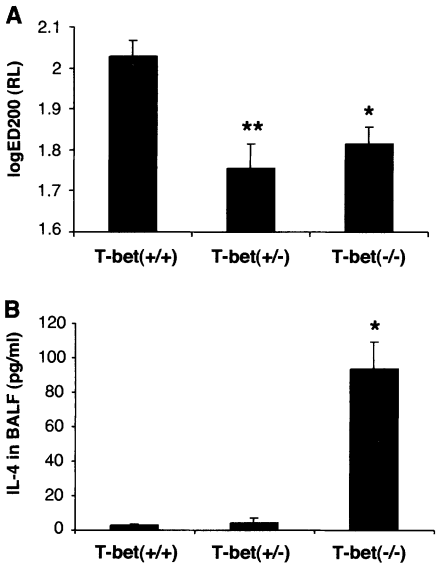

Fig. 3. Adoptive transfer of spleen CD4-OVA–specific T cells derived from T-bet–deficient mice induced increased AHR and increased IL-4 production in the airways. After OVA sensitization, CD4-positive cells were sorted from the spleen of WT or T-bet $^{-/-}$ mice by using anti-CD4 beads (Dynal, NY) (1.5×10^6 cells/mouse) and transferred intraperitoneally into histocompatible SCID mice (C.B.-17Icr $^{scid/scid}$; Taconic laboratories, Germantown, PA). **(A)** AHR in response to an increasing concentration of intravenous methacholine was determined by standard measures of pulmonary mechanics in anesthetized and mechanically ventilated mice. The effective dose of intravenous methacholine required to double pulmonary resistance is termed ED_{200} R_L; higher values of ED_{200} R_L denote lower levels of airway responsiveness (*12*). Adoptive transfer of OVA-specific CD4 cells derived from mice lacking T-bet induced AHR in SCID mice as compared with similar mice reconstituted with OVA-specific CD4 cells derived from WT matched littermates (*P = 0.05, **P = 0.0029). **(B)** An increase in IL-4 production in the BALF was observed only in SCID mice reconstituted with CD4 cells derived from T-bet$^{-/-}$) mice (*$P < 0.05$).

portion of lymphocytes that were CD4$^+$ was 38.9 ± 2.2% (WT$^{+/+}$ mice), 39.57 ± 6.48% (CD4 T-bet$^{+/-}$ mice), and 38.5 ± 5.48% (T-bet$^{-/-}$ mice). In addition, the lungs of SCID mice reconstituted with CD4$^+$ cells derived from T-bet$^{-/-}$ mice exhibited increased IL-4 in the BALF as compared with recipient mice reconstituted with spleen CD4$^+$ cells derived from WT mice (Fig. 3B), demonstrating that the AHR observed in T-bet$^{-/-}$ mice is T cell–mediated.

Our data demonstrate that targeted deletion of T-bet, in the absence of an induced inflammatory response, results in a physiological and inflammatory phenotype in murine airways similar to that created by allergen exposure in sensitized mice. T-bet–deficient mice demonstrate airway remodeling consistent with asthma that is reminiscent of the human disease. This phenotype exists in naïve mice spontaneously and is similar to that observed in mice following allergen sensitization and challenge. These data suggest that T-bet might be an attractive target for the development of anti-asthmatic drugs; the presence of the asthmatic phenotype in mice carrying only one copy of T-bet implies that subtle changes in T-bet expression or activity may provide a means of modulating the asthmatic response. In that regard, it is intriguing that in the mouse, T-bet is located on chromosome 11 in a region that has been identified as genetically linked to AHR in mice and recently reported as linked to asthma in humans.

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11. Paraffin sections from seven patients with chronic asthma were associated with histological signs of airway remodeling (collagen deposition below the basement membrane and hypertropy of the smooth muscle cells) and chronic airway inflammation (airway infiltration with eosinophils); sections from 13 histologically normal lung samples were taken at a distance from a region of lung containing nonasthmatic or allergic pathology. For immunohistochemical staining, lung sections were deparaffinized, pretreated in a microwave oven in EDTA-buffer (1 mM EDTA-NaOH, pH 8.0) for antigen retrieval, followed by incubation with 2% normal horse serum and either T-bet mAb (4B10) (1:100) or a human CD4 mAb (1:100) (Novovastra, UK). Specific binding was detected by standard methods (*18*).
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17. BALF and blood were collected and processed as described (*19*). Supernatants were analyzed by specific enzyme-linked immunosorbent assay for content of IFN-γ, IL-6, TNF-α, TGF-β, and IL-4 (Pharmingen, San Diego, CA) or IL-5 and IL-13 detection (R&D Systems).
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Distinct Effects of T-bet in T_H1 Lineage Commitment and IFN-γ Production in CD4 and CD8 T Cells

