-γ or IL-4 staining. Equal numbers of $CD4^+GFP^+$ T cells and $\gamma\delta^+GFP^+$ cells were sorted after retrovirus infection with the GATA-3-GFP vector, and their GATA-3 expression was analyzed. Similar levels of GATA-3 expression were observed in $CD4^+GFP^+$ T cells and $\gamma\delta^+GFP^+$ cells (data not shown).

Results

T-bet, but not GATA-3, is expressed in activated $\gamma\delta$ T cells

$\gamma\delta$ T cells can be divided into naive and activated phenotypes using cell surface markers and turnover rates in vivo, analogous to $CD4^+$ T cells (12, 13). We first explored the expression of T-bet in naive and activated populations of $\gamma\delta$ T cells and compared its expression to that of GATA-3. $\gamma\delta$ T cells and $CD4^+$ T cells were isolated from B6 TCR β-deficient mice and B6 wild-type mice and directly sorted into naive ($CD44^{low}$CD62 ligand^{high}) and activated ($CD44^{high}$) subsets (12, 13). The $CD4^+$ Th1 clone AE7 and the Th2 clone D10, activated with PMA plus ionomycin or left untreated, were used as controls. T-bet mRNA and protein were found in activated, but not naive, $\gamma\delta$ T cells, analogous to its expression in control cloned Th1 cells (AE7; Fig. 1, *A* and *B*, *upper panels*) or in an ex vivo sorted population of $CD4^+$ T cells (Fig. 1, *A* and *C*, *upper panels*) (3). As expected, T-bet mRNA and protein were absent in cloned Th2 cells (D10; Fig. 1, *A* and *B*, *upper panels*). IFN-γ mRNA, measured by RT-PCR, was spontaneously expressed in sorted activated, but not naive, $\gamma\delta$ T cells (data not shown), consistent with previous findings by us and others that $\gamma\delta$ T cells predominantly produce IFN-γ upon activation in vitro (11, 16). These data indicate that in $\gamma\delta$ T cells, as in $CD4^+$ T cells, T-bet protein expression is correlated with IFN-γ gene transcription. More importantly, perhaps, T-bet expression can also serve to distinguish naive and activated $\gamma\delta$ T cells identified by surface phenotype and is correlated with effector phenotype (IFN-γ expression) in the latter.

By contrast, while GATA-3 mRNA was expressed in activated $\gamma\delta$ T cells, its protein was not detected; however, both the mRNA and protein were identified in the control Th2 clone D10 (Fig. 1, *A* and *B*, *lower panels*) and in ex vivo sorted $CD4^+$ T cells, including naive $CD4^+$ T cells. This finding suggests the possibility that GATA-3 is post-transcriptionally regulated in activated $\gamma\delta$ T cells in vivo. The lack of identification of GATA-3 protein in extracts of activated $\gamma\delta$ T cells did not appear to be a consequence of a technical variable. GATA-3 mRNA was present at a lower level in naive $CD4^+$ T cells than in activated $\gamma\delta$ T cells (Fig. 1*A*, *lower panel*, compare *lane 6* to *lane 7*; note that the control ribosomal RNAs are equivalent), yet the former did express GATA-3 protein, albeit detectable in small amounts (Fig. 1*C*, *lower panel*). Of note, neither GATA-3 protein nor GATA-3 mRNA was detected in naive $\gamma\delta$ T cells, a finding different from that observed in naive $CD4^+$ T cells, which express a low level of GATA-3 mRNA and protein (compare Fig. 1, *A* and *B*, to Fig. 1*C*, *lower panels*) (5).

