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## Cutting Edge: Immunity and IFN- $\gamma$ Production during *Listeria monocytogenes* Infection in the Absence of T-bet<sup>1</sup>

Sing Sing Way\* and Christopher B. Wilson<sup>2\*</sup>

*The T-box transcription factor T-bet is an important regulator of IFN- $\gamma$  production in all cell types and is considered to be essential for the generation of CD4 Th1 T cells. IFN- $\gamma$  in turn plays a critical role in immunity to many infectious agents. In this study, we demonstrate that T-bet is not required for host resistance to primary *Listeria monocytogenes* (LM) infection. In the innate immune phase, control of LM replication, serum IFN- $\gamma$ , and numbers of IFN- $\gamma$ -producing NK cells were similar in T-bet-deficient and control mice. In the adaptive immune phase, there was no defect in bacterial clearance or in the numbers of LM-specific IFN- $\gamma$ -producing CD8 T cells in T-bet-deficient mice and only a modest, although significant, reduction in the numbers of Th1 CD4 T cells and IFN- $\gamma$  secretion by CD4 T cells. Thus, host resistance and the generation of IFN- $\gamma$ -producing cells in response to LM infection are not substantially compromised in the absence of T-bet. The Journal of Immunology, 2004, 173: 5918–5922.*

Interferon  $\gamma$  is a pleiotropic cytokine that plays a critical role in both the innate and adaptive immune responses to a variety of infectious agents. During infection with the intracellular bacterial pathogen *Listeria monocytogenes* (LM),<sup>3</sup> IFN- $\gamma$  is produced in the first few days following infection by NK cells and dendritic cells, and later as adaptive immunity develops, IFN- $\gamma$  is produced by Ag-specific CD4 Th1 and CD8 T cells (1–4). T-bet, a T-box transcription factor originally identified in Th1 CD4 T cells (5), is generally regarded as the master regulator of IFN- $\gamma$  production in CD4 T cells (6), and is also an important regulator of IFN- $\gamma$  production in CD8 T cells,  $\gamma\delta$  T cells, NK cells, and dendritic cells (7–11). Other studies have suggested that T-bet may play additional roles in optimal CD8 T and NK cell effector function independent from its ability to regulate IFN- $\gamma$  production (8, 9). Because many of these findings were derived solely in vitro, the biological significance of

T-bet-regulated IFN- $\gamma$  production for host immunity to infection in vivo is less certain.

To our knowledge, only four studies have thus far directly addressed the role of T-bet and its effects on IFN- $\gamma$  production in response to infection in vivo. A deficiency in T-bet on an otherwise resistant mouse background (C57BL/6) confers susceptibility to infection with *Leishmania major*, as determined by the degree of footpad swelling; however, differences in susceptibility in this model became evident only after the fourth week postinfection (6). Furthermore, in response to *L. major* infection, Ag-specific CD4 T cells from T-bet-deficient mice produced no IFN- $\gamma$  and increased amounts of the Th2 cytokines IL-4 and IL-5. These results support in vitro observations indicating that T-bet is required for production of IFN- $\gamma$  and to repress Th2 cytokine production by CD4 T cells (5). For Ag-specific CD8 T cells, T-bet is also required for the optimal production of IFN- $\gamma$  following lymphocytic choriomeningitis virus (LCMV) infection (8, 12). Furthermore, T-bet was required for the optimal production of IFN- $\gamma$  in the first 2 days by NK cells (9) following acute infection with murine CMV (MCMV), but viral clearance was not affected in T-bet-deficient mice. These results suggest that T-bet may be important for efficient IFN- $\gamma$  production by NK cells in the innate phase of host defense to infection and by pathogen-specific CD4 and CD8 T cells in the adaptive phase, whereas the relative contribution of T-bet in host resistance to primary infection may vary between pathogens.

LM primarily infects macrophages and hepatocytes following experimental infection in mice. The host immune mediators that confer innate and adaptive immunity following LM infection have been well characterized, and LM infection serves as a model system to examine innate and adaptive immunity to intracellular pathogens. An essential role for IFN- $\gamma$  in host resistance to LM has been clearly demonstrated by the extreme susceptibility of IFN- $\gamma$  or the IFN- $\gamma$  receptor knockout mice (13, 14). In primary LM infection, the principle cellular source of IFN- $\gamma$  in the first few days is NK cells, because of the increased susceptibility and decreased IFN- $\gamma$  production in response to

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<sup>3</sup> Abbreviations used in this paper: LM, *Listeria monocytogenes*; LCMV, lymphocytic choriomeningitis virus; MCMV, murine CMV; LLO, listeriolysin O; WT, wild type.

