Protective Immunity to Genital Herpes Simpex Virus Type 2 Infection Is Mediated by T-bet

Alexandra Svensson, Inger Nordström, Jia-Bin Sun and Kristina Eriksson

http://www.jimmunol.org/content/174/10/6266

References
This article cites 48 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/174/10/6266.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Protective Immunity to Genital Herpes Simpex Virus Type 2 Infection Is Mediated by T-bet

Alexandra Svensson,* Inger Nordström,* Jia-Bin Sun,† and Kristina Eriksson2*

We show, for the first time, that the transcription factor T-bet, which is implicated in IFN-γ production, is required for the induction of vaccine-induced antiviral immune protection. T-bet was found to be important in both the innate and acquired immune protection against genital HSV-2 infection. T-bet−/− and T-bet+/+ mice were infected vaginally with HSV-2 and examined daily for disease and mortality. T-bet−/− mice had significantly higher virus titers than T-bet+/+ mice following a primary HSV-2 infection, and succumbed significantly earlier to the infection. This result was associated with an impaired NK cell cytotoxic capacity and NK cell-mediated IFN-γ production in the T-bet−/− mice. To assess the induction of acquired antiviral immune protection, mice were vaccinated with an attenuated virus before infection. Vaccinated T-bet−/− mice could not control viral replication following an HSV-2 challenge and had significantly higher virus titers and mortality rates than vaccinated T-bet+/+ mice that remained healthy. The impaired acquired immune protection in T-bet−/− mice was associated with a significantly decreased HSV-2-specific delayed-type hypersensitivity response and a significantly reduced HSV-2-specific IFN-γ production from CD4+ T cells. However, T-bet deficiency did not impair either the IFN-γ production or the cytotoxic capacity of HSV-2-specific CD8+ T cells. We conclude that T-bet plays a crucial role in both the innate defense and the generation of vaccine-induced immunity against genital HSV-2 infection in mice. The Journal of Immunology, 2005, 174: 6266–6273.

Genital herpes infection caused by HSV-2 is the most common sexually transmitted ulcerative disease worldwide (1, 2). Host defense mechanisms against HSV-2 infection involve both natural and acquired immune responses. The natural immune response includes innate cells such as neutrophils, NK cells, NKT cells, macrophages, and dendritic cells (2–4), as well as natural Abs and cytokines (5, 6), which restrain virus replication at mucosal sites and in the nervous system. IFN-γ and NK cells appear to be especially important in the local response to primary HSV-2 infection. Both IFN-γ-deficient mice and mice lacking NK and NKT cells (IL-15−/−) are highly susceptible to genital HSV-2 infection and display an accelerated viral accumulation and disease development (7, 8). The early IFN-γ response to genital HSV-2 infection is mediated mainly by NK cells (8). Another important role for NK cells is their cytotoxic effector function, which has been seen in the innate immune response to genital HSV-2 infection (8, 9). In line with these data are our previous results showing that the IFN-γ–inducing cytokines IL-12 and IL-18 are important in innate defense (7), most likely by their ability to induce IFN-γ production and to enhance NK cell activity (10, 11).

CD4+ T cells together with IFN-γ comprise the most important components in acquired protective immunity to genital HSV-2 infection. Mice lacking either IFN-γ or CD4+ T cells cannot mount a protective immune response to HSV-2 following vaccination (6, 7). However, treating the mice with IFN-γ can bypass the requirement for CD4+ T cells, implying that the main function of CD4+ T cells in anti-HSV-2 immunity is as producers of IFN-γ (6). Accordingly, in humans, an impaired HSV-2-specific IFN-γ response by CD4+ T cells correlates with recurrent clinical disease, whereas high levels of IFN-γ are produced in T cells from individuals with an asymptomatic HSV-2 infection (12, 13). CD8+ T cells have also been ascribed a role in protective immunity to HSV-2 both in experimental animals (14–16) and in humans (17). However, in mice, CD4+ T cells apparently predominate and manifest protective functions within the vagina at an earlier time point after genital challenge (18). In humans, CD4+ T cell infiltration of the HSV-2-induced genital lesion precedes CD8+ T cell infiltration and is associated, time-wise, with the drop in infectious virus titer (19, 20).

The dominant factor for the induction of IFN-γ is generally held to be IL-12 (21), which acts in synergy with IL-18 (22). However, immune responses induced by viral infections appear to circumvent the requirement of these cytokines in the development of antiviral IFN-γ production and Th1 immunity. Thus, neither IL-12 nor IL-18 is required for the induction of protective immunity to genital HSV-2 infection (7), and protective immune responses can be induced in the absence of IL-12 also during other viral infections (23).