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T-bet is a member of the T-box family of transcription factors that appears to regulate lineage commitment in CD4 T helper (T_H) lymphocytes in part by activating the hallmark T_H1 cytokine, interferon-γ (IFN-γ). IFN-γ is also produced by natural killer (NK) cells and most prominently by CD8 cytotoxic T cells, and is vital for the control of microbial pathogens. Although T-bet is expressed in all these cell types, it is required for control of IFN-γ production in CD4 and NK cells, but not in CD8 cells. This difference is also apparent in the function of these cell subsets. Thus, the regulation of a single cytokine, IFN-γ, is controlled by distinct transcriptional mechanisms within the T cell lineage.

IFN-γ, a pleiotropic cytokine produced principally by CD4 T_H1 cells, CD8 T cells, and NK cells, is essential for both innate and adaptive immunity. It acts by binding to the IFN-γ receptor expressed on nearly all cell types (*1*, *2*) that is coupled to the Jak-STAT signaling pathway (*2*, *3*). Mice lacking IFN-γ, the IFN-γ receptor, or Stat1 display a profound disruption of both innate and adaptive immunity, resulting in death from infection by microbial pathogens and viruses (*4*–*7*). Humans with inactivating mutations in components of the IFN-γ signaling die at an early age from uncontrolled mycobacterial

infections (*8–11*). Although much is known about the structure, function, and signaling pathways of the IFN-γ receptor complex, the control of IFN-γ expression in immune system cells is relatively poorly understood.

We recently identified T-bet, a member of the T-box family of transcription factors (*12*, *13*). T-bet, whose expression is primarily limited to the immune system, is rapidly induced in early developing T_H1 cells and is absent in developing T_H2 cells (*14*, *15*). Introduction of T-bet into polarized CD4 T_H2 cells and their CD8 counterparts, Tc2 primary T cells, results in the conversion of these cells into T_H1 and Tc1 cells, respectively, as evidenced by their production of IFN-γ and repression of IL-4 and IL-5 production. In these studies, T-bet appeared to simultaneously induce T_H1 and Tc1 differentiation and inhibit T_H2 and Tc2 differentiation. T-bet expression correlates with IFN-γ expression in all cells examined, and T-bet can transactivate the IFN-γ gene and induce both endogenous IFN-γ production and chromatin remodeling of individual IFN-γ alleles (*16*). Thus, T-bet may activate T_H1 and Tc1 genetic programs in part by directly controlling IFN-γ gene transcription. Here we show that T-bet is required for IFN-γ production and lineage commitment of CD4 T cells but, unexpectedly, not of CD8 T cells.

To further examine the role of T-bet in IFN-γ production and in T cell development and differentiation, we disrupted the T-bet gene in mice by homologous recombination. Mice homozygous for the T-bet deletion (T-bet$^{-/-}$) were born at the expected Mendelian ratios and appeared phenotypically normal (*17*). Previously, retrovirally transduced T-bet strongly induced IFN-γ production from CD4 T cells (*14*, *16*). To examine whether endogenous T-bet controls CD4 T cell IFN-γ production, we purified CD4 T cells from the lymph nodes of wild-type (T-bet$^{+/+}$) mice and from mice homozygous (T-bet$^{-/-}$) or heterozygous (T-bet$^{+/-}$) for deletion of T-bet; stimulated the cells with plate-bound antibodies to CD3 (anti-CD3) and anti-CD28; and measured IFN-γ production during primary stimulation. A marked decrease in IFN-γ production by T-bet$^{-/-}$ CD4 T cells was observed even in the presence of IL-12, a potent inducer of IFN-γ production (Fig. 1A). These results demonstrate that T-bet is required for CD4 T cell IFN-γ production.