LM infection in mice depleted of NK cells (1). Therefore, to better define the role of T-bet in the regulation of IFN- $\gamma$  production in the innate and adaptive phases of the immune response to infection, we examined the importance of T-bet for proper IFN- $\gamma$  production and host resistance to primary LM infection.

## Materials and Methods

### Mice

T-bet-deficient (6) mice used in this study were kindly provided by Dr. L. H. Glimcher (Harvard School of Public Health, Cambridge, MA) and had been backcrossed either onto the BALB/c (H-2<sup>d</sup>) or C57BL/6 (H-2<sup>b</sup>) background for three or more generations before use. For studies involving F<sub>1</sub> mice, Tbet<sup>-/-</sup> (BALB/c) and Tbet<sup>-/-</sup> (C57BL/6) mice were intercrossed, and normal (BALB/c  $\times$  C57BL/6)F<sub>1</sub> (The Jackson Laboratory, Bar Harbor, ME) mice were used as controls.

### LM infections

Wild-type (WT) LM strain 10403s was provided by Dr. D. Portnoy (University of California, Berkeley, CA). For infection, LM was grown to mid-log phase ( $A_{600}$ , 0.1) at 37°C, diluted in 200  $\mu$ l of saline, and injected into mice via the lateral tail vein as described previously (15). The numbers of bacteria in lysates of infected spleens and livers were determined as described elsewhere (15). All experiments were performed under Institutional Animal Care and Use Committee-approved protocols.

### Intracellular cytokine staining

Intracellular cytokine staining was performed as described using reagents from BD Biosciences (San Jose, CA). For direct detection of ongoing cytokine production, whole splenocytes were incubated with GolgiStop for 5 h directly ex vivo and subjected to cell surface and intracellular cytokine staining. For enumeration of LM-specific T cells, splenocytes were stimulated with the indicated LM-specific peptide ( $10^{-6}$  M) in the presence of GolgiStop for 5 h. Cells were stained with the indicated cell surface Abs, permeabilized with Cytoperm solution, and stained with anti-IFN- $\gamma$  Ab. Stained cells were then analyzed on an LSR flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

### Cytokine production

The concentrations of IFN- $\gamma$ , IL-13, IL-4, IL-2, IL-10, and TNF- $\alpha$  in the supernatants of splenocyte cultures were determined 72 h after peptide stimulation by ELISA using reagents from R&D Systems (Minneapolis, MN).

### RNA isolation and real-time RT-PCR

RNA was isolated from FACS-purified cells using TRIzol reagent, digested with DNaseI (DNA free; Ambion, Austin, TX), and reverse transcribed to cDNA using random primers and SuperScript II (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturers' instructions. The expression of *Eomesodermin* (Eomes) was quantified by real-time RT-PCR using SYBR Green QPCR reagents (Stratagene, La Jolla, CA) and normalized to the expression of  $\beta$ -actin as described previously (16). Real-time primers used for  $\beta$ -actin (16) have been published, and the following primers were used for quantification of murine Eomes (XM135209): Eomes forward, 5'-cacggcttcagaaaatgaca-3'; reverse, 5'-ctctgttgggggtgagaggac-3'