The transcription factor T-bet (T box expressed in T cells) controls the IFN-γ production early during the differentiation of naive CD4+ T cells into Th1 cells (24–26) in an IL-12-independent pathway (27). T-bet expression is augmented by signals mediated through the TCR and STAT-1 (28, 29). Elevated T-bet expression induces IL-12Rβ2 expression and IL-12 activation via STAT4 and thereby expression of IFN-γ. IFN-γ may extend a positive feedback to enhance T-bet expression in an autocrine and endocrine fashion, which gives rise to an amplification loop that stabilizes T-bet expression and the Th1 differentiation program (29). T-bet has been shown to be important during viral, bacterial, and parasitic infections. T-bet controls both the Th1 cell differentiation and the CD8+ T cell response in lymphocytic choriomeningitis virus-
infected mice. T-bet-deficient mice could not generate a sufficient CTL effector function in response to the viral infection, and they also displayed a decreased IFN-γ production (24). T-bet also plays an important role in protection against Staphylococcus aureus infection and subsequent joint inflammation, which is associated with an impaired IFN-γ response (30). T-bet has also been shown to play a crucial role in the clearance of infection with Leishmania major, which was again accompanied by a diminished IFN-γ production (31).

This study was undertaken to evaluate the influence of T-bet on the innate and adaptive immune responses to genital HSV-2 infection. For this purpose, we infected T-bet-deficient mice with HSV-2 and measured both viral replication and the disease progression. To assess the ability of T-bet−/− mice to develop a protective acquired immune response, mice were immunized with an attenuated strain of HSV-2, lacking the gene for thymidine kinase (TK−),3 before infection (32, 33).

Materials and Methods

Mice

Female 6- to 10-wk-old mice were used for all experiments. Naïve C57BL/6 mice (T-bet−/−) were purchased from B&K Universal and Charles River Breeding Laboratories. T-bet knockout mice (T-bet−/−) were a kind gift from L. H. Glimcher (Harvard Medical School, Boston, MA). The T-bet-deficient mice were generated via traditional gene targeting methods in C57BL/6 × 129 chimeras and confirmed to be deficient in T-bet protein, and were then backcrossed to C57BL/6 (25). The mice were kept in ventilated cages under pathogen-free conditions at the Department of Experimental Biomedicine, Göteborg University (Göteborg, Sweden). These studies were approved by the ethical committee for animal experiments (Göteborg, Sweden).

Virus and HSV-2 Ag preparation

HSV-2 strain 333 (34) and the attenuated strain Lyons (35), which contains a partial deletion of the gene for TK, were grown in African green monkey kidney cell (GMK-AH1) monolayers. At 2–3 days after infection the cells and the supernatants were harvested and the virus was retrieved by UV light inactivated whole virus. Depletion of CD8 T cells was done by incubating the cells in Iscove’s complete medium with 5 μg/ml mouse anti-mouse CD8 (cat. no. 553027), B220 (cat. no. 553084), and I-Ab (cat. no. 553621) Abs (BD Pharmingen) for 45 min at 4°C. The cells were centrifuged and then resuspended in complete Iscove’s medium containing anti-mouse magnetic beads (Dynal Biotech), incubated for 30 min at 4°C, and then placed on a magnet. Nonresorbed cells were then immediately used as effector cells. Whole spleen cell suspensions from T-bet−/− and T-bet+/+ mice were activated for 48 h with 300 ng/ml murine recombinant IL-2 before use as effector cells.

Effector cells were dispersed in triplicates at different cell densities in 96-well U-bottom microtiter plates (Nunc) together with 106 51Cr-labeled YAC-1 cells, in a total of 200 μl of complete Iscove’s medium. The plates were incubated for 16 h at 37°C. Supernatants were collected and assayed for radioactivity in a gamma counter. Maximum 51Cr release was determined in target cell cultures treated with Triton X-100. NK cell cytotoxicity was calculated as a percentage of cell killing according to the following formula: 100 × [(experimental release − spontaneous release)/maximum release − spontaneous release] = percentage of specific killing.

HSV-specific CD8 T cell cytotoxicity

Spleen cells from T-bet−/− mice and T-bet+/+ mice were activated for 48 h with 1 μg/ml H-2Kd restricted glycoprotein B (gB-2) peptide SSIEFARL (KJ Ross-Petersen) (37) and 10 ng/ml murine recombinant IL-2. A total of 106 MC-57 fibroblast target cells were labeled for 1 h with 150 μCi 51Cr (Amersham Biosciences) and 10 μg/ml SSIEFARL-activated spleen cells from T-bet−/− mice and T-bet+/+ mice were dispersed in triplicate at different cell densities in 96-well U-bottom microtiter plates (Nunc) together with 106 target cells, in a total of 200 μl of complete Iscove’s medium. The plates were incubated for 6 h at 37°C. Supernatants were collected by a tissue collecting system (Amersham) and assayed for radioactivity in a gamma counter. Maximum 51Cr release was determined in target cell cultures treated with Triton X-100. CD8 T cell cytotoxicity was calculated as a percentage of cell killing according to the following formula: 100 × [(experimental release − spontaneous release)/maximum release − spontaneous release] = percentage of specific killing.