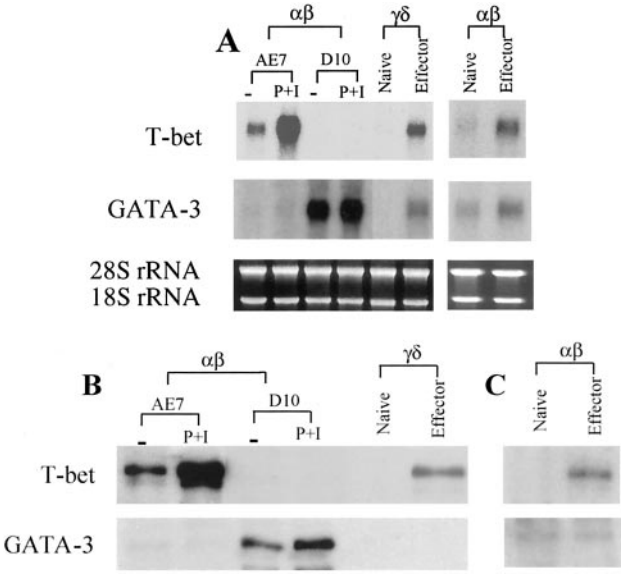

FIGURE 1. T-bet, but not GATA-3, is expressed in activated $\gamma\delta$ T cells. *A*, Total cellular RNA from naive and activated $\gamma\delta$ T cells was prepared for T-bet and GATA-3 mRNA analysis by Northern blots. *B*, Nuclear extracts from naive and activated $\gamma\delta$ cells were harvested, and the same membrane was used for T-bet and GATA-3 protein expression as measured by immunoblots. *C*, Nuclear extracts from naive and activated $CD4^+$ T cells were used for immunoblot. The membrane was first probed with anti-T-bet Abs, then stripped and reprobed for GATA-3 protein expression. The data shown are representative of three experiments.

T-bet expression directly correlates with IFN-γ production in $\gamma\delta$ T cells

T-bet expression is directly correlated with IFN-γ production in $CD4^+$ T cells (3). Based upon our previous observations that $\gamma\delta$ T cells predominantly produce IFN-γ upon stimulation regardless of cytokines in the priming environment (i.e., even in the presence of IL-4) (11), we next investigated whether T-bet expression would correlate with IFN-γ production in $\gamma\delta$ T cells. Toward this end, naive $\gamma\delta$ T cells sorted by flow cytometry were activated with anti-CD3 and anti-CD28 under a neutral condition (IL-2 alone) or under Th1 (IL-2 plus IL-12 and anti-IL-4) or Th2 (IL-2 plus IL-4 and anti-IFN-γ) priming conditions for 3 days. Cells were activated as described above and restimulated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h, followed by intracellular IFN-γ and T-bet staining. Upon restimulation, the percentages of $\gamma\delta$ T cells that produced IFN-γ were 35, 57, or 24% under neutral, Th1, or Th2 priming conditions, respectively, similar to our previous results (11). As expected, >99% of cells in the IFN-γ-positive gate under all three conditions expressed the T-bet

protein as determined by intracellular staining (Fig. 2*A*). Note that only double-positive cells are shown in the histogram, as only IFN-γ-positive cells were gated. T-bet was expressed in the nucleus (green fluorescence) of IFN-γ-producing cells, as determined by confocal microscopy (Fig. 2*B*). These data add weight to the linkage between T-bet expression and IFN-γ production in γδ T cells.

Activation of naive γδ T cells in the presence of IL-12 or IL-4 leads to T-bet expression

Recent work has shown that T-bet is selectively expressed in Th1, but not Th2, αβ T cells (3). By contrast, however, we have shown that γδ T cells produce IFN-γ upon activation, even in the presence of IL-4 and in the absence of IL-12, conditions that induce Th2 polarization in $CD4^+$ T cells (11). To explore this potential dichotomy and to determine the expression pattern of T-bet in recently primed T cells, naive γδ and $CD4^+$ T cells sorted by flow cytometry were activated with anti-CD3 and anti-CD28 and stimulated with cytokines under neutral, Th1, or Th2 priming conditions. As noted above, neither T-bet nor GATA-3 mRNA or protein was detected in naive γδ T cells (see Fig. 1, *A* and *B*). T-bet protein was induced by TCR signaling and was strongly enhanced in the presence of IL-12 (Fig. 3, *upper panel*). Moreover, TCR signaling, even in the presence of IL-4 and in the absence of exogenously added IL-12, led to T-bet expression in γδ T cells, a finding in accord with our previous observation that these same conditions promoted IFN-γ production (11). Notably, TCR signaling in the presence of IL-4 also led to a low level of T-bet synthesis in $CD4^+$ T cells (Fig. 3, *upper panel*). The latter result is at odds with the previous finding that T-bet protein expression can be extinguished in the presence of IL-4; however, this finding probably stems from differences in the background strains of mice used in these experiments (B6 mice used herein vs BALB/c mice used in the earlier work (3)).

