to find new agents that can regulate melanogenesis from natural resources.

We investigated one plant having the ability to inhibit melanogenesis. An extract from the herb *T. hirsuta* considerably inhibited melanogenesis without cytotoxicity (Fig. 1). After 48 h of treatment with the extract, B16 cells were still completely viable (Fig. 1b), which is important if this plant will be used for medicine or cosmetics. If the *T. hirsuta* extract is cytotoxic even on murine cells, it would be difficult to market it as a product. Our findings showing an antimelanogenesis effect of the extract without cytotoxicity can lead to new treatment technologies.

In addition, the *T. hirsuta* extract downregulated the tyrosinase expression level after more than 24 h of treatment (Fig. 2b). The melanogenesis reaction is regulated by tyrosinase, a key enzyme and tyrosinase-related protein-1 and -2 (1–3). *Thymelaea hirsuta* extract downregulated the tyrosinase expression level and this effect continued for more than 48 h. This suggests that the actual compound that inhibits melanogenesis and downregulates tyrosinase expression might be the same and has a very strong influence on these activities. This is because the melanogenesis inhibition by the extract was observed after 24 h of treatment, and tyrosinase expression was also inhibited after 24 h. Furthermore, we found that tyrosinase expression almost completely disappeared even after 48 h. These results showing a long and continuous effect after only one treatment are useful for the management of disease or cosmetic problems, because the antimelanogenesis effect can be observed with a very low concentration of the extract applied only once.

Recently, the relationship between MAPK phosphorylation and melanogenesis inhibition has been shown (26–28). The antimelanogenesis effect of some agents has been shown to be associated with the phosphorylation of ERK1/2, one of the main molecules in the MAPK intracellular signal transduction cascade (29–31). Furthermore, transforming growth factor (TGF)-β1 has been shown to decrease melanin synthesis via ERK1/2 phosphorylation (32). Our results also showed ERK1/2 phosphorylation by the extract in B16 cells (Fig. 2a). This effect was observed after 0.5–5 h of treatment with the extract. The TGF-β1 effect was previously shown to be continuous for 6 h. The *T. hirsuta* extract target molecule and cascade might be the same as the TGF-β1 target, because our result is almost similar to the report in which ERK1/2 phosphorylation continued to occur after 6 h (31). On the other hand, it was clearly shown that corticotrophin-releasing hormone (CRH) phosphorylated ERK1/2 through CRH receptors in B16 cells within 15 min after treatment (33). Although it was shown that CRH treatment induces B16 cell migration via ERK1/2 phosphorylation (33) and it is quit different from our observation, it is possible that cell migration and antimelanogenesis share one signal transduction pathway. From these reports and our results, we propose three hypotheses for the ERK1/2 phosphorylation: (i) a compound in the *T. hirsuta* extract can phosphorylate ERK1/2 via the same cascade as that of TGF-β1, (ii) a compound in the *T. hirsuta* extract can phosphorylate ERK1/2 via the activation of CRH receptors; and (iii) a small compound in the *T. hirsuta* extract can phosphorylate ERK1/2 directly. As the extraction was carried out using organic solvent, however, the agent inducing this phosphorylation is predicted to be a small molecule. If indeed so, this actual component could pass through the cell membrane of B16 cells and phosphorylate the ERK1/2 directly. This is one reason behind the early phosphorylation of ERK1/2. These results suggest that the effect of tyrosinase on melanogenesis is downstream of the MAPK cascade.

To determine the main compound in the extract that is responsible for the antimelanogenesis effect, we carried out further fractionation and quantification of the melanin synthesized by B16 cells treated with each fraction. Results showed that one fraction of the EtOAc layer partitioned from the *T. hirsuta* extract, *Th*-EtOAc-11-3, has strong antimelanogenesis activity (Fig. 3). We further found that this fraction has two daphnanes, genkwadaphnin and gnidicin, as the main components. These results indicate that the antimelanogenesis effect of the extract on B16 cells might be attributable to these daphnanes. Daphnanes have been previously isolated from *Thymelaeaceae*, and it was previously reported that daphnanes can induce apoptosis in human leukaemia cells and also have neurotrophic and anticancer effects (34–36). However, the antimelanogenesis effect of daphnanes on B16 melanoma cells has never been reported. In addition, effects on intracellular signal transduction and tyrosinase expression of daphnanes have not been reported. From all of our results, we could hypothesize that daphnanes phosphorylate ERK1/2 at first, and tyrosinase expression is then downregulated by this signal via MAPK, followed by melanin synthesis inhibition. This is the first report showing that daphnanes might function as an antimelanogenesis agent through ERK1/2 phosphorylation and downregulation of tyrosinase in B16 murine melanoma cells.

We showed here for the first time that an herb grown in Tunisia has the ability to maintain skin homeostasis, particularly as an antimelanogenic agent. As another effect of the extract, we also observed that the extract induced cell shape change in B16 cells by changing the F-actin polymerization and distribution (Fig. 4). In the cells treated with the extract, phalloidin signals were strongly detected in the dendritic extensions (Fig. 4, lower-left panel), whereas phalloidin signals were detected widely in the cytoplasm of control cells (Fig. 4, upper-left panel). Moreover, while the control cells were clumped (Fig. 3, upper panels), the

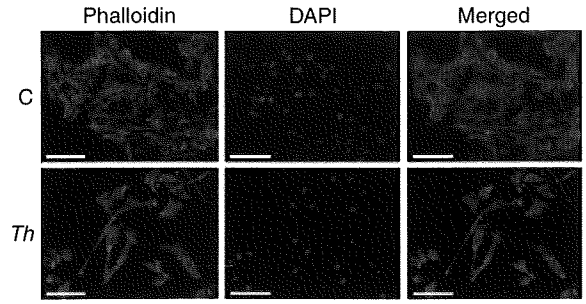

Figure 4. F-actin polymerization in B16 murine melanoma cells treated with *T. hirsuta* extract. B16 cells were seeded onto four-well chamber slides at 4×10^4 cells per well. After overnight incubation, the cells were treated with (*Th*) or without (C) *T. hirsuta* extract at 1000-fold dilution for 24 h. The cells were stained with rhodamine-phalloidin for the actin cytoskeleton (left panels) and with DAPI for the nuclei (middle panels). Merged images are shown in the right panels. All photographs were taken at 400× magnification and each bar represents 50 μm.

treated cells were separated (Fig. 3, lower panels). It is known that normal melanocytes in the basal layer of the epidermis have a dendritic cell shape and are scattered. We might be observing the effects of daphnanes on not only antimelanogenesis but also on some other important phenomena. To confirm the other effects of the extract, more analysis should be performed and more details of the mechanism should be clarified. We are now analysing the other effects of the *T. hirsuta* extract in detail.