Naïve CD4 T cells differentiate into distinct T_H cell subsets, called T_H1 and T_H2 (*18*, *19*), that are defined by distinct cytokine profiles and effector functions. To determine whether T-bet plays a central role in T helper cell development, we generated T_H effector populations from T-bet$^{+/+}$, T-bet$^{+/-}$, and T-bet$^{-/-}$ CD4 T cells stimulated through the T cell receptor (TCR) under neutral conditions or under conditions that induce either a T_H1 or T_H2 phenotype and upon restimulation, cytokine production assayed by enzyme-linked immunosorbent assay (ELISA) and intracellular cytokine staining (ICC). Under neutral conditions, T-bet$^{-/-}$ CD4 T cells produced substantially less IFN-γ than control T-bet$^{+/+}$ CD4 T cells (Fig. 1B). This decrease in IFN-γ production was accompanied by an increase in production of the T_H2-specific cytokines IL-4 and IL-5. Even when stimulated under T_H1-inducing conditions, T-bet$^{-/-}$ cells continued to produce very low levels of IFN-γ and were unable to suppress production of IL-4 and IL-5 (Fig. 1B). ICC analysis showed a marked decrease in the number of IFN-γ–producing cells in the absence of T-bet and a corresponding increase in IL-4– and IL-5–producing cells (*17*). Thus, T-bet controls not only immediate cytokine production from naïve CD4 T cells but also profoundly affects long-term T helper differentiation. In the absence of T-bet, CD4 T cells fail to differentiate into the T_H1 lineage and default to a T_H2 fate. Thus, T-bet not only induces T_H1 development but also actively suppresses T_H2 differentiation.

We also observed that heterozygous T-bet$^{+/-}$ CD4 T cells, whose absence of one T-bet allele yielded a corresponding decrease in T-bet mRNA and protein (*17*), displayed an intermediate phenotype of cytokine production (Fig. 1B). ICC analysis revealed that 81% of wild-type (WT) T_H1 cells were high-level IFN-γ producers, whereas the intermediate phenotype observed in the heterozygous T cells was the result of about half the number of cells producing WT levels of IFN-γ (42% high-level producers) (*17*). Therefore, the ability of T-bet to control IFN-γ production is highly dosage sensitive, a finding consistent with the known function of other T-box family genes in which haploid insufficiency of Tbx3 and Tbx5 leads to the genetic disorders ulnar mammary and Holt-Oram syndromes, respectively (*20*, *21*). Alternatively, the expression of T-bet may be monoallelic, rather than biallelic, as documented for certain cytokine genes (e.g., IL-2 and IL-4) (*22*, *23*).

Immunization with protein antigens typically induces a mixed T_H1/T_H2 response that leads to B cell immunoglobulin heavy-chain isotype switching to immunoglobulin G2a (IgG2a) and IgG1, respectively. IFN-γ–deficient T-bet$^{-/-}$ mice might produce an altered pattern of Ig isotypes following protein antigen immunization. Immunization of WT and T-bet$^{-/-}$ mice with TNP-KLH (2,4,6-trinitrophenol–keyhole limpet hemocyanin) revealed that T-bet$^{-/-}$ mice produced decreased amounts of TNP-specific IgG2a and a small increase in TNP-specific IgG1 as compared with controls at day 12 (Table 1). Additionally, we have recently determined a role for T-bet in B cells in controlling the transcription of germ line IgG2a (*24*). CD4 T cells isolated from TNP-KLH–immunized mice failed to produce IFN-γ in response to KLH and produced higher levels of IL-4 and IL-5 as compared with control mice (Fig. 1C). These results provide in vivo confirmation of our in vitro data demonstrating that T-bet$^{-/-}$ CD4 cells fail to generate T_H1 responses and default to the T_H2 pathway.

A critical function of CD4 T cells in vivo is to combat infection by certain pathogens. Infection with the intracellular protozoan *Leishmania major* (*L. major*) is a well-characterized model for studying the in vivo differentiation and function of CD4 T cells. Inbred mouse strains such as C57BL/6 (B6) control infection by developing a curative T_H1 response, whereas genetically susceptible mice such as BALB/c develop a noncurative T_H2 response and fail to control the infection (*25*). We therefore tested the ability of the normally resistant B6 strain to control *L. major* infection in the absence of T-bet. After infection with *L. major*, popliteal lymph node cells from T-bet$^{-/-}$ B6 mice produced very little IFN-γ as compared with littermate controls (Fig. 1D) (*17*). T-bet$^{-/-}$ B6 mice failed to cure a *L. major* infection, and their susceptibility to infection was similar to that of the naturally susceptible BALB/c strain as assessed by lesion size (Fig. 1E), parasite burden, and cellular infiltrates (*17*, *24*). These results demonstrate that T-bet is central to the in vivo control of T_H cell lineage commitment and subsequent T_H cell effector function.