Interestingly, GATA-3 protein was detected in both CD4 and γδ T cells after TCR triggering in the presence of IL-4 (Th2-priming condition); however, the protein level was substantially less in γδ T cells than in $CD4^+$ T cells (Fig. 3, *lower panel*). Moreover, the ratio of T-bet:GATA-3 protein expression after TCR triggering in the presence of IL-4 was reversed in γδ cells compared with $CD4^+$ T cells (Fig. 3, compare *upper* and *lower panels*). Here, we note that the same numbers of both types of cells were used to prepare nuclear extracts for immunoblots and the relative loading amounts were standardized, as the same membrane was first probed for T-bet expression then stripped and reprobed for analysis of GATA-3 protein levels. These findings further emphasize that, in contrast to $CD4^+$ T cells, naive γδ T cells largely default toward T-bet expression after TCR signaling despite the presence of IL-4.

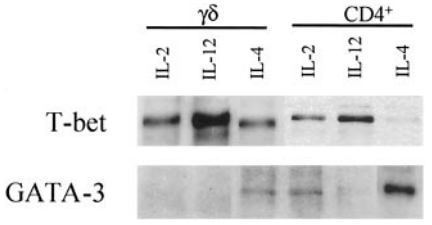

FIGURE 3. Activation of naive γδ T cells in the presence of IL-12 or IL-4 leads to T-bet expression. Naive γδ and $CD4^+$ T cells were sorted from TCR β-deficient and wild-type B6 mice, respectively, by flow cytometry, and activated with anti-CD3 and anti-CD28 in the presence of IL-2 (neutral condition), IL-12 plus anti-IL-4 (Th1 condition), or IL-4 plus anti-IFN-γ (Th2 conditions). After 3 days of culture, cells were collected for the preparation of nuclear extracts, and the same membrane was used for analysis of T-bet and GATA-3 by immunoblots. The data shown are representative of two experiments.

Overexpression of GATA-3 induces IL-4 production but fails to down-regulate IFN-γ secretion by γδ T cells

The above results indicate that γδ T cells predominantly express T-bet and produce IFN-γ even in the presence of IL-4. One possibility to help explain the functional dominance of this transcription factor in γδ cells is that the expression level of GATA-3, even after IL-4 signaling, is too low to counterbalance the T-bet effect. To test this hypothesis, we infected primary γδ T cells with a bicistronic retrovirus expressing GATA-3 and GFP, a system that has been previously used in $CD4^+$ T cells to demonstrate that overexpression of GATA-3 blocks IFN-γ production but augments IL-4 synthesis (4–6). After retrovirus infection, two fractions of GFP-positive cells were present, GFP^{high} and GFP^{low}. In preliminary studies GFP intensity correlated with GATA-3 levels (data not shown). GATA-3 overexpression (GFP^{high}) in $CD4^+$ T cells significantly reduced IFN-γ production under all three priming conditions compared with GFP^{low} cells, with a significant reduction in the percentage of IFN-γ-secreting cells (Fig. 4*A* and Table I; $p < 0.001$), consistent with published results (4–6). The results from this experiment were representative of three performed. By contrast, overexpression of GATA-3 (GFP^{high}) had significantly less effect on IFN-γ production by γδ T cells, with no significant reduction in the percentage of IFN-γ-producing cells (comparison of GFP^{high} to GFP^{low} cells; Fig. 4*A* and Table I). Again, these data are representative of three separate experiments. Here, it should be