From our results, we found that the *T. hirsuta* extract can inhibit melanin synthesis in B16 cells and this antimelanogenesis effect occurs via ERK1/2 phosphorylation and inhibition of tyrosinase expression. Furthermore, we suggested that these phenomena could be attributable to the daphnanes in the *T. hirsuta* extract. However, we do not discount the possibility that other minor compounds in the extract may also function as antimelanogenesis agents or that antimelanogenesis may be the result of a synergistic effect of some compounds in the extract. Nonetheless, our results suggest that compounds from the *T. hirsuta* extract can be useful antimelanogenesis agents.

Acknowledgements

This research was performed under the General Framework Agreement on Science and Technology Cooperation between the Ministry of Scientific Research, Technology and Competency Development (MRSTDC) of the Republic of Tunisia and the Alliance for Research on North Africa (ARENA) of the University of Tsukuba, Japan. The agreement, which was signed on the 6th of June 2005, is in line with the UN convention on Biological Diversity.

The authors express their gratitude to Dr Terence P.N. Talorete of the University of Tsukuba for his English correction.

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Blockade of NKG2D on NKT cells prevents hepatitis and the acute immune response to hepatitis B virus

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Communicated by Arthur Weiss, University of California School of Medicine, San Francisco, CA, September 20, 2007 (received for review July 12, 2007)

Hepatitis B virus (HBV) is a hepadnavirus that is a major cause of acute and chronic hepatitis in humans. Hepatitis B viral infection itself is noncytopathic, and it is the immune response to the viral antigens that is thought to be responsible for hepatic pathology. Previously, we developed a transgenic mouse model of primary HBV infection and demonstrated that the acute liver injury is mediated by nonclassical natural killer (NK)T cells, which are CD1d-restricted, but nonreactive to α-GalCer. We now demonstrate a role for NKG2D and its ligands in this nonclassical NKT cell-mediated immune response to hepatitis B virus and in the subsequent acute hepatitis that ensues. Surface expression of NKG2D and one of its ligands (retinoic acid early inducible-1 or RAE-1) are modulated in an HBV-dependent manner. Furthermore, blockade of an NKG2D–ligand interaction completely prevents the HBV- and CD1d-dependent, nonclassical NKT cell-mediated acute hepatitis and liver injury. This study has major implications for understanding activation of NKT cells and identifies a potential therapeutic target in treating hepatitis B viral infection.

Infection with hepatitis B virus (HBV) is a major cause of liver disease worldwide. More than 350 million people are persistently infected with HBV (1, 2). Hepatitis B viral infection itself is noncytopathic, and it is the immune response to the viral antigens that is thought to be responsible for the necroinflammatory process involved in chronic infection, cirrhosis, and hepatocellular carcinoma (3, 4). Thus, understanding the pathogenesis of acute and chronic HBV infection mandates understanding the immune responses underlying these processes. Unfortunately, the study of HBV immunopathogenesis has been problematic because natural hepadnaviral infections occur only in outbred species whose immune systems are difficult to experimentally manipulate.

We have established a transgenic mouse model of primary HBV infection that allows the study of mechanisms underlying the immunopathogenesis of hepatitis B virus-induced disease. To generate this mouse model of primary HBV infection, we used either mice that express the small, middle, and large envelope proteins of HBV as transgenes in the liver by using an albumin promoter (hereafter designated HBV-Env$^+$) (5) or mice that express a terminally redundant HBV DNA construct as a transgene and display intrahepatic HBV replication (hereafter designated HBV-Replication$^+$) (6). We ablated the resident adaptive immune system of these HBV-transgenic mice, in which the B and T cells are tolerant to HBV, by crossing the HBV-transgenic mice to mice with mutations in the recombinase activating genes (*RAG-1*) (7). We then reconstituted the immune system in these mice by the adoptive transfer of unimmunized splenocytes isolated from syngeneic, wild-type mice. In this way, a liver with HBV-expressing hepatocytes is exposed to a healthy, untolerized, naïve immune system, just as in acute HBV infection of humans. This system results in a biphasic illness, with a rapid acute hepatitis, followed by a smoldering chronic hepatitis (8).

In this model of HBV infection, a spontaneous, natural immune response to hepatitis B virus is generated. Because this experimental system mimics primary HBV infection, we can uniquely study innate and adaptive immune responses to HBV. Our previous results using this model demonstrated that natural killer (NK)T cells (and not NK cells or conventional αβ-TcR T cells) alone are sufficient to induce hepatitis when adoptively transferred into HBV transgenic RAG$^{-/-}$ mice. Furthermore, we demonstrated, using several different experimental approaches, that this population of NKT cells that mediates acute hepatitis is nonclassical in that these cells do not recognize the classic NKT cell ligand, α-galactosylceramide CD1d and do not express the canonical T cell receptor Vα14; but activation of these cells depends on expression of CD1d and HBV.

Innate immune effector cells mediate the acute hepatitis in our model, although the mechanism of activation of these cells in response to the presence of HBV in liver is unclear. Our previous data suggest that the presence of HBV leads to alterations in the class I-like molecule CD1d and, subsequently, to the activation of nonclassical NKT cells and hepatitis (8). NKT and NK cells share many of the same activating and inhibitory receptors. One of the activating receptors is NKG2D, a type II transmembrane-anchored glycoprotein, which has been shown to be an activating or costimulatory receptor expressed on the surface of all NK cells, activated CD8$^+$ T lymphocytes, and most γ/δ T cells, both in mice and humans (9–11). Although NKG2D is known to also be expressed on the surface of NKT cells (12, 13), a role for NKG2D in NKT cell activation has never been demonstrated.

NKG2D binds to a family of ligands with structural homology to MHC class I molecules. In contrast to classical MHC molecules, NKG2D ligands do not require association with β_2 microglobulin for expression or function, and do not bind antigenic peptides (14). In mice, NKG2D ligands include the retinoic acid early-inducible 1 family of proteins (RAE-1α, β, γ, δ, ε), H60, and MULT1 (15–17). The ligands of NKG2D are known to be "stress-inducible" molecules, whose expression is triggered by cellular transformation, viral infection (9), and/or DNA damage (18).

In this study, we addressed the question of whether NKG2D and its ligands play a role in the nonclassical NKT cell-mediated immune response to HBV and the subsequent acute hepatitis that ensues. Our results demonstrate that NKG2D is modulated on NK and NKT cells at the time of acute hepatitis; and the presence of HBV in the livers of our transgenic mice can lead to an increase in RAE-1 surface expression on hepatocytes. Furthermore, blockade

Author contributions: S.V., L.L.L., and J.L.B. designed research; S.V. and S.N. performed research; K.O. and L.L.L. contributed new reagents/analytic tools; S.V., S.N., L.L.L., and J.L.B. analyzed data; and S.V. and J.L.B. wrote the paper.