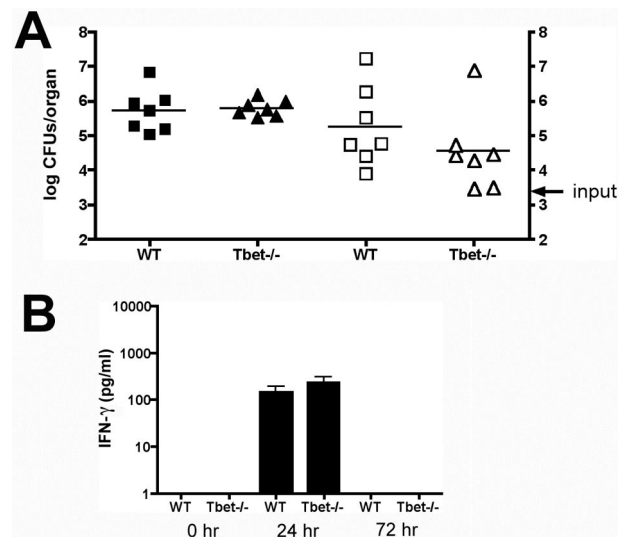
### Statistics

The differences in geometric mean CFUs, the percentages and numbers of activated splenocytes, and cytokine concentrations were evaluated with the non-parametric Mann-Whitney *U* test (GraphPad, San Diego, CA), and with *p* < 0.05 taken as statistically significant.

## Results

### T-bet-independent innate resistance and IFN- $\gamma$ production following LM infection

Since T-bet is required for the optimal production of IFN- $\gamma$  by NK cell and dendritic cells (6, 7) and IFN- $\gamma$  plays a crucial role in innate host resistance to LM infection, we hypothesized that T-bet-deficient mice would be highly susceptible to primary LM infection. Surprisingly, they were not (Fig. 1). After i.v. inoculation with 0.3 LD<sub>50</sub> (3000 CFUs), no mortality was observed for any T-bet-deficient (*n* = 8) or BALB/c control (*n* =

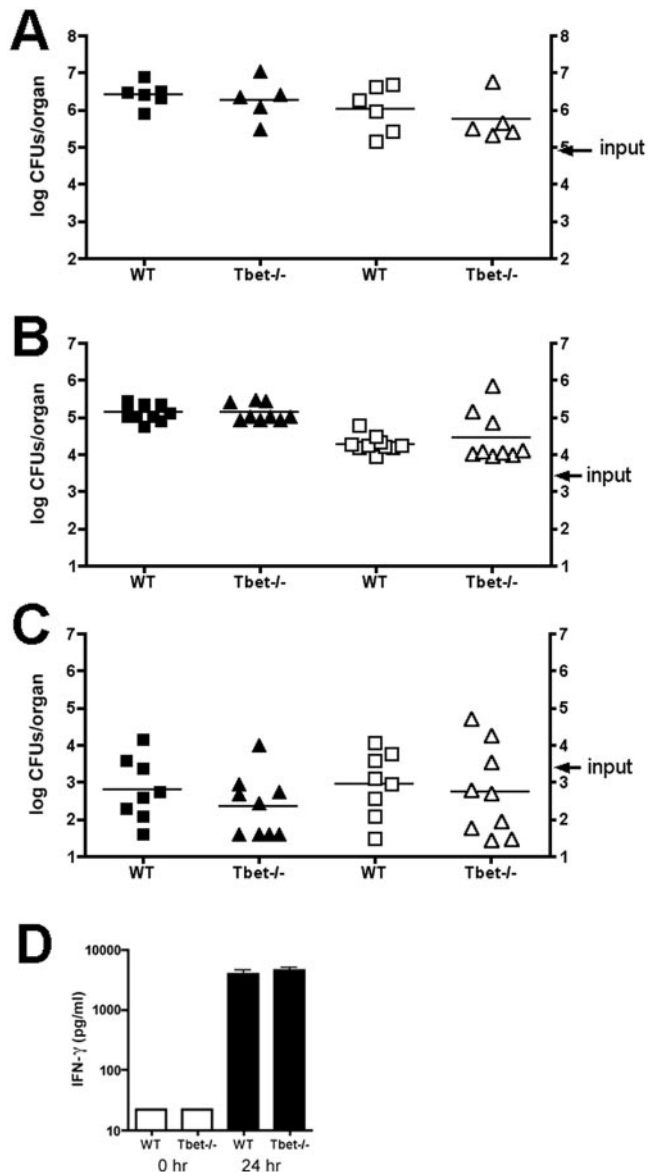


**FIGURE 1.** *A*, Recoverable CFU from the spleens (■ and ▲) and livers (□ and △) day 3 after i.v. infection with LM in BALB/c mice (WT, ■ and □) or T-bet-deficient mice (Tbet<sup>-/-</sup>, ▲ and △) on the BALB/c background. *B*, Serum concentration of IFN- $\gamma$  in BALB/c (WT) or T-bet-deficient (Tbet<sup>-/-</sup>) mice on the BALB/c background at the indicated times postinfection. These data represent a total of six to eight mice per group from two separate experiments.