IFN-γ measurements

Measurement of IFN-γ production from NK cell-enriched spleen cell suspensions (i.e., spleen cell suspensions depleted of CD4+, CD8+, and CD20+ cells, as previously described) was performed by incubating NK cell-enriched spleen cells from T-bet−/− mice and T-bet+/+ mice with YAC-1 cells. Supernatants were harvested after 24 h and analyzed for IFN-γ content using a murine IFN-γ duoset ELISA (cat. no. DY485; R&D Systems). Measurement of IFN-γ production from CD4+ and CD8+ spleen cells was performed using a cell ELISA with the Abs from the murine IFN-γ duoset ELISA (cat. no. DY485; R&D Systems). CD4+ T cell activation was performed with CD8-depleted spleen cell cultures in the presence of inactivated whole virus. Depletion of CD8+ T cells was done by incubating the cells in Iscove’s complete medium with 5 μg/ml mouse anti-mouse CD8 Ab (cat. no. 553027; BD Pharmingen) for 45 min at 4°C. This was followed by anti-mouse magnetic beads (Dynal Biotech) for 30 min at 4°C and then separation on a magnet. CD8+ T cell activation was analyzed in whole spleen cell cultures using the HMC class I-restricted peptide SSIEFARL.
For CD8$^+$ T cells, mononuclear spleen cell suspensions were seeded in duplicate at different cell densities in complete Iscove's medium containing 10 μg/ml SSIEFARL peptide. Spleen cells depleted of CD8$^+$ T cells were seeded in duplicate at different cell densities in complete Iscove's medium containing UV-inactivated HSV-2 Ag diluted 1/50 corresponding to 2 × 10$^6$ PFU/ml. Plates were then incubated at 37°C for 24 h. Plates were washed in 0.05% Tween 20 and biotinylated anti-mouse IFN-γ was added (1 μg/ml) and incubated at 4°C overnight. This was followed by 45 min incubation with peroxidase-labeled avidin (Sigma-Aldrich) (diluted 1/200 in 0.5% BSA in PBS) at room temperature. Plates were developed for 20 min using 0.1 mg/ml tetramethylbenzidine in 0.05% phosphate-citrate buffer pH 5.0 and 0.04% H$_2$O$_2$. Development was stopped with 25 μl of 1 M H$_2$SO$_4$ and absorbance was measured at 450 nm. The concentration of IFN-γ was determined by extrapolation from a standard curve obtained using recombinant IFN-γ. The sensitivity of the assay was 50 pg/ml. Results are expressed as the concentration of IFN-γ secreted per 1 × 10$^6$ spleen cells.

**FACS analysis**

The number of SSIEFARL-specific CD8$^+$ T cells were determined in vaccinated T-bet$^{-/-}$ and T-bet$^{+/+}$ mice. Spleen cells were used either directly following isolation or after a 24 h incubation with 10 ng/ml IL-2 and 1 μg/ml SSIEFARL in Iscove's complete medium. Cells were stained with FITC-labeled anti-mouse CD8 Ab (BD Biosciences) diluted 1/100 and with PE-labeled SSIEFARL pentamer (Proimmune) used undiluted. Cells were stained for 30 min in 4°C PBS and then washed twice in PBS with 1% FCS. Analysis was performed using CellQuest software (BD Biosciences).

The number of B cells in spleens from naive T-bet$^{-/-}$ and T-bet$^{+/+}$ mice were determined using a PE-labeled anti-mouse B220 marker (BD Biosciences). The cells were stained and analyzed as described.

**HSV-specific Ab measurements**

The deoxy-solubilized membrane fraction of HSV-1-infected cells used in the ELISA was prepared from HSV-1-infected BHK cells as described (38). Briefly, cells were mixed with 0.025 M Tris-hydrochloride (pH 8.0), homogenized with a Dounce homogenizer, and centrifuged at 1500 g for 15 min at 4°C. The membranes remaining in the supernatant were pelleted at 160,000 × g for 1 h, washed with 0.1 M glycine-sodium hydroxide buffer (pH 8.8), and 1.1 ml of 10% sodium deoxycholate (Merck) was added with stirring. The mixture was homogenized in a Dounce homogenizer and centrifuged at 160,000 × g for 1 h. The HSV Ags were retrieved from the supernatant.