Conflict of interest statement: The authors declare a conflict of interest (such as defined by PNAS policy). University of California (San Francisco, CA) has licensed intellectual property rights relating to this research for potential therapeutic development.

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; NK, natural killer; RAE-1, retinoic acid early-inducible-1; MULT1, murine ULBP-like transcript 1; RAG$^{-/-}$, recombinase activating gene-1.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0708968104/DC1.

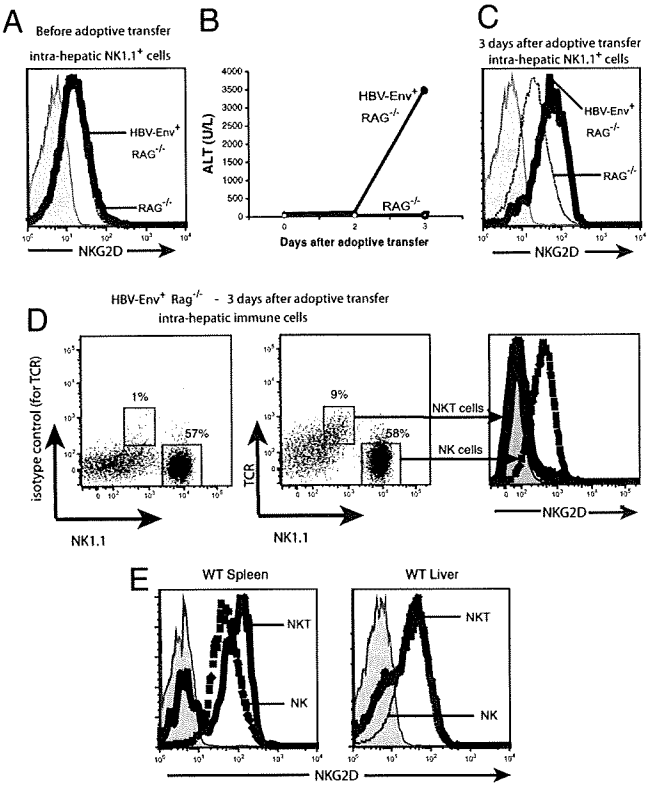

Fig. 1. Modulation of the NKG2D receptor during acute hepatitis. (*A* and *C*) NKG2D expression on the surface of NK1.1$^+$ cells from HBV-Env$^+$ RAG$^{-/-}$ (heavy black line) and RAG$^{-/-}$ (dotted line) before (*A*) and 3 days after (*C*) adoptive transfer of 10^8 splenocytes. (*B*) Hepatic necrosis in these animals was assessed by the measurement of ALT in the sera of RAG$^{-/-}$ (○) or HBV-Env$^+$ RAG$^{-/-}$ (●) mice. ALT values are shown as mean ± SEM. (*D*) Surface expression of NKG2D on intrahepatic NKT (heavy black line) and NK cells (dashed line) from HBV-Env$^+$ RAG$^{-/-}$ mice 3 days after adoptive transfer. The left dot plot depicts the isotype-matched control Ig staining of TCR on NKT cells. (*E*) Surface expression of NKG2D on NKT cells (heavy black line) and NK cells (dashed line) from the spleen and liver of wild-type mice. The shaded histograms depict staining using an isotype-matched control rat IgG1. All experiments were repeated at least three times, and representative data are shown.

of an NKG2D–ligand interaction completely prevents the HBV-specific and CD1d-dependent, nonclassical NKT cell-mediated acute hepatitis and liver injury.

Results

NKG2D Expression Is Modulated on Intrahepatic Immune Cells from HBV-Env$^+$ RAG$^{-/-}$ Mice with Acute Hepatitis. Our previous studies have demonstrated that activation of nonclassical NKT cells is necessary for the acute hepatitis to develop and that NK cells or conventional T cells alone cannot initiate the acute hepatitis (8). NK1.1$^+$ cells from the livers of HBV-Env$^+$ RAG$^{-/-}$ and RAG$^{-/-}$ mice before adoptive transfer of syngeneic naïve splenocytes expressed equivalent amounts of NKG2D on their surface (Fig. 1*A*). However, when we analyzed the expression of NKG2D on liver lymphoid cells during the acute immune response and hepatitis seen in the livers of HBV-expressing mice with reconstituted immunity (Fig. 1*B*), we found that NK1.1$^+$ cells from HBV-Env$^+$ RAG$^{-/-}$ mice (which include both resident and donor NK cells and donor NKT cells) expressed higher levels of NKG2D than the same population eluted from RAG$^{-/-}$ mice that also had reconstituted immunity (Fig. 1*C*).

We next analyzed the surface expression of NKG2D on the NKT and NK populations in the liver at the peak of acute hepatitis. We found that NK cells eluted from the livers of HBV-Env$^+$ RAG$^{-/-}$ mice with acute hepatitis expressed high levels of NKG2D (Fig. 1*D*), but the majority of activated NKT cells expressed very low levels of NKG2D on their cell surface (Fig. 1*D*). Because the majority of NKT cells in the spleen (the cells adoptively transferred) and liver of wild-type mice expressed high levels of NKG2D (Fig. 1*E*), this result suggests that NKT cells eluted from the livers of the HBV-Env$^+$ RAG$^{-/-}$ mice have down-regulated the surface expression of NKG2D. This is consistent with the fact that NKG2D is known to be internalized after interaction with its ligands (8, 14, 19). Taken together, these results suggest that NKG2D expression is up-regulated on the NK cells, and down-regulated on the NKT cells, during acute hepatitis.