8) mice. Although the numbers of recoverable CFUs revealed that substantial bacterial replication had occurred in both the spleens and livers of T-bet-deficient and control mice by day 3 postinfection, there were no differences in CFUs in either organ (Fig. 1*A*). Consistent with this normal level of resistance, serum concentrations of IFN- $\gamma$  in the first 3 days of infection were similar in T-bet-deficient and control mice (Fig. 1*B*). To determine whether these results were unique to T-bet-deficient mice on the LM-susceptible BALB/c background, we also tested the susceptibility of T-bet-deficient and control (C57BL/6  $\times$  BALB/c)F<sub>1</sub> mice to both moderate (3000 CFUs) and high ( $1 \times 10^5$  CFUs, 1 LD<sub>50</sub>) LM inocula. Again, resistance (Fig. 2, *A* and *B*) and induction of serum IFN- $\gamma$  (Fig. 2*D*) in response to LM infection were similar in these mice. These data demonstrate that contrary to results reported for primary infection with MCMV, during LM infection there are T-bet-independent pathways that result in unaltered innate host resistance and IFN- $\gamma$  production.

### T-bet-independent production of IFN- $\gamma$ by NK1.1<sup>+</sup> cells following LM infection

NK and dendritic cells are considered to be the principle cell types that produce IFN- $\gamma$  in the first days of a primary LM infection (1, 3, 4). To determine the cellular source of IFN- $\gamma$  during the innate immune response to LM infection in T-bet-deficient mice, ex vivo-derived whole splenocytes were subjected to intracellular IFN- $\gamma$  and cell surface staining 21 h following LM infection. Using this method, NK1.1<sup>+</sup> cells were found to be the predominant cell type producing IFN- $\gamma$  in both T-bet-deficient and control mice, as >70% of IFN- $\gamma$ <sup>+</sup> cells were NK1.1<sup>+</sup>, while <6% of IFN- $\gamma$ <sup>+</sup> cells were either CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, or CD11c<sup>+</sup> (Fig. 3). The production of IFN- $\gamma$  by NK1.1<sup>+</sup> cells in T-bet-deficient and control mice was clearly a response to LM infection, since IFN- $\gamma$ <sup>+</sup> cells were detected at only baseline levels in uninfected mice. Thus, there are



**FIGURE 2.** *A*, Recoverable CFU from the spleens (■ and ▲) and livers (□ and □) on day 3 (*A* and *B*) or day 7 (*C*) after i.v. infection with either  $1.0 \times 10^5$  (*A*) or  $3.0 \times 10^3$  (*B* and *C*) CFUs of LM in (B6  $\times$  BALB/c)<sub>F1</sub> mice (WT, ■ and □) or T-bet-deficient mice (Tbet<sup>-/-</sup>, ▲ and △) on the (B6  $\times$  BALB/c) F<sub>1</sub> background. *D*, Serum concentration of IFN- $\gamma$  in (B6  $\times$  BALB/c)<sub>F1</sub> WT or T-bet-deficient mice (Tbet<sup>-/-</sup>) at the indicated times after infection with  $1.0 \times 10^5$  CFUs of LM.

T-bet-independent pathways present in NK cells that lead to the normal production of IFN- $\gamma$  during LM infection. As recently reported by others (9), we observed an  $\sim 50\%$  reduction in percentage and total numbers of NK1.1<sup>+</sup>CD3<sup>-</sup> and NK1.1<sup>+</sup>CD3<sup>+</sup> cells in T-bet-deficient compared with control mice (data not shown).

#### *T-bet-independent resolution of primary LM infection*

The normal level of resistance to primary LM infection in T-bet-deficient mice was not limited to the first 3 days following infection. By day 7 postinfection, T-bet-deficient and control mice effected a similar  $\sim 2\text{--}3$  log<sub>10</sub> reduction in the numbers of bacteria in livers and spleens compared with the numbers recovered on day 3 (Fig. 2*C*). Since clearance of bacteria between

days 3 and 7 is mediated by Th1 CD4 and Tc1 CD8 T cells, these data suggested that the adaptive immune response was functionally intact in T-bet-deficient mice.