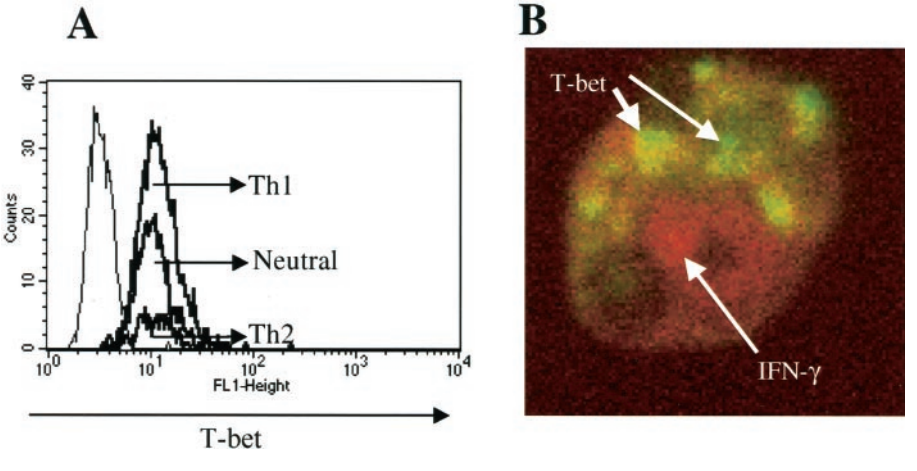

FIGURE 2. T-bet expression directly correlates with IFN-γ production in γδ T cells. Naive γδ T cells were activated as described in *Materials and Methods.* After 5 days in culture cells were restimulated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h. Cells were then used for intracellular IFN-γ and T-bet staining. *A*, T-bet-positive cells within the IFN-γ-positive gate are shown as a histogram. *B*, One example of double-positive cells analyzed with confocal microscopy is shown. The data shown are representative of five experiments.

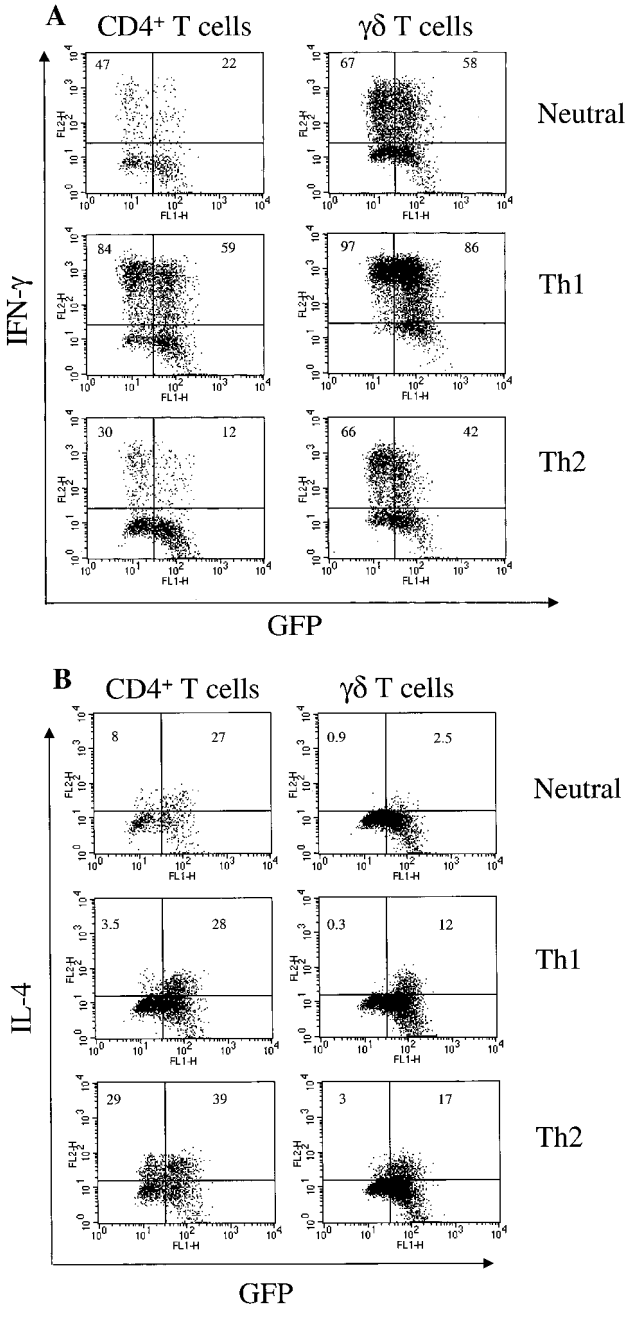

FIGURE 4. Differential regulation of IFN-γ production by GATA-3 in $CD4^+$ compared with γδ T cells. Purified $CD4^+$ T cells or γδ T cells (by FACS sorting) were activated with anti-CD3 and anti-CD28 under neutral, Th1-, or Th2-priming conditions for 24 h. Cells were then infected with GATA-3-GFP, as described in *Materials and Methods*. Cells were sorted on day 7 and further activated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h for IFN-γ or IL-4 staining. The percentage of IFN-γ-positive cells (*A*) and IL-4-positive cells (*B*) in GFP^{high} vs GFP^{low} cells are shown. The data shown are representative of three experiments.