Constitutive Surface Expression of RAE-1 on Hepatocytes Is Elevated Specifically on HBV-Env$^+$ Hepatocytes. In light of these data, we examined the expression of NKG2D ligands on wild-type nontransgenic hepatocytes and on HBV-Env$^+$ hepatocytes. In the genetic background of the HBV-transgenic mice (B10.D2 and C57BL/6), the NKG2D ligands expressed are *RAE-1δ*, *RAE-1ε*, and MULT1 (14). Although RAE-1 is not expressed in most tissues isolated from healthy, adult mice, RAE-1 is transcribed preferentially in liver of healthy, adult mice (http://source.stanford.edu/cgi-bin/source/sourceSearch). We examined the expression of these NKG2D ligand proteins on primary hepatocytes and intrahepatic immune cells of HBV-Env$^+$ RAG$^{-/-}$ and wild-type nontransgenic RAG$^{-/-}$ mice before adoptive transfer. We found constitutive low-level surface expression of RAE-1 on hepatocytes from RAG$^{-/-}$ mice, which was increased specifically on the surface of HBV-Env$^+$ hepatocytes (Fig. 2*A*). This constitutive expression of RAE-1 on hepatocytes, and increased expression in the HBV-Env$^+$ transgenic mice, was also found in wild-type mice that were not crossed to RAG$^{-/-}$ mice (data not shown). We found no expression of RAE-1 on splenocytes or on intrahepatic immune cells from HBV-Env$^+$ RAG$^{-/-}$ mice, RAG$^{-/-}$ mice, or wild-type mice (Fig. 2*B* and data not shown). The constitutive surface expression of RAE-1 on hepatocytes is an interesting finding, because the expression of RAE-1 family members is strictly regulated in normal cells, and little expression is found on healthy adult tissue (14). Increased RAE-1 expression on hepatocytes from HBV-Env$^+$ RAG$^{-/-}$ mice demonstrates that RAE-1 can be modulated on hepatocytes in an HBV-specific manner. We did not detect MULT1 expression, or a change in either MHC class I or CD1d expression, on primary hepatocytes derived from either HBV-Env$^+$ RAG$^{-/-}$ or RAG$^{-/-}$ mice or on intrahepatic immune cells (data not shown).

Constitutive expression of RAE-1 on primary hepatocytes from RAG$^{-/-}$ mice was confirmed by quantitative PCR of reverse-transcribed RAE-1 mRNA normalized to the expression of *Hprt* transcripts (Fig. 2*B*). Increased RAE-1 expression on hepatocytes from HBV-Env$^+$ RAG$^{-/-}$ mice, as compared with RAG$^{-/-}$ mice, was also confirmed by quantitative RT-PCR. There was an almost 7-fold increase in RAE-1 mRNA from HBV-Env$^+$ RAG$^{-/-}$ hepatocytes, as compared with RAG$^{-/-}$ hepatocytes (Fig. 2*B*).

Blocking of an NKG2D–Ligand Interaction *in Vivo* Prevents the Acute Immune Response to Human HBV. In view of the fact that NKG2D and one of its ligands are modulated during the acute immune response to HBV, we studied the effects of blocking this interaction on the onset of the acute hepatitis by using an anti-mouse NKG2D monoclonal antibody (CX5), which efficiently blocks the binding of NKG2D to its ligands and does not deplete NKG2D-bearing cells *in vivo* (20). HBV-Env$^+$ RAG$^{-/-}$ recipient mice were treated with 200 μg of anti-NKG2D mAb (CX5) or control rat IgG the day before and 4 days after adoptive transfer of syngeneic naïve splenocytes. Blocking the NKG2D receptor completely prevented acute liver injury in all HBV-Env$^+$ RAG$^{-/-}$ mice, whereas the control antibody had no effect as all mice showed signs of massive acute hepatitis, as revealed by the elevated serum ALT values at days 3 and 4 after adoptive transfer (Fig. 3*A*). Histological analyses

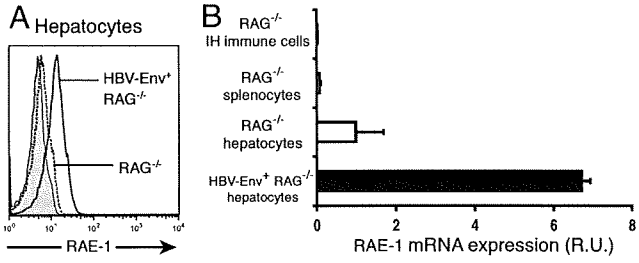

Fig. 2. Constitutive surface expression of the NKG2D ligand RAE-1 on hepatocytes is up-regulated specifically on HBV-Env-expressing hepatocytes, before adoptive transfer. (*A*) Surface expression of RAE-1 on hepatocytes from HBV-Env$^+$ RAG$^{-/-}$ (solid line) and RAG$^{-/-}$ (dotted line) mice. Shaded histograms depict staining using the appropriate isotype-matched control Ig. (*B*) RAE-1 mRNA expression in hepatocytes from HBV-Env$^+$ RAG$^{-/-}$ (black bar) and RAG$^{-/-}$ (white bar) mice and from intrahepatic (IH) immune cells and splenocytes from RAG$^{-/-}$ mice in comparison with HPRT expression. All data are representative of at least three independent experiments.

of liver sections also showed that mice treated with control IgG developed a severe hepatitis, pathologically characterized by parenchymal inflammation, hepatocellular damage, and portal inflammation and necrotic hepatocytes at day 4 after adoptive transfer (Fig. 3*B*). These histological abnormalities were absent at the same time point in all mice treated with anti-NKG2D mAb (Fig. 3*B*). These results demonstrate a fundamental role played by NKG2D in the acute immune response to HBV-expressing hepatocytes, and the consequent development of hepatitis and hepatic necrosis.

HBV-Env$^+$ RAG$^{-/-}$ mice have an HBV-dependent increase in the frequency of IFN-γ- and IL-4-producing cells in their livers 3 days after adoptive transfer (8). Because NKT cells mediate this cytokine burst detected at the time of acute hepatitis, we investigated the cytokine profile of lymphoid cells in anti-NKG2D or control IgG-treated HBV-Env$^+$ RAG$^{-/-}$ mice. We quantified the number of IFN-γ- and IL-4-producing intrahepatic immune cells by Elispot at days 3 and 4 after adoptive transfer of syngeneic wild-type splenocytes. Three days after the adoptive transfer, the number of IFN-γ- and IL-4-producing cells increased by 8- and 7-fold, respectively, in mice that received control IgG (and developed hepatitis) as compared with NKG2D-blocked mice (Fig. 3*C*). A similar difference was observed on day 4 after adoptive transfer. These data demonstrate that blocking of NKG2D also severely impaired the production of cytokines by intrahepatic immune cells in mice expressing HBV antigens. Flow cytometric analysis of the intrahepatic immune cells derived from the anti-NKG2D or control IgG-treated HBV-Env$^+$ RAG$^{-/-}$ mice revealed that the absolute number of NK cells eluted from both groups of mice was similar, whereas the absolute number of NKT cells was reduced by 2- and 3-fold in the mice that received the anti-NKG2D treatment and did not develop hepatitis [supporting information (SI) Table 1]. This specific reduction in the number of NKT cells, but not NK cells, in the livers of the anti-NKG2D-treated mice suggests that the antibody is specifically affecting the NKT cells.