Greiner 96F microwell plates (655061; Labora) were coated with 100 μl of the HSV-1 deoxy-solubilized membrane fraction overnight at 4°C in 0.05 M carbonate buffer at pH 9.6. The plates were blocked with 2% BSA in PBS for 30 min in 37°C. Serial dilutions of sera obtained 4 wk post-vaccination with attenuated HSV-2 TK$^-$ were incubated for 1 h at 37°C. After washing with 0.05% Tween 20, the plates were incubated for 1 h at 37°C with goat anti-mouse IgG1 or IgG2a coupled to HRP (1/1000 South- ern Biotechnology Associates) in 1% BSA in PBS. The plates were washed as described and developed for 20 min using 50 μl of 0.1 mg/ml tetramethylbenzidine in 0.05% phosphate-citrate buffer pH 5.0 and 0.04% H$_2$O$_2$. Development was stopped with 25 μl of 1 M H$_2$SO$_4$. Absorbance was then measured at 450 nm. The IgG1 and IgG2a titers were defined as the reciprocal sample dilution giving an absorbance of 0.4 above the background level.

**HSV-specific delayed-type hypersensitivity (DTH) responses**

DTH reactions were elicited by injection of UV-inactivated HSV-2 (corresponding to 7 × 10$^6$ PFU in 30 μl of medium) in the left footpad (experimental footpad) and 30 μl of PBS in the right footpad (control footpad). The virus-specific footpad swelling was determined 48 h later for each animal and is expressed as the mean difference between the increased thickness of experimental and control footpads.

**Statistical methods**

Statistical analyses were done by Student’s two-tailed t test. Survival data were analyzed by Fisher’s exact test and Kaplan-Meier survival test.

**Results**

**Impaired innate defense against primary HSV-2 infection in T-bet-deficient mice**

To examine the influence of T-bet in the induction of an innate protective immune response against lethal genital HSV-2 infection, T-bet$^{-/-}$ mice and T-bet$^{+/+}$ mice were inoculated intravaginally with a lethal dose of HSV-2 strain 333. We found that T-bet$^{-/-}$ mice had significantly higher HSV-2 titers in vaginal washings obtained 48 h postchallenge, compared with T-bet$^{+/+}$ controls (Fig. 1A). The viral amount in the CNS was measured 8 days postvaginal challenge (Fig. 1B). The viral amount in the CNS was measured 8 days postvaginal challenge (Fig. 1B).

**FIGURE 1.** Viral replication and disease progression in HSV-2-infected T-bet$^{-/-}$ mice. T-bet$^{-/-}$ mice and T-bet$^{+/+}$ mice were challenged intravaginally with HSV-2. A, HSV-2 replication was determined in vaginal fluid obtained 48 h postvaginal HSV-2 challenge. Data are expressed as the mean PFU ± SEM for 12 animals per group. B, HSV-2 titers were determined in CNS 8 days postvaginal challenge. Data are expressed as the mean PFU ± SEM for six animals per group. Animals were scored daily for pathologic symptoms (C) and mortality (D). Disease progression was scored as 0, healthy; 1, mild inflammation; 2, moderate genital inflammation; 3, genital lesion and/or generally bad condition; 4, hind-limb paraplegis; 5, death or sacrifice due to paralysis. Data represent the mean score from 15 animals per group. *, p < 0.05; **, p < 0.01; ***, p < 0.001 using Student’s t test (A–C) and Fisher’s exact test (D).
days after challenge in bone marrow from spinal cord. We also found that the amount of HSV-2 in the CNS was significantly higher among T-bet−/− mice as compared with T-bet+/+ mice (Fig. 1B). Furthermore, T-bet−/− mice succumbed significantly more rapidly to the disease. Macroscopic signs of disease started earlier and developed faster in T-bet−/− mice (Fig. 1C) and the T-bet−/− animals died significantly earlier than T-bet+/+ controls (Fig. 1D). The survival curves for T-bet−/− mice and T-bet+/+ mice differed significantly (p < 0.001) using Kaplan-Meier analysis.