noted that overexpression of GATA-3 only changed the percentage of IFN-γ-producing cells, but not the mean fluorescence intensity, as an indicator of cytokine production per single cell (Fig. 4*A*). These results indicate that while IFN-γ synthesis in γδ cells is dependent upon T-bet expression, production of this cytokine is not cross-regulated by GATA-3 to the degree found in $CD4^+$ cells (6, 7).

Notably, GFP^{high}, but not GFP^{low}, γδ T cells primarily produced IL-4, indicating a dependence on the dose of GATA-3 in IL-4 production by γδ T cells (Fig. 4*B*). Thus, GATA-3 can promote IL-4 synthesis in γδ T cells; however, as noted above, it does not effectively block IFN-γ production as in $CD4^+$ T cells.

T-bet deficiency significantly reduced IFN-γ production and enhanced IL-4 production by γδ T cells

T-bet-deficient mice were recently produced by gene targeting and were described in detail elsewhere (20). To firmly establish the role of T-bet in γδ T cell differentiation, naive γδ T cells were sorted from T-bet-deficient mice and wild-type littermate controls and activated with anti-CD3 and anti-CD28 in the presence of different priming conditions as described above (neutral, Th1, or Th2). After 4 days of culture, cells were restimulated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h and stained for IFN-γ and IL-4. The percentage of γδ T cells from T-bet-deficient mice that produced IFN-γ was significantly less under all priming conditions compared with cells from wild-type mice (Fig. 5, compare *upper three panels* to *lower three panels*), with no significant change in mean fluorescence intensity, indicating an impairment of γδ T cell differentiation in T-bet-deficient mice. Strikingly, IL-4 secretion was significantly higher in T-bet-deficient γδ T cells compared with wild-type cells. These results indicate that T-bet is a critical factor in abrogation of IL-4 production by γδ T cells, contributing to the default pathway of IFN-γ production by these cells after TCR triggering. It also promotes IFN-γ production but is not absolutely required for synthesis of this cytokine.

Discussion

T-bet is a Th1-specific transcription factor, inducing IFN-γ production and promoting $CD4^+$ Th1 development though binding to the promoter of the gene for this cytokine (3, 17, 20). By contrast, GATA-3 is a Th2-specific transcription factor that can promote Th2 cytokine secretion and inhibit IFN-γ production (4–6). The balance between T-bet and GATA-3 is critical for the outcome of $CD4^+$ T cell differentiation (18); however, the precise role of T-bet and GATA-3, and the relationship between these two transcription factors, in γδ T cell differentiation are unknown. We and others have previously demonstrated that murine γδ T cells predominantly produce IFN-γ upon activation (11, 16). In the present study we showed that the molecular basis for default production of IFN-γ by splenic γδ cells, in comparison to $CD4^+$ T cells, is their high level of T-bet expression vs that of GATA-3, with failure of the latter to effectively inhibit production of this cytokine.

Given the phenotype of γδ T cells, we hypothesized they would strongly express T-bet, in contrast to GATA-3, and that the former would promote IFN-γ production and inhibit IL-4 secretion by these cells. Several of our findings support this hypothesis. First, activated splenic γδ T cells analyzed ex vivo expressed T-bet protein, but not GATA-3, although both T-bet and GATA-3 mRNA were present in this population of γδ T cells (Fig. 1). Moreover, the expression pattern of T-bet in γδ T cells upon activation was also compatible with their default toward a Th1 pattern of cytokine production. T-bet protein, not expressed in naive γδ T cells, was up-regulated upon TCR signaling and further enhanced by IL-12 and was directly correlated with IFN-γ secretion, as determined by flow cytometry and confocal microscopy (Fig. 2). Third, T-bet was not down-regulated in the presence of IL-4 (Fig. 3), consistent with the microscopic and flow cytometric observations that cells primed in this manner also had simultaneous expression of T-bet and IFN-γ, and consistent with our previous observation that IL-4 does not extinguish IFN-γ production in γδ T cells at the population level (11). This finding is in sharp contrast to that seen $CD4^+$ T cells, where T-bet expression is decreased in the presence of Th2