NK cells are an essential early component of the host response to infection and secrete large amounts of IFN-γ in response to cytokines such as IL-12 and IL-18. We observed the coordinate induction of T-bet with IFN-γ secretion in several NK cell lines (*14*, *24*). To determine whether T-bet was essential for IFN-γ production from primary NK cells, we purified splenic NK cells from T-bet$^{-/-}$, T-bet$^{+/-}$, and T-bet$^{+/+}$ mice using the DX5 marker (*26*) and measured IFN-γ production 72 hours after the indicated cytokine treatment. A decrease in IFN-γ production was observed from T-bet$^{-/-}$ and T-bet$^{+/-}$ NK cells (Fig. 2A), which correlated with a decreased percentage of individual IFN-γ–producing cells (*24*). Thus, similar to CD4 T cells, T-bet is essential for optimal IFN-γ production from purified NK cells. T-bet$^{-/-}$ NK cells were also markedly impaired in their ability to spon-

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taneously lyse the NK-sensitive target cell line, YAC-1 (Fig. 2B, left). This diminished cytolytic function of T-bet$^{-/-}$ NK cells was more severe than observed in the IFN-γ$^{-/-}$ NK cells (*6*). To determine if there was a global defect in T-bet$^{-/-}$ NK cytolytic activity, we injected T- bet$^{-/-}$ or control mice with poly(I:C) 24 hours before harvesting splenocytes, which preactivates NK cells exclusively through the IFN-α signaling pathway (*27*). T-bet$^{-/-}$ and T-bet$^{+/+}$ NK cells when activated in vivo with poly(I:C) lysed tumor cell targets equivalently (Fig. 2B, right). These results demonstrate that T-bet is required for both normal NK cell IFN-γ production and effector function and suggest that T-bet may control other genes involved in NK cytolytic activity that are likely to overlap with IFN-α–induced genes.

IFN-γ production from cytotoxic CD8 T cells is a key mechanism by which these cells combat viral infections. Northern blot analysis of purified CD8 T cells that were activated with plate-bound anti-CD3 and anti-CD28 and the indicated cytokines demonstrates the coordinate expression and induction of both T-bet and IFN-γ (Fig. 3A), as occur in CD4 T cells. We next examined whether IFN-γ production in CD8 T cells was affected by T-bet deficiency. Unexpectedly, we found no difference in the level of IFN-γ produced nor in the number of IFN-γ–producing cells among the three genotypes (Fig. 3, B and C). Thus, although retroviral transduction of T-bet into CD8 Tc2 cells converts them into Tc1 cells (*14*), in a physiological setting, T-bet is not required for IFN-γ gene transcription in the CD8 T cell lineage. This finding was also reflected in the capacity of T-bet$^{-/-}$ CD8 T cells to display equivalent cytotoxic activities (Fig. 3D). However, the presence of T-bet in CD8 T cells, and the regulation of T-bet expression by signals emanating