Reduced NK cell function in T-bet-deficient mice

To evaluate whether the increased viral replication and disease progression in T-bet−/− mice were associated with a diminished NK cell function, we analyzed the ability of NK cells from T-bet−/− and T-bet+/+ mice to kill YAC-1 target cells in vitro. We found that NK cells from T-bet−/− mice were impaired in their cytotoxic activity. The specific killing obtained both with freshly isolated cells (Fig. 2A) and with in vitro-activated cells from T-bet−/− mice (data not shown) was statistically significantly lower than the killing obtained with NK cells from T-bet+/+ mice. We also measured IFN-γ production by YAC-1-activated NK cells. NK cell-enriched spleen cell suspension (i.e., spleen cells depleted of CD4+, CD8+, and B220+ cells) from T-bet−/− mice produced significantly lower levels of IFN-γ in response to YAC-1 cells compared with T-bet+/+ mice (Fig. 2B). Furthermore, FACS analysis revealed that NK cells from T-bet−/− mice did not express the NK marker NK1.1, in contrast to T-bet+/+ NK cells. A 6.7% (range from 6.2 to 8.2%, n = 4) of NK cells from T-bet+/+ mice expressed the NK1.1 marker, whereas the expression in NK cells from T-bet−/− mice was <0.96% (range from 0.44 to 1.3%, n = 5).

T-bet is required for the development of acquired immunity to HSV-2

To examine the influence of T-bet in the induction of an acquired protective immune response, T-bet−/− and T-bet+/+ mice were vaccinated intravaginally with HSV-2 TK− followed by a genital HSV-2 challenge 4 wk later. Vaccinated T-bet−/− mice were unable to control viral replication. Vaccinated T-bet−/− mice challenged with virulent HSV-2 had significantly elevated levels of HSV-2 in vaginal fluids as compared with vaccinated T-bet+/+ mice (Fig. 3A). Furthermore, 44% of the vaccinated T-bet−/− mice developed macroscopic signs of disease starting at day 5 postchallenge, whereas only one of 16 T-bet+/+ mice developed any signs of genital disease. These differences were statistically significant from day 9 onward (Fig. 3B). Finally, 37% (6/16) of the T-bet−/− mice but none of the T-bet+/+ animals died within 25 days of the viral challenge (Fig. 3C). The survival curves for T-bet−/− mice and T-bet+/+ mice differed significantly (p < 0.001) using Student's t test (A and B) and Fisher's exact test (C).

FIGURE 2. NK cell function in T-bet−/− mice. A, Cytotoxic killing of YAC-1 cells by NK cell-enriched spleen cell suspensions obtained from T-bet−/− (●) and T-bet+/+ (○) mice. Data represent the mean ± SEM from three animals per group. B, IFN-γ production by NK cell-enriched spleen cell suspensions obtained from T-bet−/− and T-bet+/+ mice incubated for 24 h with YAC-1 cells. Data represent the mean ± SEM from three animals per group. *, p < 0.05; **, p < 0.01 using Student’s t test.

FIGURE 3. Viral replication and disease progression in vaccinated T-bet−/− mice following a vaginal HSV-2 challenge. T-bet−/− mice and T-bet+/+ mice were vaccinated intravaginally with 2 × 10⁶ PFU HSV-2 TK− and challenged 4 wk later with a lethal dose of HSV-2. A, HSV-2 replication was determined in vaginal fluid obtained 48 h postvaginal HSV-2 challenge. Data are expressed as the mean ± SEM virus load (PFU/ml washing fluid) and represent 12 mice per group. Animals were scored daily for pathologic symptoms (B) and mortality (C). Disease progression was scored from 0 to 5 as in Fig. 1. Data represent the mean score from 15 animals per group. *, p < 0.05; **, p < 0.01 using Student’s t test (A and B) and Fisher’s exact test (C).
Kaplan-Meier analysis. The different disease outcome between vaccinated T-bet/H11002 and T-bet/H11001 mice was not due to a difference in replication efficacy of the HSV-2 TK/H11002 vaccine in the two strains of mice, as no significant differences in virus amount between T-bet/H11001 and T-bet/H11002 mice could be detected in the vaginal fluids obtained 48 h after vaccination with HSV-2 TK/H11002 (data not shown).

Mice were also given murine recombinant IFN-/H9253 at the time of vaccination, but no differences in disease development or mortality could be seen between the treated and untreated T-bet/H11002 mice (data not shown). Together these data suggest that T-bet plays a role in the acquired immunity to genital HSV-2 infection.

HSV-2-specific immune responses in T-bet-deficient mice

To investigate whether the reduced immune protection in T-bet⁻/⁻ mice was associated with impairments in the humoral and cell-mediated immune responses, we measured HSV-2-specific T cell responses in vivo and in vitro, 4 wk after HSV-2 TK⁻ vaccine.