Table I. *Differential regulation of IFN-γ production by GATA-3 in CD4$^+$ compared to γδ T cells*[a]

Priming Condition	γδ T Cells			CD4$^+$ T Cells		
	GFP^{low}	GFP^{high}	Decrease (%)	GFP^{low}	GFP^{high}	Decrease (%)
Neutral	63 ± 5	55 ± 7	13 ± 6	44 ± 6	20 ± 3	67 ± 18[b]
Th1	94 ± 3	80 ± 8	15 ± 8	82 ± 4	56 ± 5	31 ± 4[b]
Th2	62 ± 3	45 ± 5	28 ± 6	28 ± 2	13 ± 3	54 ± 11[b]

[a] Purified CD4$^+$ T cells or γδ T cells were activated with anti-CD3 and anti-CD28 under neutral, Th1-, or Th2-priming conditions for 24 h. Cells were then infected with GATA-3-GFP, as described in *Materials and Methods.* Cells were sorted at day 7 and further activated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h for IFN-γ or IL-4 staining. The mean ± SD percentage of IFN-γ-positive cells out of corresponding GFP^{high} or GFP^{low} cells, and the mean ± SD percentage of decrease in GFP^{high} versus GFP^{low} cells are shown.

[b] Values of $p < 0.001$ for the comparison of the percentage of IFN-γ$^+$CD4$^+$ T cells between the GFP^{high} and GFP^{low} groups. There was no statistically significant reduction in the percentage of IFN-γ$^+$γδ$^+$ T cells between the GFP^{high} and GFP^{low} groups.

priming conditions (3, 17). Finally, genetic depletion of T-bet led to significant impairment of Th1 differentiation, with reduction in the percentage of IFN-γ-positive cells, concomitant with greatly enhanced IL-4 secretion (Fig. 5), suggesting that T-bet functions to both promote IFN-γ production and inhibit IL-4 production in γδ cells. Although T-bet inhibits GATA-3 expression in CD4$^+$ T cells (20), and overexpression of T-bet directly suppresses IL-4 production in established Th2 cells (3), we have not yet analyzed the level of GATA-3 in γδ cells from T-bet-deficient mice. Thus, at present we cannot exclude the possibility that T-bet inhibits IL-4 production indirectly through its promotion of IFN-γ production.

GATA-3 is a critical transcription factor for CD4$^+$ Th2 cell development (4–6). Transfection of GATA-3 at an early stage into Th1-primed CD4$^+$ T cells results in induction of Th2 cytokines and inhibition of IFN-γ production, suggesting that IFN-γ is cross-regulated by GATA-3 in CD4$^+$ T cell differentiation (6, 7). Given these observations, in the face of our previous finding that γδ T cells, despite their predominant Th1-like phenotype, express GATA-3 mRNA at a level similar to that in CD4$^+$ T cells in the presence of IL-4 (11), we sought to determine GATA-3 protein expression in relation to that of T-bet and its function in cytokine production in the former lineage.

We identified GATA-3 mRNA, but not its protein, in activated γδ T cells, suggesting post-transcriptional regulation of GATA-3 in γδ T cells. The lack of identification of protein in the extracts of activated γδ T cells did not appear to be a consequence of loading