NKG2D Receptor Expression on NKT Cells Is Required for Efficient Disease Induction. NKG2D is expressed on ≈60% of NKT cells in the spleen (Fig. 1*E*). To determine whether donor NKT cells expressing NKG2D are responsible for induction of the acute hepatitis after transfer into HBV-Env$^+$ RAG$^{-/-}$ mice, splenocytes from wild-type mice were depleted of NKG2D$^+$ lymphocytes by flow cytometric cell sorting. NKG2D-depleted splenocytes were adoptively transferred into HBV-Env$^+$ RAG$^{-/-}$ recipients. In this way, the transferred donor NKT cells would not express surface NKG2D, but resident NK cells in the recipient mice would still express NKG2D. HBV-Env$^+$ RAG$^{-/-}$ mice received NKG2D-

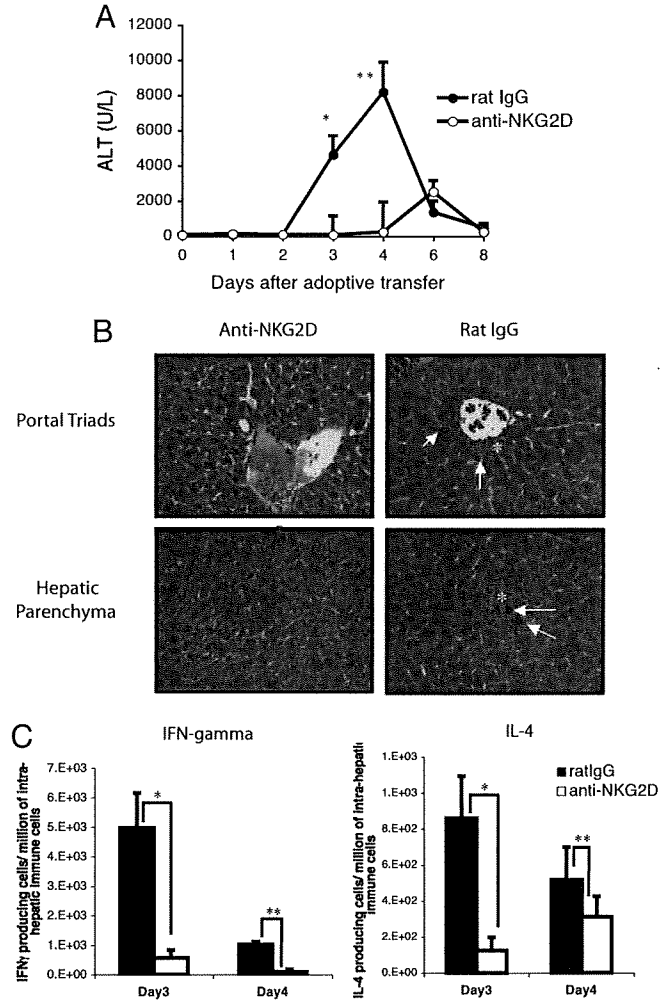

Fig. 3. Blocking NKG2D *in vivo* prevents the liver injury caused by the acute immune response to HBV. (*A*) Serum ALT levels of HBV-Env$^+$ RAG$^{-/-}$ mice treated with anti-NKG2D mAb (○) or rat IgG (●). The ALT values as mean ± SEM are shown. Student's *t* test analyses: *, $P < 0.02$; **, $P < 0.01$. (*B*) Hematoxylin and eosin-stained section (magnification ×20) of portal triads (*Upper*) and hepatic lobular parenchyma (*Lower*) from HBV-Env$^+$ RAG$^{-/-}$ mice treated with anti-NKG2D mAb (*Left*) or rat IgG (*Right*), 4 days after the adoptive transfer of 10^8 splenocytes. Arrows point to necrotic hepatocytes, and asterisks indicate inflammatory infiltrate. (*C*) Elispot analyses of IFN-γ- and IL4-producing intrahepatic immune cells from HBV-Env$^+$ RAG$^{-/-}$ mice treated with control rat IgG (black bars) or anti-NKG2D mAb (white bars) at days 3 and 4 after adoptive transfer. Representative data are shown as mean ± SD. Student's *t* test analyses: *, $P < 0.005$; **, $P < 0.02$. All data are representative of at least three independent experiments.

depleted splenocytes or appropriate controls for the total number of splenocytes, or total number of NK and NKT cells transferred. The HBV-Env$^+$ RAG$^{-/-}$ recipient mice received one of three different populations of donor splenocytes: 50 million NKG2D-depleted splenocytes (which included 1.25×10^5 NKG2D$^-$ NKT cells, and no NK cells); 50 million unsorted splenocytes (which included 2.5×10^5 unsorted NKT cells and 1.25×10^6 NK cells); or 33 million NK cell-depleted splenocytes (which included 1.25×10^5 unsorted NKT cells, and no NK cells). In this latter group, the NK cells were depleted from the donor mice by injection of anti-asialoGM1 antisera, which is known to deplete NK cells, but not NKT cells (21).

The depletion of NKG2D$^+$ NKT cells, but not the depletion of NKG2D-bearing NK cells, from donor splenocytes significantly diminished the acute liver injury and cytokine burst seen in the

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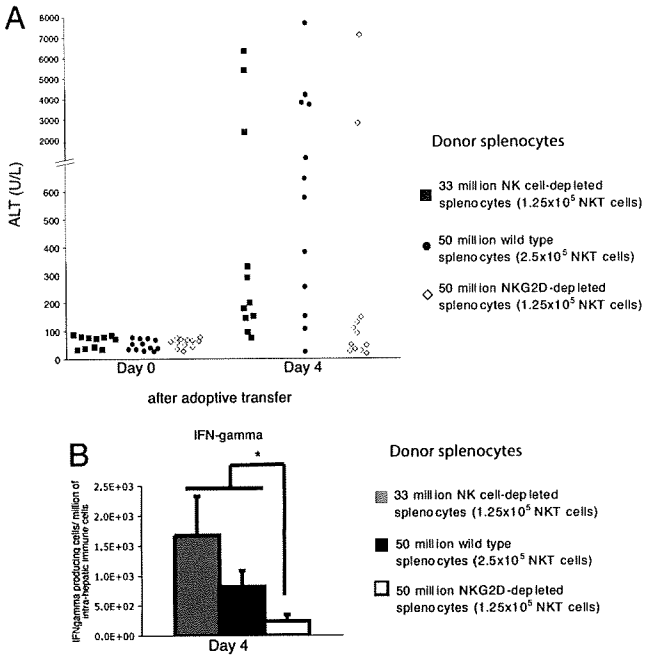