CD4 responses. In vivo CD4⁺ T cell response was studied by measuring footpad swelling 24 h after injection of irradiated HSV-2. Virus-specific DTH responses were impaired in T-bet⁻/⁻ mice. The footpad swelling in response to HSV-2 Ags was almost negligible in T-bet mice, in sharp contrast to the strong T

FIGURE 4. HSV-2-specific CD4⁺ T cell immune responses in vaccinated T-bet⁻/⁻ mice. T-bet⁻/⁻ mice and T-bet⁺/⁺ mice were vaccinated intravaginally with 2 × 10⁶ PFU HSV-2 TK⁻ and analyzed 4 wk later. A, DTH reaction was elicited by injection of UV-inactivated HSV-2 in the left footpad and mock Ag in the right footpad. The specific footpad swelling was determined 48 h later and is expressed as the mean difference ± SEM between the increased thickness of experimental and control footpads for T-bet⁻/⁻ and T-bet⁺/⁺ mice with five animals per group. B, HSV-2-specific IFN-γ production by CD8⁺ T cell-depleted spleen cell suspensions from vaccinated T-bet⁻/⁻ and T-bet⁺/⁺ mice was analyzed by a cell ELISA using whole UV-inactivated HSV-2 as specific Ag. Data are presented as the mean ± SEM of IFN-γ produced per 1 × 10⁶ cells with three animals per group. *, p < 0.05 using Student’s t test.

CD8 responses. In vitro CD8⁺ T cell cytotoxicity in in vitro-activated spleen cell cultures from vaccinated T-bet⁻/⁻ mice and T-bet⁺/⁺ mice. Data are expressed as the mean specific killing percentage (%) of SSIEFARL-labeled MC-57 fibroblast target cells by CD8⁺ T cells using five mice per group. B, HSV-2-specific IFN-γ production by CD8⁺ T cells was analyzed by a cell ELISA using SSIEFARL peptide as specific Ag. Data are presented as the mean ± SEM of IFN-γ produced per 10⁶ cells with five animals per group. C and D, SSIEFARL-specific CD8⁺ T cells in spleen cells analyzed immediately after isolation (C) or after 24 h in vitro activation (D) with SSIEFARL peptide and IL-2. Data are presented as the mean percentage ± SEM of CD8⁺ cells reacting with SSIEFARL-labeled pentamers using three animals per group. *, p < 0.05; **, p < 0.01 using Student’s t test.
cell-mediated inflammatory response obtained in T-bet\(^{+/+}\) mice (Fig. 4A).

HSV-2-specific IFN-\(\gamma\) production from CD4\(^+\) cells was analyzed in spleen cell suspensions depleted of CD8\(^+\) T cells, using the cell ELISA method. The IFN-\(\gamma\) production obtained in response to irradiated HSV-2 virus was also impaired in T-bet\(^{-/-}\) mice. CD4\(^+\) T cells from T-bet\(^{-/-}\) mice produced significantly lower levels of IFN-\(\gamma\) in response to HSV-2 compared with T-bet\(^{+/+}\) mice (Fig. 4B).

**CD8 responses.** HSV-specific cytotoxicity CD8\(^+\) T cell-mediated killing of HSV-2-infected cells was measured using a cytotoxicity assay where in vitro-activated spleen cell cultures from vaccinated mice were incubated together with SSIEFARL peptide-labeled target cells. Surprisingly, the CD8\(^+\) T cells from T-bet\(^{-/-}\) mice had a significantly higher specific killing capacity than the CD8\(^+\) T cells from T-bet\(^{+/+}\) mice (Fig. 5A).

HSV-2-specific IFN-\(\gamma\) production from CD8\(^+\) cells was also analyzed in whole spleen cell suspensions in response to SSIEFARL peptide, using the cell ELISA method. No significant differences were found in IFN-\(\gamma\) production from CD8\(^+\) T cells between T-bet\(^{-/-}\) mice and T-bet\(^{+/+}\) mice (Fig. 5B).

SSIEFARL-specific pentamer staining revealed that the number of SSIEFARL-specific CD8\(^+\) T cells were slightly higher in T-bet\(^{-/-}\) mice than in T-bet\(^{+/+}\) mice (Fig. 5C). These differences were amplified after an overnight incubation with SSIEFARL peptide yielding significantly higher numbers of SSIEFARL-specific CD8\(^+\) T cells in the spleen cell suspensions obtained from T-bet\(^{-/-}\) mice compared with T-bet\(^{+/+}\) mice (Fig. 5D).