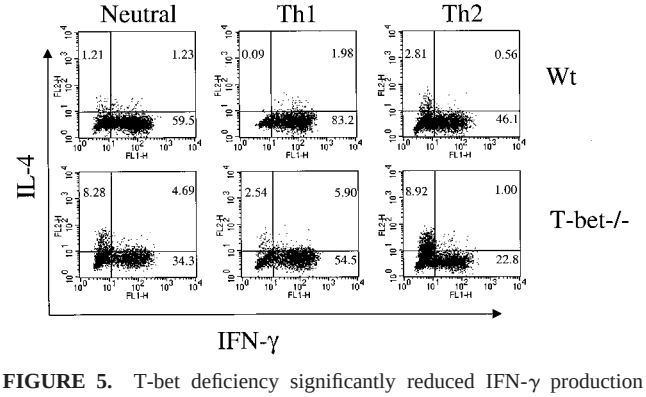

FIGURE 5. T-bet deficiency significantly reduced IFN-γ production and enhanced IL-4 secretion by γδ T cells. Naive γδ T cells were sorted from splenocytes of T-bet-intact (Wt) or T-bet-deficient mice, and activated with anti-CD3 and anti-CD28 in the presence of IL-2 (neutral condition), IL-12 plus anti-IL-4 (Th1 condition), or IL-4 plus anti-IFN-γ (Th2 condition). After 5 days in culture, cells were restimulated with anti-CD3 and anti-CD28 in the presence of brefeldin A for 6 h. Cells were then used for intracellular IFN-γ and IL-4 staining. The data shown are representative of two experiments.

variability or a too-short exposure time, because extracts were loaded (the same membrane was used for analysis of both T-bet and GATA-3 protein expression), and because the GATA-3 protein was easily detected in extracts of the control Th2 clone using the same exposure time of the membrane. Moreover, GATA-3 mRNA was detected at a lower level in naive CD4$^+$ T cells in comparison with effector γδ T cells (compare *lane 6* to *lane 7*, *lower panel* of Fig. 1*A*), yet the latter cells expressed the protein product (Fig. 1*C*, *lower panel*).

GATA-3 was only detectable in the presence of both IL-4 and a strong TCR signaling in vitro, as in CD4$^+$ T cells (Fig. 3). However, even under these conditions, T-bet expression was stronger than that of GATA-3, and the reverse was true in CD4$^+$ T cells. While it is difficult to draw quantitative conclusions about the relative levels of T-bet and GATA-3 protein based upon analyses using immunoblots, their differential expression in γδ vs CD4$^+$ T cells provides an additional clue to help explain the predominant Th1-like phenotype of the former: the ratio of T-bet:GATA-3 in IL-4-primed naive γδ T cells was the reverse of that found in naive CD4$^+$ T cells activated under the same priming conditions (Fig. 3). While our experiments with overexpression of GATA-3 clearly show that this factor strongly influences IL-4 secretion in γδ T cells akin to that seen in CD4$^+$ T cells (data not shown), GATA-3 nevertheless fails to negatively influence IFN-γ production in γδ T cells compared with that seen in CD4$^+$ T cells (Fig. 4*A* and Table I), presumably due to its inability to antagonize T-bet function. Although we do not know whether overexpression of GATA-3 reduces T-bet levels in γδ T cells, GATA-3 fails to cross-regulate T-bet-mediated IFN-γ production, unlike CD4$^+$ T cells. This lack of cross-regulation, presumably in conjunction with the relative paucity of GATA-3 in these cells compared with CD4$^+$ T cells, accounts for the default production of IFN-γ by the former. We would add the cautionary note that these studies are with cell populations, and it is not yet clear whether T-bet and GATA-3 are expressed in the same cell after IL-4 exposure.

It has to be emphasized that although it is very well established that T-bet is a critical Th1 transcription factor for CD4$^+$ T cells, genetically depletion of T-bet has no effect on CD8$^+$ T cell IFN-γ production (20), strongly arguing that the effect of T-bet in CD4$^+$ T cells cannot be simply copied to the other lineage of T cells. Similarly, CD4$^+$ T cells and CD8$^+$ T cells have different requirements for IL-12 (19). Our results further support the lineage difference of regulation and function of T-bet in the light of different cross-regulation with GATA-3.

In summary, we have shown that the high level of T-bet expression dictates the phenotype of murine splenic γδ T cells toward default production of IFN-γ. By contrast, the paucity of IL-4 synthesis is secondary to the low level of GATA-3 expression in

these cells. However, while an increase in GATA-3 expression is able to augment IL-4 levels in γδ T cells, it fails to concomitantly down-regulate IFN-γ production, unlike that seen in $CD4^+$ T cells, and an uncoupling of the functional antagonism between GATA-3 and T-bet together form the molecular basis for the default production of IFN-γ by γδ T cells. Because γδ T cells play an important role in regulating αβ T cells, probably through their cytokine production early after activation, understanding the molecular mechanisms of γδ T cell differentiation will likely shed light on the proper control of immune responses.

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