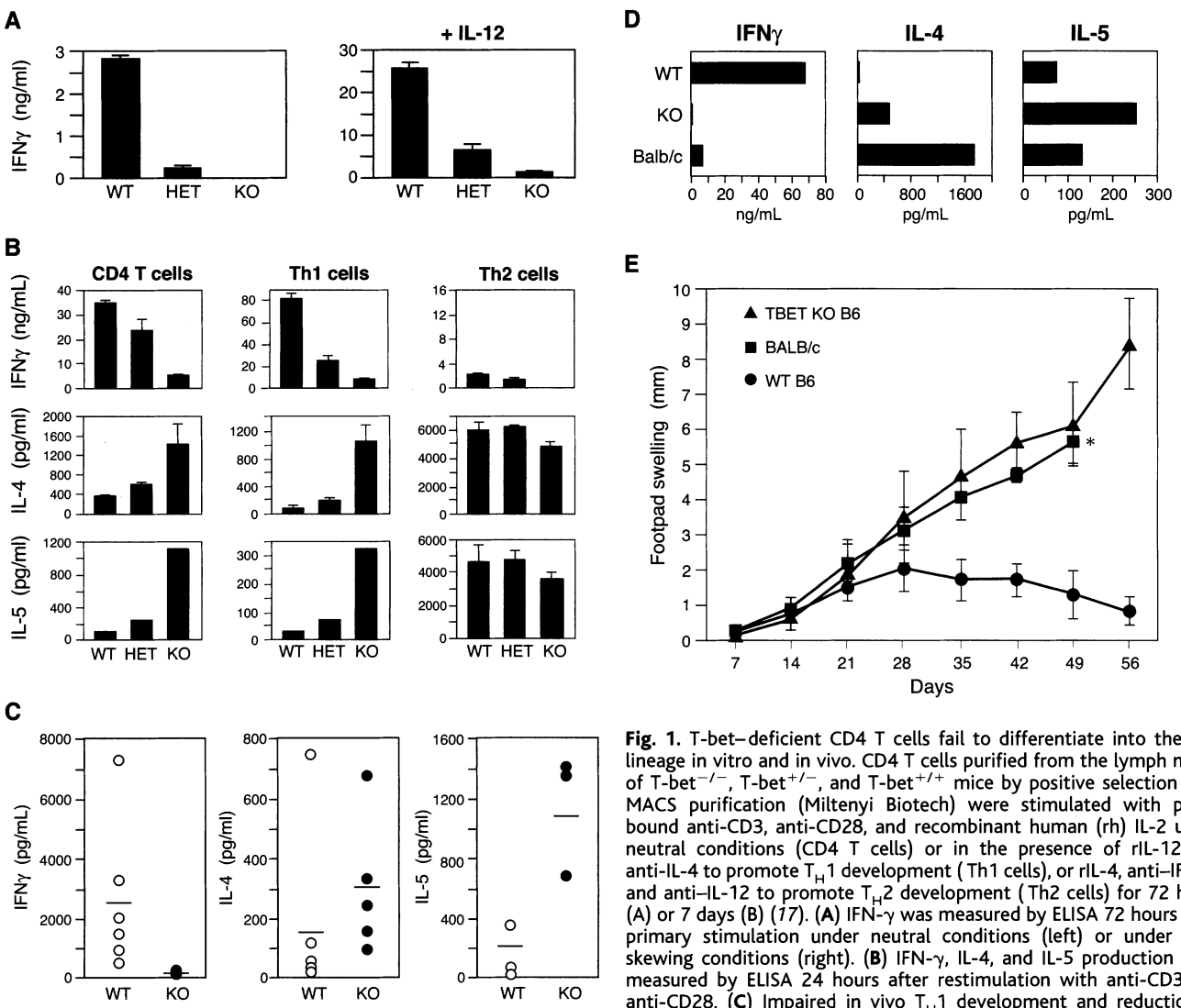

Fig. 1. T-bet–deficient CD4 T cells fail to differentiate into the T_H1 lineage in vitro and in vivo. CD4 T cells purified from the lymph nodes of T-bet$^{-/-}$, T-bet$^{+/-}$, and T-bet$^{+/+}$ mice by positive selection with MACS purification (Miltenyi Biotech) were stimulated with plate-bound anti-CD3, anti-CD28, and recombinant human (rh) IL-2 under neutral conditions (CD4 T cells) or in the presence of rIL-12 and anti-IL-4 to promote T_H1 development (Th1 cells), or rIL-4, anti–IFN-γ, and anti–IL-12 to promote T_H2 development (Th2 cells) for 72 hours (A) or 7 days (B) (*17*). (**A**) IFN-γ was measured by ELISA 72 hours after primary stimulation under neutral conditions (left) or under T_H1-skewing conditions (right). (**B**) IFN-γ, IL-4, and IL-5 production were measured by ELISA 24 hours after restimulation with anti-CD3 and anti-CD28. (**C**) Impaired in vivo T_H1 development and reduction of IgG2a levels in T-bet–deficient mice. Three (IL-5) or six (IFN-γ, IL-4, IgG1, and IgG2a) 6- to 8-week-old T-bet$^{-/-}$ and T-bet$^{+/+}$ mice were immunized at the base of the tail with 100 μg of TNP-KLH emulsified in complete Freund's adjuvant and hapten-specific Ig isotype titers determined by ELISA at day 12 (Table 1) (*29*). CD4 T cells purified from popliteal lymph nodes were stimulated in vitro with KLH antigen (100 μg/ml) and irradiated C57BL/6 splenocytes as antigen-presenting cells (APCs). IFN-γ, IL-4, and IL-5 production was measured after 48 hours. (**D** and **E**) Enhanced susceptibility to *L. major* infection in T-bet$^{-/-}$ C57BL/6 mice. Six 4- to 5-week-old T-bet$^{-/-}$ mice (fourth backcross to C57BL/6), C57BL/6 littermate controls, and WT BALB/c mice were infected in the hind right footpad with 2×10^6 stationary-phase *L. major* promastigotes (LV39). (D) Decreased *L. major*–specific T cell IFN-γ production. CD4 T cells purified from popliteal lymph nodes from infected mice were stimulated in vitro with leishmania antigen and irradiated C57BL/6 splenocytes as APCs. IFN-γ, IL-4, and IL-5 production measured after 48 hours. (E) Increased lesion size from C57BL/6 T-bet$^{-/-}$ mice. Lesion size was measured with a dial-gauge micrometer at 7-day intervals up to day 49 for BALB/c mice (asterisk denotes that mice were killed) or day 56 for T-bet$^{-/-}$ C57BL/6 (TBET KO B6) and littermate controls (WT B6). Footpad swelling was determined by calculating the increase in thickness between the infected and uninfected contralateral footpad as described (*30*).