**Ab responses.** To investigate whether T-bet might influence the isotype distribution of the HSV-2-specific Ab response, the HSV-2-specific IgG1 and IgG2a titers were measured 4 wk after vaccination with HSV-2 TK\(^{+}\). Sera obtained from vaccinated T-bet\(^{-/-}\) mice contained lower titers of both HSV-specific IgG1 and IgG2a (Fig. 6A) and IgG2a (Fig. 6B) than sera from vaccinated T-bet\(^{+/+}\) mice. In accordance with this result, we found that the frequencies of spleen B cells were significantly lower in T-bet\(^{-/-}\) mice. The frequency of B220\(^+\) spleen cells was 55% (range from 54 to 57%, \(n = 3\)) in T-bet\(^{-/-}\) and 48% (range from 46 to 49%, \(n = 3\)) in T-bet\(^{+/+}\) mice (\(p < 0.01\)) (data not shown).

**Discussion**

We show that T-bet is important both in innate defense and IFN-\(\gamma\)-mediated acquired immune protection against genital HSV-2 infection. T-bet\(^{-/-}\) mice were more susceptible to a primary HSV-2 infection. This was associated with a significant impairment in NK cell function in T-bet\(^{-/-}\) mice. Furthermore, T-bet\(^{-/-}\) mice could not mount a protective immune response to HSV-2 following vaccination. We thus show, for the first time, that T-bet is required for efficient vaccination against a viral disease. Vaccinated T-bet\(^{-/-}\) mice were unable to control viral replication and one-third of the animals did not survive the genital HSV-2 challenge. The impaired immune protection in T-bet\(^{-/-}\) mice was associated with a reduced function of CD4\(^+\) but not CD8\(^+\) T cells.

It has previously been shown that IL-12 and in particular IL-15 and IL-18 were important in the innate immune response to genital HSV-2 infection in mice, most likely through their involvement in NK cell function. Both IL-15 and IL-18 are required for the development of functional NK cells (10, 39). When present, all three cytokines promote NK cell activity by inducing or enhancing NK cell activation and by promoting IFN-\(\gamma\) production (7, 8, 10, 11). We show that IL-12 plays a crucial role in the activation of NK cells and thus in the innate immune response to primary genital HSV-2 infection. NK cells from T-bet\(^{-/-}\) mice had a distinct reduction in cytotoxic effector function and also showed a marked decrease in IFN-\(\gamma\) production and in their expression of the NK1.1 receptor. This supports previous findings showing that T-bet-deficient NK cells have an impaired IFN-\(\gamma\) production (28) as well as NK1.1 expression (40). The reduced production of IFN-\(\gamma\) is believed to be a direct consequence of the T-bet deficiency (28). Whether NK1.1 expression is regulated directly by T-bet or through IFN-\(\gamma\) remains to be determined. However, both IFN-\(\gamma\) and NK1.1 expression are correlated to NK cell cytotoxic capacity (41, 42). The impaired NK cell cytotoxic potential in T-bet\(^{-/-}\) mice could thus be caused directly by T-bet deficiency or be a secondary effect due to the lack of NK1.1 and to the reduced IFN-\(\gamma\) production. We favor the second hypothesis considering the fact that the cytotoxic potential of CD8\(^+\) T cells was not reduced by T-bet deficiency.

NK1.1 is expressed by a minority of inbred mouse strains, including C57BL/6 but not 129 mice, which leave a possibility that NK1.1 deficiency could have been favored in the selection process when the chimeras were backcrossed to C57BL/6 mice. However, we exclude this possibility as the genes for T-bet and NK1.1 are located on different chromosomes (chromosome 11 and chromosome 6, respectively). Thus, NK1.1 is most likely down-regulated in our T-bet\(^{-/-}\) mice because mice backcrossed to C57BL/6 mice have a probability of 98% to carry the gene for a functional NK1.1 on at least one of the chromosomes.

T-bet-deficient mice were not able to mount a protective acquired immune response after vaccination, despite the fact that the attenuated vaccine strain replicated equally well in T-bet\(^{+/+}\) and T-bet\(^{-/-}\) mice. The lack of adequate acquired immunity allowed the virus to replicate and cause disease, which caused mortality in a large proportion of the animals. The CD4\(^+\) T cell responses were...
particularly affected, evidenced by an impaired HSV-2-specific DTH response in vivo and by significantly lowered IFN-γ production from CD8^{+} T cells in vitro. The impaired vaccine-induced immunity in T-bet^{−/−} mice was not rescued by the addition of exogenous IFN-γ at the time of vaccination implying that it is indeed T-bet expression that is crucial for the development of protective immunity. This is, to our knowledge, the first study to show that T-bet plays a major role in the induction and the effector phase of a CD4^{+} T cell- and IFN-γ-dependent acquired immune response to a genital viral infection.