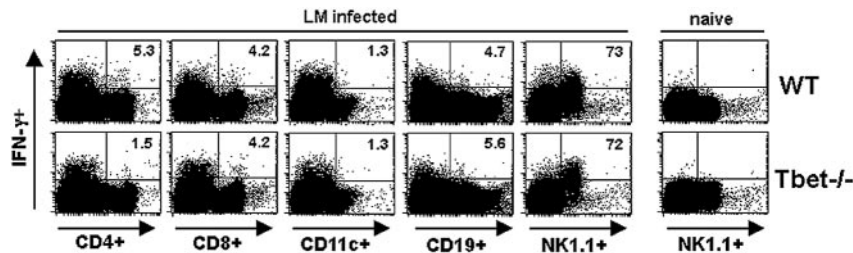
#### *Generation of LM-specific Th1 CD4 and Tc1 CD8 cells does not require T-bet*

T-bet is critical for the differentiation of Ag-specific CD4 T cells into Th1 effectors and makes an important contribution to the differentiation of CD8 T cells into Tc1 cells following infection with *L. major* and LCMV, respectively. These data suggest that T-bet is critically important for IFN- $\gamma$  production by Ag-specific CD4 and CD8 T cells upon reactivation by Ag. However, since LM infection is able to induce normal levels of early IFN- $\gamma$  in a T-bet-independent fashion, we further examined the requirement for T-bet in the generation of LM-specific IFN- $\gamma$ -producing Th1 CD4 and Tc1 CD8 T cells. At the peak of the LM-specific adaptive immune response (day 7 following infection), the LM-specific CD8 and CD4 T responses in T-bet-deficient and control mice were examined by stimulation of splenocytes with either of two LM-specific MHC class I-restricted peptides (listeriolysin O (LLO<sub>91-99</sub>, p60<sub>217-225</sub>) or the class II-restricted peptide (LLO<sub>189-201</sub>). Ag-specific IFN- $\gamma$  production was analyzed both by intracellular cytokine staining (after stimulation for 5 h) and by ELISA measurement of culture supernatants (after stimulation for 72 h), while the Ag-specific production of IL-4 and IL-13 was analyzed by ELISA. Based on the previous reports cited above, we predicted that the generation of IFN- $\gamma$ -producing LM-specific CD4 and CD8 T cells would be largely abolished and substantially reduced, respectively.

In contrast to these predictions, following peptide stimulation for 5 h, the percentage (Fig. 4*A*) and total numbers (Fig. 4*B*) of LM-specific IFN- $\gamma$ -producing CD8 were modestly but significantly increased (1.3- to 2.0-fold,  $p < 0.01$ ) for T-bet-deficient compared with control mice. Moreover, the numbers of IFN- $\gamma$ -producing LM-specific CD4 T cells were on average only reduced  $\sim 50\%$  ( $p < 0.01$ ) in T-bet-deficient compared with control mice (Fig. 4, *A* and *B*), although in T-bet-deficient mice there was more variability in the LM-specific CD4 T cell response. Consistent with results reported by others for CD8 T cells from T-bet-deficient mice (8), IFN- $\gamma$ <sup>+</sup> CD8 T cells from T-bet-deficient mice uniformly did not down-regulate CD8 upon activation to the extent seen in CD8 T cells from normal mice (Fig. 4*A*).

In agreement with the  $\sim 50\%$  reduction in the fraction and total numbers of IFN- $\gamma$ -producing CD4 T cells following stimulation with LM class II peptide for 5 h, 72-h culture supernatants of class II peptide-stimulated cells from T-bet-deficient mice contained  $\sim 80\%$  less IFN- $\gamma$  than supernatants from control cells ( $p < 0.01$ ). Culture supernatants from splenocytes stimulated for 72 h with the LLO<sub>91-99</sub> class I peptide also contained somewhat less IFN- $\gamma$  (Fig. 4*C*), but this difference was not significant. IL-2 production by peptide-stimulated CD4 T cells from T-bet-deficient mice was somewhat greater than that from controls ( $1096 \pm 336$  and  $588 \pm 170$  ng/ml, respectively). Furthermore, there was no detectable Ag-specific production of IL-4, IL-10, IL-13, or TNF- $\alpha$  in the supernatants from class I or class II peptide-stimulated cell cultures.