Fig. 4. NKG2D$^+$ NKT cells are required for efficient disease induction and cytokine production during the acute immune response to HBV. (*A*) Hepatic injury of HBV-Env$^+$ RAG$^{-/-}$ mice at day 4 after adoptive transfer of 50 × 10^6 NKG2D-depleted splenocytes (which included 1.25 × 10^5 NKG2D$^-$ NKT cells, and no NK cells) (open diamonds) was compared with hepatic injury in HBV-Env$^+$ RAG$^{-/-}$ mice that received the same total number of unsorted wild-type splenocytes (50 × 10^6, which included 2.5 × 10^5 unsorted NKT cells, and 1.25 × 10^6 NK) (filled circles) (Mann–Whitney test analyses: $P < 0.02$) or that received the same total number of unsorted NKT cells and NK cells (33 × 10^6, which included 1.25 × 10^5 unsorted NKT cells, and no NK cells) (filled squares). (Mann–Whitney test analyses: $P < 0.03$). (*B*) Elispot analyses of IFN-γ producing intrahepatic immune cells from HBV-Env$^+$ RAG$^{-/-}$ mice depicted in *A*. Representative data are shown as mean ± SD. Student's *t* test analyses: *, $P < 0.001$.

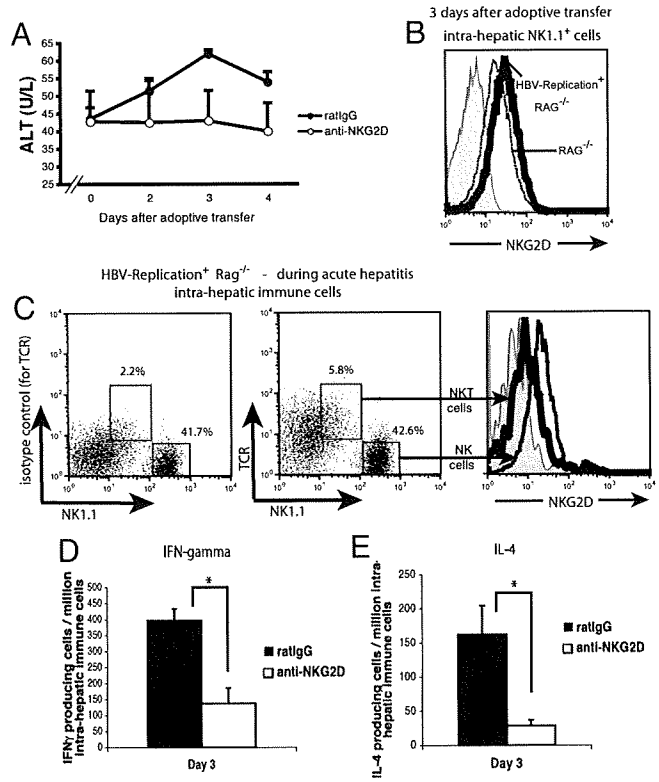

Fig. 5. Blocking an NKG2D–ligand interaction in HBV-Replication$^+$ RAG$^{-/-}$ mice prevents liver injury and cytokine production mediated by the acute immune response to HBV. (*A*) Serum ALT levels of HBV-Replication$^+$ RAG$^{-/-}$ mice treated with anti-NKG2D mAb (○) or rat IgG (●) at 2, 3, and 4 days after adoptive transfer of 1 × 10^8 syngeneic splenocytes are shown as mean ± SEM. (*B*) NKG2D surface expression on intrahepatic NK1.1$^+$ cells from HBV-Replication$^+$ RAG$^{-/-}$ mice (heavy black line) as compared with RAG$^{-/-}$ mice (dotted line) at day 3 after the adoptive transfer of syngeneic naïve splenocytes. Shaded histogram depicts staining using the appropriate isotype-matched control Ig (rat IgG1). (*C*) Surface expression of NKG2D on intrahepatic NKT (heavy black line) and NK cells (lighter black line) from HBV-Replication$^+$ RAG$^{-/-}$ mice 3 days after adoptive transfer. The left dot plot depicts the isotype-matched control Ig staining of TCR on NKT cells. (*D* and *E*) Elispot analyses of IFN-γ (*D*) and IL-4-producing (*E*) intrahepatic immune cells from HBV-Replication$^+$ RAG$^{-/-}$ mice treated with rat IgG (black bars) or anti-NKG2D mAb (white bars) at day 3 after adoptive transfer. Representative data are shown as mean ± SD. Student's *t* test analyses: *, $P < 0.001$. All data are representative of at least two independent experiments.

HBV-Env$^+$ RAG$^{-/-}$ mice as compared with either control group (Fig. 4 *A* and *B*). Thus, NKG2D receptor expression on NKT cells and not on NK cells is required for efficient disease induction. The finding that depletion of NKG2D-bearing cells did not completely eliminate all disease in all recipient mice could be accounted for the fact that the NKG2D$^-$ NKT cells to begin to express surface NKG2D after adoptive transfer (data not shown). Further evidence that NKG2D expression only on NKT cells is necessary for disease induction comes from the finding that depletion of NKG2D$^+$ NKT cells from donor splenocytes completely prevented induction of acute hepatitis in the HBV-Env$^+$ RAG$^{-/-}$ mice (SI Fig. 6). Thus, NKG2D expression on NKT cells, and not on any other cell types, is necessary for induction of hepatitis.

NKG2D Blockade Prevents Acute Hepatitis and the Cytokine Burst Seen in HBV-Replication$^+$ RAG$^{-/-}$ Transgenic Mice. To assess the relevance of our observations to responses to authentic human HBV infection, we characterized the role of NKG2D in the acute immune response developed in HBV-Replication$^+$ RAG$^{-/-}$ mice, which display intrahepatic HBV replication and produce infectious virions (6). Reminiscent of the usual initial presentation of human HBV infection, these mice develop a mild, subclinical hepatitis after adoptive transfer of naïve splenocytes. Analogous to our observations in the HBV-Env$^+$ RAG$^{-/-}$ mice, this hepatitis is mediated by nonclassical NKT cells in an HBV-specific and CD1d-dependent manner, leading to cytokine production (ref. 8 and unpublished data). Although the severity of hepatitis seen in the two lines of HBV-transgenic mice is different, owing to an increase in hepatocyte sensitivity to cytotoxic effects of IFN-γ in the HBV-Env$^+$ mice (22), a similar disease pattern is seen in both lines of HBV-transgenic mice. Specifically, a biphasic ALT rise that is seen in the HBV-Env$^+$ RAG$^{-/-}$ mice is also observed in the HBV-Replication$^+$ RAG$^{-/-}$ mice, but, as expected, the ALT rise is much more modest than that seen in the HBV-Env$^+$ mice, typically, serum transaminases were elevated no more than 2-fold above background, which is a clinically significant finding in human HBV disease.