CD8^{+} T cells secreting IFN-γ are required for an efficient acquired immune response to genital HSV-2 infection (6, 7, 9, 43). Our previous data show that the HSV-2-specific IFN-γ responses induced by an attenuated viral vaccine appear to operate independently of IL-12 and IL-18 (7). Instead, as shown in this report, the induction of IFN-γ production by HSV-2-specific CD4^{+} T cells and thus appropriate antiviral effector functions requires T-bet for its development. Thus, T-bet is required for a DTH response to occur and for the production of IFN-γ in response to HSV-2. These findings are in line with earlier studies, showing that T-bet is crucial for the production of IFN-γ and for Th1-mediated pathogenic clearance (25, 31).

The requirement for T-bet for appropriate CD8 effector function appears to vary in different experimental setups. In our hands, T-bet deficiency did not reduce the cytotoxic function or the IFN-γ production by CD8^{+} T cells. This implies that T-bet is not required for adequate CD4 T cell-mediated help to virus-specific CD8^{+} T cells, at least not in this particular animal model. Similarly, studies have shown that T-bet is not required for IFN-γ production and cytotoxicity by CD8^{+} T cells stimulated with plate-bound anti-CD3 and anti-CD28 (31). However, T-bet^{−/−} mice infected with lymphocytic choriomeningitis virus do not develop the required CD8^{+} response. T-bet^{−/−} CD8^{+} T cells failed to acquire an effector phenotype and showed a diminished IFN-γ production (24).

Surprisingly, we found that CD8^{+} T cells from T-bet^{−/−} mice had a significantly higher cytotoxic potential than CD8^{+} T cells from T-bet^{+/−} mice. This was however due to the accelerated expansion of SSIEFARL-specific CD8^{+} T cells from T-bet^{−/−} mice during the 24 h activation with SSIEFARL peptide and IL-2. There was no difference in the frequencies of SSIEFARL-specific CD8^{+} T cells between freshly isolated T-bet^{−/−} and T-bet^{+/−} spleens. After activation, the frequency of SSIEFARL-specific CD8^{+} T cells from T-bet^{−/−} mice had increased by 290% whereas the frequency of the SSIEFARL-specific CD8^{+} T cells from T-bet^{+/−} mice did not increase at all. We do not know why the SSIEFARL-specific T-bet-deficient CD8^{+} T cells were selectively favored during the in vitro activation.

Vaccinated T-bet^{−/−} mice were not protected against a genital HSV-2 challenge despite having highly functional HSV-2-specific CD8^{+} T cells. Thus, in this particular animal model using attenuated virus as vaccine, the CD8^{+} T cells are not the major protective effector cells. This is in line with previous studies showing that CD8^{+} T cells are not required for acquired immunity to genital HSV-2 infection (6, 9, 44, 45). However, CD8^{+} T cells do play a major role in acquired immune protection in other vaccination models, e.g., when using the gB-derived CTL peptide SSIEFARL expressed either in vaccinia virus or given together with unmethylated CpG-containing oligonucleotide sequences as adjuvant (14, 46). Thus, the lack of protection by CD8^{+} T cells observed in our model should be treated with caution, as these cells obviously are of major importance following other means of vaccination. Mice immunized intranasal with a vaccinia or influenza virus expressing the single CTL recognition epitope HSVgB498–505 induced both primary and memory CTL responses, indicating that the induction of a single epitope-specific CTL response confers a protective immunity to lethal HSV-2 infection (14). Thus, it appears that the importance of an appropriate CD8^{+} T cell response might depend on the vaccination protocol of the animal model.

We show that the HSV-2-specific Ab response obtained in T-bet^{−/−} mice was impaired both in IgG1 and IgG2a production. This differs from a previous study in which T-bet deficiency was associated with a reduced Th1 (IgG2a) and enhanced Th2 (IgG1 and IgE) Ab repertoire in response to a hapten-protein conjugate (25). We believe that the reduced HSV-specific IgG1 production observed could reflect the reduced frequencies of spleen B cells in T-bet^{−/−} mice. Still, it is unclear whether the reduced IgG1 responses obtained in T-bet-deficient mice contribute to the impaired protection against HSV-2. Several studies in mice show that B cells are not required for a protective immune response (6, 47). Furthermore, studies in humans show that high titers of HSV-2-specific Abs do not protect against infection, even though they might have a beneficial effect on the disease profile (48). In conclusion we show that T-bet is important both in innate defense and for the generation of protective Th1 immunity against genital HSV-2 infection in mice, mainly through a decreased function of NK cells and CD4^{+} T cells, respectively.

Disclosures
The authors have no financial conflict of interest.

References
7. Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired T-cell-mediated protective immunity. J. Virol. 75: 6705–6709.

Downloaded from http://www.jimmunol.org/ by guest on September 25, 2017