from the TCR and cytokine receptors, suggest that this transcription factor may play a distinct role in CD8 T cells.

The analysis of the immune system in mice that lack T-bet, as described above, establishes T-bet as a transcription factor required for T_H1 lineage commitment. T-bet$^{-/-}$ CD4 T cells fail to produce the hallmark T_H1 cytokine, IFN-γ, even upon deliberate polarization of the culture conditions and instead produce the T_H2-specific cytokines IL-4 and IL-5. Mice that lack T-bet cannot generate a functional T_H1 response in vivo to protein immunization and fail to control a T_H1-dependent protozoan infection. Given the evidence suggesting a pathogenic role of T_H1 cells in autoimmunity and a protective role in asthma and cancer, these observations have important implications for the treatment of human disease. Indeed, as might be predicted from this shift in the T_H1/T_H2 balance, preliminary studies from our laboratory reveal that T-bet–deficient mice are completely protected from developing inflammatory bowel disease (*24*) and, conversely, develop spontaneous airway hyperreactivity and asthma (*28*).

In contrast to its role in CD4 T and NK cells, T-bet is not involved in controlling IFN-γ production in the other major subset of T cells, the cytotoxic CD8 T cell. This unexpected observation demonstrates that CD4 and CD8 T cells, although closely related and arising from a common progenitor in the thymus, have nevertheless evolved distinct mechanisms for transcriptional control of IFN-γ production. Potentially, this divergence may have occurred to maximize the ability of an organism to mount a protective immune response against a diverse range of microorganisms, protozoans, and viruses.

Table 1. TNP-specific serum IgG levels.

Ig isotype	WT, arbitrary units (±SD)	KO, arbitrary units (±SD)
TNP-specific IgG2a	10,270,750 (4,615,332)	3,023,933 (1,105,319)
TNP-specific IgG1	193,533 (145,897)	458,333 (157,872)

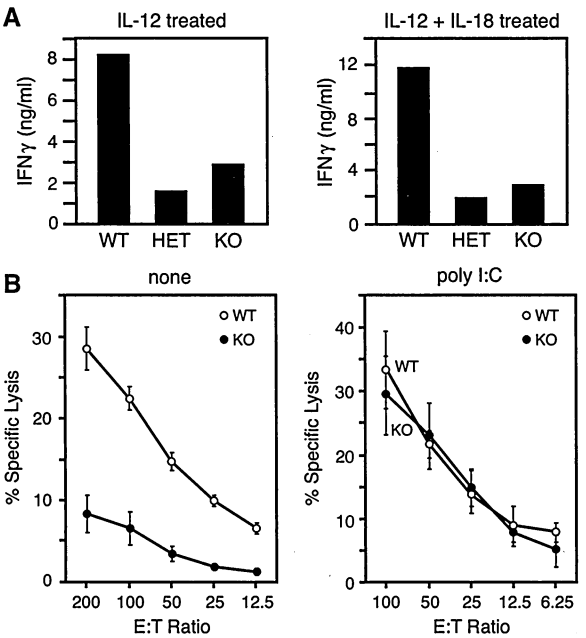

Fig. 2. Reduced IFN-γ production and effector function in T-bet$^{-/-}$ NK cells. (**A**) DX5$^+$ splenic NK cells were purified from T-bet$^{-/-}$, T-bet$^{+/-}$, and T-bet$^{+/+}$ mice by positive selection with MACS purification and IFN-γ production measured by ELISA 72 hours after treatment with IL-12 alone or rIL-12 and rIL-18. (**B**) Diminished spontaneous tumor cell lysis by T-bet$^{-/-}$ NK cells. (Left) Unfractionated splenocytes were incubated for 4 hours with ^{51}Cr-labeled NK-sensitive YAC-1 target cells at the indicated effector-to-target ratios. (Right) T-bet$^{-/-}$ and T-bet$^{+/+}$ mice were injected intraperitoneally with 100 μg of poly(I:C) 24 hours before splenocyte isolation as described above. Results are the mean ± SEM of three mice in each group and are representative of two independent experiments.