HBV-Replication$^+$ RAG$^{-/-}$ mice were treated the day before the adoptive transfer of syngeneic naïve splenocytes with 200 μg of anti-NKG2D or control IgG, and we monitored the serum ALT levels. We found that the modest rise in the serum ALT in the HBV-Replication$^+$ RAG$^{-/-}$ mice treated with control IgG was not evident in mice treated with anti-NKG2D (Fig. 5*A*). Together with this modest rise of ALTs, we observed that 3 days after the adoptive transfer, the number of IFN-γ- and IL-4-producing cells increased by 3- and 6-fold, respectively, in mice that received control IgG as compared with NKG2D-blocked mice (Fig. 5 *D* and *E*). To evaluate the role of NKG2D in acute hepatitis developed in HBV-

Replication$^+$ mice, we examined the expression of NKG2D on NK1.1$^+$ cells from HBV-Replication$^+$ RAG$^{-/-}$ mice 3 days after adoptive transfer. Just as we observed in the HBV-Env$^+$ RAG$^{-/-}$ mice, NK cells from the livers of HBV-Replication$^+$ RAG$^{-/-}$ mice had increased expression of NKG2D during acute hepatitis, as compared with RAG$^{-/-}$ mice; and the NKT cells had down-regulated NKG2D (Fig. 5 *B* and *C*).

Discussion

Collectively, our findings clearly establish a role for NKG2D in the HBV-specific, CD1d-restricted nonclassical NKT cell-mediated acute hepatitis and cytokine production seen in both the HBV-Env$^+$ RAG$^{-/-}$ and HBV-Replication$^+$ RAG$^{-/-}$ mice. These results demonstrate a role for an NKG2D–ligand interaction in NKT cell activation. NKG2D is expressed on several cell types in the liver. However, NKG2D-bearing NK cells alone, or splenocytes depleted only of NKG2D$^+$ NKT cells do not induce acute hepatitis in the HBV-Env$^+$ RAG$^{-/-}$ and HBV-Replication$^+$ RAG$^{-/-}$ mice (ref. 8 and SI Fig. 6).

A direct effect of NKG2D blockade on NKT cell activation in our studies is suggested by several lines of evidence. First, anti-NKG2D mAb treatment efficiently prevented production of IL-4, which is expressed by the HBV-activated NKT cells but not NK cells. Second, anti-NKG2D mAb treatment decreased the number of NKT cells, but not the number of NK cells, in the livers of mice with hepatitis. Finally, NKG2D receptor expression on NKT cells, and not on other cells types, is required for efficient disease induction in our transgenic model of primary HBV infection.

For these reasons, we propose a model in which nonclassical NKT cells are first activated in a HBV-specific, CD1d-restricted and NKG2D-dependent manner, leading to production of cytokines, which in turn activate NK cells. NKG2D ligand interaction can function to directly activate cells or function as a costimulatory molecule (9–11). That the activation of nonclassical NKT cells in our mouse model of HBV infection requires expression of HBV and CD1d, in addition to an NKG2D-ligand interaction, suggests that nonclassical NKT cell activation requires a CD1d-dependent signal through its T cell receptor and that NKG2D may function as a costimulatory molecule. Further studies will be required to address whether NKG2D is also important in the subsequent NK cell activation. While our studies were under review, Chen *et al.* (23) reported the ability of NKG2D blockade to diminish hepatitis in HBV-transgenic mice; however, in these experiments, induction of the disease required the injection of the mitogen Con A, which polyclonally activates all T cells and possibly other cell types such as NK cells in the host.

We detected the NKG2D ligand, RAE-1, on the surface of all hepatocytes in normal, wild-type as well as HBV transgenic mice. This constitutive expression of RAE-1 was increased on hepatocytes from the HBV-Env$^+$ RAG$^{-/-}$ mice. Unlike the HBV-Env$^+$ RAG$^{-/-}$ mice, the amount of RAE-1 on hepatocytes from the HBV-Replication$^+$ RAG$^{-/-}$ mice was not elevated compared with nontransgenic hepatocytes (data not shown). NKG2D is nonetheless necessary for the nonclassical NKT cell activation and onset of hepatitis in both the HBV-Env$^+$ RAG$^{-/-}$ mice and HBV-Replication$^+$ RAG$^{-/-}$ mice because disease was completely prevented and cytokine production was greatly diminished by anti-NKG2D blockade. Therefore, we hypothesize that the constitutive, basal levels of RAE-1 on the hepatocytes are sufficient to trigger the HBV-specific, CD1d-restricted, NKG2D-dependent, nonclassical NKT cell-mediated hepatitis. This constitutive surface expression of RAE-1 on hepatocytes is also an interesting finding, because the expression of RAE-1 family members is strictly regulated in normal cells, and little expression is found on healthy adult tissue.

Because the HBV-Env$^+$ RAG$^{-/-}$ mice have increased expression of one of the three isoforms of HBV envelope protein (large or L protein) that is retained in the endoplasmic reticulum, these mice display increased sensitivity to the cytotoxic effects of IFN-γ (22). The up-regulation of RAE-1 in the liver of these mice may be a direct or indirect consequence of increased large envelope expression. Increased expression and accumulation of envelope proteins is also one of the pathophysiologic consequences of HBV infection in humans (24). Because expression of HBV large envelope genes is zonal (6, 25), it is possible that RAE-1 is increased on some hepatocytes that have higher expression of envelope protein, but we cannot detect the increased expression of RAE-1 because these cells are a minority of the total hepatocyte population.

Our present findings reveal a mechanism by which human HBV activates the innate immune system and sets up the cytokine milieu in which the subsequent adaptive immune response develops. The question of whether HBV alerts the innate immune system and what role the innate immune system plays in HBV pathogenesis is controversial. Studies of acute HBV infection in primates and humans reveal an initial quiescent phase of $\approx$4–7 weeks before HBV starts to replicate vigorously, reaching levels of 10^9 to 10^{10} copies per milliliter (26–28). Activation of components of the innate immune system are likely to play a central role in control of this initial HBV burst because HBV-DNA quantity decreases by almost 90% well in advance of the appearance of an antigen-specific CD8$^+$ T cell response and hepatopathology (27–31). However, identification of the individual components of the innate immune system responsible for this rapid down-regulation of viral replication, and the mechanism of activation, has been elusive. NK cells have been implicated in this process, because there is an increase in the number of peripheral NK cells before the peak of viral replication (31). However, Northern blot and gene expression analysis of total liver RNA derived from core liver biopsies during this period have failed to reveal evidence of activation of innate immune effector pathways, leading to the hypothesis that HBV does not alert the innate immune system (32, 33).