To explore the basis for IFN- $\gamma$  production by CD4 T cells in response to LM in the absence of T-bet, the relative expression of Eomes was determined by real-time RT-PCR before and on



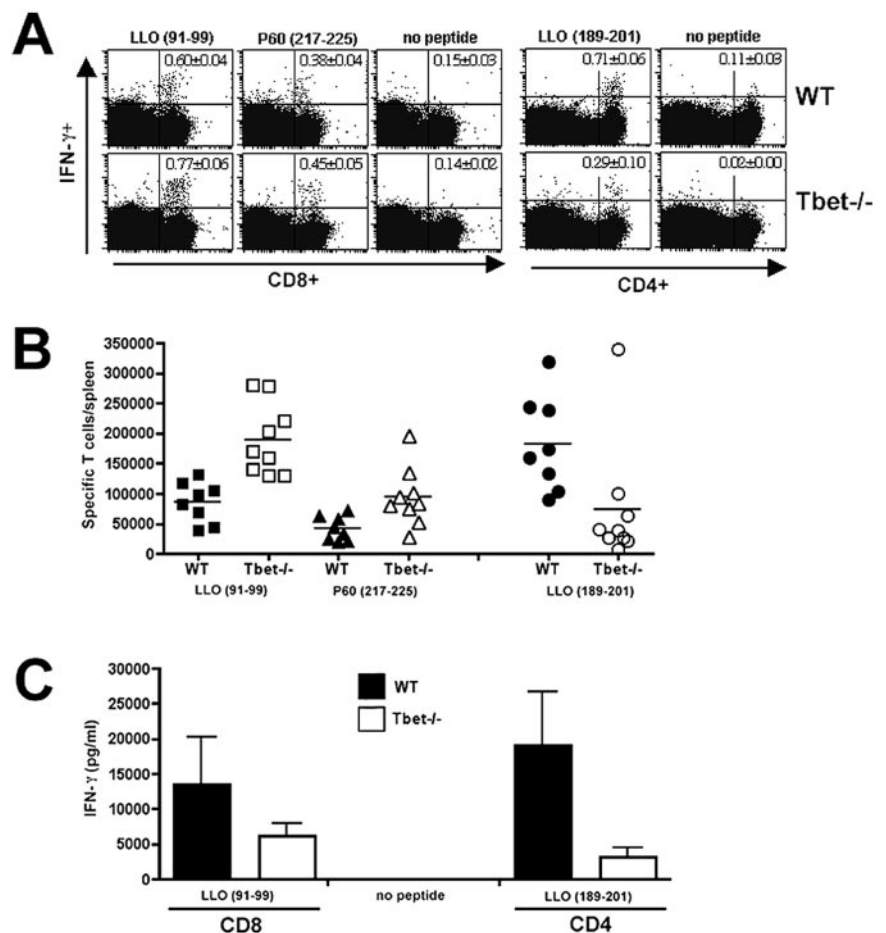
**FIGURE 3.** IFN- $\gamma$  production by splenocytes from (B6  $\times$  BALB/c)<sub>F1</sub> WT or Tbet<sup>-/-</sup> mice following LM infection. Splenocytes from uninfected (naive) or LM-infected mice were harvested 21 h postinfection, incubated directly ex vivo with GolgiStop for 5 h, and then analyzed by cell surface and intracellular IFN- $\gamma$  staining. The numbers at the upper right in each plot indicate the percentage of IFN- $\gamma$ -producing cells of each type for either WT or Tbet-deficient mice. These data represent five to six mice per group from two separate experiments.

day 7 after LM infection, which normally induces a strong Th1 and Tc1 cellular response in CD4 and CD8 T cells (17), respectively. Consistent with previously published findings (17), Eomes mRNA was expressed in CD8 T cells, but we did not detect Eomes mRNA in CD4 T cells regardless of LM infection or Tbet deficiency (data not shown). These results strongly suggest that for LM infection, neither Tbet nor Eomes is required for the generation of IFN- $\gamma$ -producing Ag-specific CD4 T cells.