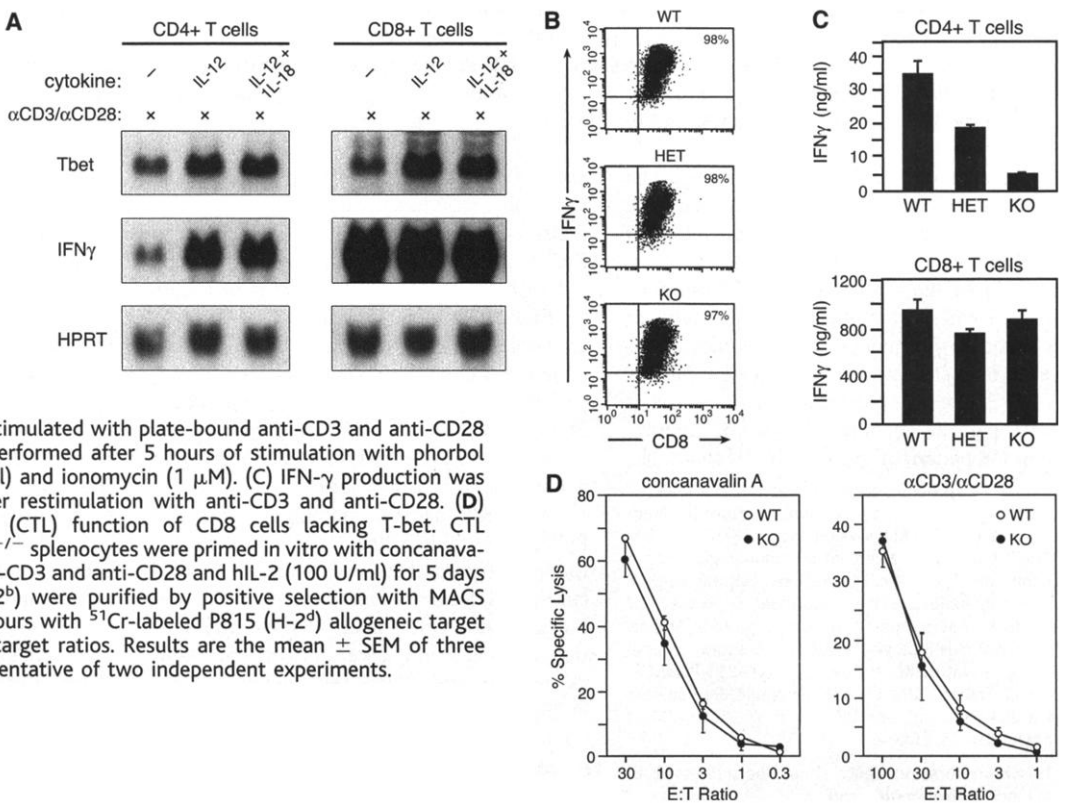

Fig. 3. Unimpaired IFN-γ production and CTL effector function in T-bet$^{-/-}$ CD8 T cells. (**A**) T-bet is expressed in both CD4 and CD8 T cells. Purified CD4 and CD8 T cells were stimulated for 72 hours with plate-bound anti-CD3, anti-CD28, rIL-12 and rIL-18, RNA prepared and Northern blot analysis performed with T-bet, IFN-γ, and hypoxanthine phosphoribosyltransferase probes. (**B** and **C**) Defective IFN-γ production is restricted to T-bet$^{-/-}$ CD4 T cells. CD8 T cells and CD4 T cells purified from T-bet$^{-/-}$, T-bet$^{+/-}$, and T-bet$^{+/+}$ lymph nodes were stimulated with plate-bound anti-CD3 and anti-CD28 for 7 days. (B) ICC analysis was performed after 5 hours of stimulation with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μM). (C) IFN-γ production was measured by ELISA 24 hours after restimulation with anti-CD3 and anti-CD28. (**D**) Normal cytotoxic T lymphocyte (CTL) function of CD8 cells lacking T-bet. CTL precursors from T-bet$^{+/+}$ or T-bet$^{-/-}$ splenocytes were primed in vitro with concanavalin A (5 μg/ml) or plate-bound anti-CD3 and anti-CD28 and hIL-2 (100 U/ml) for 5 days (*31*). On day 5, CD8 T cells (H-2^b) were purified by positive selection with MACS purification and incubated for 4 hours with ^{51}Cr-labeled P815 (H-2^d) allogeneic target cells at the indicated effector-to-target ratios. Results are the mean ± SEM of three mice in each group and are representative of two independent experiments.

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Stage-Specific Transcription of Distinct Repertoires of a Multigene Family During *Plasmodium* Life Cycle

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Members of a multigene family in the rodent malaria parasite *Plasmodium yoelii yoelii* code for 235-kilodalton proteins (Py235) that are located in the merozoite apical complex, are implicated in virulence, and may determine red blood cell specificity. We show that distinct subsets of *py235* genes are expressed in sporozoites and hepatic and erythrocytic stages. Antibodies to Py235 inhibited sporozoite invasion of hepatocytes. The switch in expression profile occurred immediately after transition from one stage to another. The results suggest that this differential expression is driven by strong biological requirements and provide evidence that hepatic and erythrocytic merozoites differ.

Invasive stages (oökinete, sporozoite, and merozoite) of the malaria parasite penetrate specific host cell types at different stages of the life cycle. The 235-kD rhoptry proteins (Py235) of the rodent parasite *Plasmodium yoelii yoelii* are implicated in the type of erythrocyte (normocyte or reticulocyte) invaded by merozoites and in parasite virulence (*1*–*3*). There are ~35 copies of *py235* genes in the parasite genome. Analysis of the transcription pattern of *py235* in blood stages has revealed a mechanism of clonal phenotypic variation (*4*): Merozoites from a single infected erythrocyte differ with respect to Py235 in their rhoptries, suggesting a unique survival strategy (*4*, *5*). Homologs of Py235 are found in other malaria species (*6*–*13*), and antibodies to both Py235 and a *P. falciparum* homolog inhibit merozoite invasion (*1*, *2*, *13*). We investigated the transcription pattern of *py235* during the different stages of the parasite's life cycle and the effect of specific antibodies on cell invasion.

We used a panel of antibodies specific to Py235 to establish that Py235 proteins are found in sporozoites and infected hepatocytes. A 235-kD protein was detected in extracts of sporozoites (Fig. 1A). By immunofluorescence, staining was only obtained with the pAb-S6 and pAb-F sera, indicating that Py235 proteins in pre-erythrocytic stages differ from those in erythrocytic parasites. All sporozoites were labeled, with diffuse staining outlining each cell and regions of more intense label at both ends (Fig. 1B). Infected hepatocytes were labeled with a patchy pattern that may correspond to developing merozoites (Fig. 1C). Evidence that sporozoite Py235 proteins have a functional role was obtained from antibody inhibition of sporozoite invasion of cultured primary hepatocytes (*14*). A Py235-specific antibody reactive with the proteins in sporozoites inhibited invasion, but an antibody to Py235 expressed only during blood stages did not inhibit invasion (Fig. 1D).

We analyzed *py235* transcripts using nested reverse transcription–polymerase chain reaction (RT-PCR). The size-polymorphic 3′-end of *py235* (*4*, *15*) (Fig. 2A) was amplified with RNA purified from 10 to 100 oocysts (found on a single midgut) or from 10,000 to 100,000 salivary gland sporozoites (265BY line) (Fig. 2B). A single-sized fragment was consistently amplified from early (5-day) and mature (10-day) oocysts (Fig. 2B, + lanes) and from different batches of salivary gland sporozoites (Fig. 2B, + lanes); in contrast, multiple-sized products were obtained with RNA purified from an equivalent number of erythrocytic parasites (Fig. 2C). Sequencing of about 200 different cloned fragments derived from at least three independent RT-PCRs showed that these single products all had the same sequence (Fig. 2D, type IIb). A single band was also consistently amplified from RNA extracted from liver-stage parasites grown in vitro or in vivo (Fig. 2C), and sequence analysis of about 100 cloned products also showed that they had the identical sequence. No transcript was detected in very early hepatic trophozoites (in liver biopsies 3 hours after sporozoite inoculation), indicating that the sporozoite *py235* mRNA is degraded very soon after hepatocyte invasion.

Although multiple *py235* genes are transcribed in the erythrocytic stages (*4*, *15*), it is not known how soon this pattern is established after initiation of blood infection by hepatic merozoites. Therefore, we prepared RNA from blood samples of sporozoite-in-

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