### Discussion

IFN- $\gamma$  is critical for resistance to primary LM infection. Targeted deletion of the *Ifng* gene results in an  $\sim$ 1000-fold decrease in the LD<sub>50</sub> for LM, such that even 10 CFUs is lethal in

the absence of this cytokine (13). In the early phase of LM infection, the major cellular source of IFN- $\gamma$  is NK cells. We confirmed the recent finding by Glimcher and colleagues (9) that the numbers of mature NK cells in the spleen are reduced in Tbet-deficient mice. They also reported that although the initial release of IFN- $\gamma$  by NK cells in vitro in response to IL-12 and IL-18 is normal, sustained release of IFN- $\gamma$  in vitro and IFN- $\gamma$  secretion in the first 2 days of acute MCMV infection in vivo is substantially reduced (9). However, during LM infection, innate immunity and the early production of IFN- $\gamma$  by NK cells were not detectably affected by the absence of Tbet. These data demonstrate that LM infection is able to induce normal amounts of IFN- $\gamma$  by NK cells through a Tbet-independent mechanism. Although others have reported that dendritic



**FIGURE 4.** *A*, IFN- $\gamma$  production by CD8 and CD4 T cells from WT and Tbet-deficient mice following stimulation with the indicated LM peptides or no peptide control for 5 h. Splenocytes were obtained 7 days after infection with LM. The numbers in the upper right quadrants indicate the mean percentage  $\pm$  SE of IFN- $\gamma$ -producing CD8 or CD4 T cells from eight to nine mice per group with one representative dot plot shown. *B*, Total numbers of IFN- $\gamma$ -producing CD8 and CD4 T cells per mouse spleen following stimulation with the indicated MHC class I (LLO<sub>91-99</sub>, p60<sub>217-225</sub>)- or class II-restricted (LLO<sub>189-201</sub>) LM peptides. *C*, IFN- $\gamma$  production in culture supernatants by CD8 and CD4 T cells from WT and Tbet-deficient mice following stimulation with the indicated LM peptides or no peptide control for 72 h. Bar, 1 SE.

cells are a source of innate IFN- $\gamma$  (3, 4), we found that NK cells were the dominant cell type producing IFN- $\gamma$  in the early innate phase of the response to LM infection when assayed directly *ex vivo*, while the fraction of IFN- $\gamma$ <sup>+</sup> dendritic cells was not above the baseline observed in uninfected mice. There was also no defect in the generation of IFN- $\gamma$ -producing Ag-specific CD8 T cells in response to LM infection in the absence of T-bet. This result is in sharp contrast to the ~80% reduction in Ag-specific CD8 T cells observed following primary LCMV infection (8, 12).

Although little to no IFN- $\gamma$  is produced by T-bet-deficient CD4 T cells following nonspecific activation *in vitro* or in response to *L. major* infection (6), we observed only a modest ~50% reduction in total numbers of IFN- $\gamma$ -producing Ag-specific CD4 T cells in T-bet-deficient mice following LM infection. Secretion of IFN- $\gamma$  into culture supernatants by Ag-specific T-bet-deficient CD4 T cells was also readily detectable, albeit reduced by ~80% compared with controls, suggesting that T-bet is more important for sustained secretion of IFN- $\gamma$  than the induction of IFN- $\gamma$ -producing CD4 T cells. Since no Eomes mRNA was detected in CD4 T cells even in LM-infected T-bet-deficient mice, these data demonstrate that there are T-bet-independent and likely Eomes-independent pathways for IFN- $\gamma$  production in CD4 T cells that play more prominent roles in response to specific infectious agents (i.e., LM) than others (i.e., *L. major*). The only other context in which substantial IFN- $\gamma$  production by CD4 T cells in the absence of T-bet has been described previously is in cells from Fas-deficient lupus prone *lpr* mice (18). These findings, and the data we present in this study, imply that the requirement for T-bet in IFN- $\gamma$  production by CD4 T cells is not absolute and varies depending on the context of certain infectious or noninfectious (Fas deficiency) parameters.

Since STAT-4 signaling can induce IFN- $\gamma$  in CD4 T cells *in vitro* in the absence of T-bet (19, 20), it is possible that STAT-4 signaling in response to IL-12 (and perhaps IL-23) along with IL-18 released by cells of the innate immune system in response to LM may allow for the induction of IFN- $\gamma$  from T-bet-deficient CD4 T cells. However, because T-bet reinforces its own expression and IFN- $\gamma$  production by signaling through STAT-1, loss of T-bet would be expected to impair sustained IFN- $\gamma$  secretion into cell culture supernatants (19), as we observed.

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