Our current data demonstrating that nonclassical NKT cells are activated to produce cytokines in an HBV-specific, CD1d-restricted, and NKG2D-dependent manner is consistent with a role for these cells in the initial response to HBV. The finding that activation of these nonclassical NKT cells leads to a cytokine burst in the absence of overt hepatocellular injury in the HBV-Replication$^+$ mice is consistent with the usual initial subclinical presentation of HBV infection. Using real-time PCR analysis on whole liver biopsies, we, like others, cannot detect an innate immune response (the presence of T cell receptor, IL-4, or IFN-γ transcripts) in the HBV-transgenic RAG$^{-/-}$ mice 3 days after adoptive transfer of syngeneic splenocytes (SI Fig. 7). In contrast, we clearly demonstrate the presence of NKT cells (using flow cytometry) and the production of IL-4 and IFN-γ (using ELispot assays) in the eluted lymphocytes from the same livers used in the real-time PCR experiments, as depicted in Figs. 1, 3, and 5 (data not shown). Thus, our data suggest that innate immune responses to HBV infection exist, and likely have been previously unappreciated because NKT cells represent only a small fraction of the total cell mass of the liver; thus, any NKT cell transcripts are diluted by the overwhelming abundance of hepatocyte RNA.

These mouse models of HBV infection lay the foundation for directed studies analyzing the role of NKT cells, NK cells, NKG2D, and its ligands in human HBV infection. In addition, because the activation of innate effector cells has also been implicated in hepatic flares in chronic HBV infection (34), our models offer the opportunity to examine the role of NKG2D and its ligands in chronic HBV infection, and suggest possible new strategies for therapeutic intervention in this disease.

Methods

Mice and Disease Model. HBV-Env$^+$ transgenic mice: mouse lineage 107-5D [official designation Tg (Alb-1.HBV) Bri66; inbred B10.D2, H-2^d) (5) and HBV-Replication$^+$ mice: lineage 1.3.46 [official designation, Tg (HBV 1.3 genome) chi46; inbred C57BL/6H-2^b] (6)

crossed to RAG-1 $^{-/-}$ mice. HBV-Tg × Rag-1$^{-/-}$ mice (8-to 10-week-old) were intravenously injected with donor splenocytes from 6- to 10-week-old wild-type B10.D2 or C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, ME), respectively. Mice were bled by tail vein at the described intervals, and sera were collected. Other mice were killed at the indicated time points, and livers were perfused or collected for histology. All mice were kept in a pathogen-free facility at University of California (San Francisco, CA).

Alanine Aminotransferase (ALT). Serum ALT was measured by the standard photometric method by using a COBAS MIRA plus autoanalyzer.

Isolation of Hepatocytes and Intrahepatic Immune Cells. To obtain hepatocytes, livers were perfused via the thoracic portion of the inferior vein cava with a commercial liver perfusion medium (GIBCO, Carlsbad, CA) for 5 min, followed by incubation in a digestion media (DMEM Low Glucose 50%/F-12 50% mixture and 0.12–0.2 mg/ml collagenase) for 8 min. Livers were cut into small pieces and filtered through a 70-μm nylon cell strainer and centrifuged at 30 × g for 3 min. Immune cells were obtained as described (8).

Flow Cytometry. Fc-block (2.4G2 anti-CD16/32 mAb) and fluorochrome-conjugated antibodies against TCRβ (H57), NK1.1 (PK136), CD1d (1B1), H-2K^{d} (SF1–1.1), or the appropriate isotype-matched control Ig were purchased from BD (Franklin Lakes, NJ). PE-labeled anti-NKG2D (CX5) antibody (rat IgG1 isotype) was purchased from eBioscience (San Diego, CA). Purified antibodies against RAE-1, which recognizes all known RAE-1 proteins (rat IgG2a isotype), and MULT1 were purchased from R & D Systems (Minneapolis, MN) (35). Cells were analyzed on a LSR II (BD) by using FlowJo software.

Cell Sorting. Splenocytes were stained with anti-NKG2D mAb CX5 and the negative lymphocytes isolated. In some experiments, splenocytes were costained with anti-NKG2D (CX5), anti-TCRβ (H57), and anti-NK1.1 (PK136). Cells expressing all three cell surface markers (NKG2D^{+} NKT cells) were depleted. All flow cytometry sorting experiments showed >98% purity by using a FACS Aria cell sorter (BD).

TaqMan Quantitative RT-PCR. Quantitative (real-time) RT-PCR was carried out by using an ABI 7300 according to the manufacture's instructions. Specific primers and probes were used for HPRT (19), pan-RAE-1 (20), IFNγ, TCRβ, and IL-4.

***In Vivo* Antibodies.** A neutralizing, nondepleting rat anti-mouse NKG2D mAb, clone CX5 (rat IgG1), generated as described (19), recognizes the NKG2D extracellular domain and blocks the binding of NKG2D to its ligands. We injected i.p. 200 μg of CX5 or control rat IgG (Sigma, St. Louis, MO) per recipient mouse the day before and 4 days after the adoptive transfer of syngeneic naïve splenocytes. To deplete NK cells, but not NKT cells, from donor splenocytes, we used a depleting rabbit anti-mouse/rat asialo GM1 polyclonal antibody purchased from Cedarlane Laboratories (21). Depletion of NK cells (<0.1%) was verified by flow cytometry before the adoptive transfer was performed.

ELISpot Assay. Intrahepatic immune cells were eluted from mice at day 3 and/or day 4 after adoptive transfer. Cells were counted and immediately plated in an anti-cytokine mAb-coated 96-well microplate (ELISpot mouse IFN-γ and IL-4 kits; BD). Eight serial 2- or 3-fold dilutions were done in duplicate, per condition. Spots were counted automatically by using an AID ELISpot Reader.

Histology. Liver was fixed, embedded in paraffin, and stained with hematoxylin and eosin. Liver sections were scored by an unbiased pathologist, according to the histopathologic standard scale for assessing viral hepatitis (36).

We thank Laura Vilarinho for technical advice; Gerald Willkom and Anna Bogdanova for technical support; João P. Pereira for technical advice and manuscript comments; and William Seaman, Samuel Baron, T. S. Benedict Yen, Stewart Cooper, and Don Ganem for critically reading the manuscript. This work was supported in part by the Burroughs Wellcome Fund, the American Liver Foundation, the Cancer Research Institute, and the University of California Liver Center (San Francisco, CA) (P30-DK26743). S.V. is supported by Portuguese Foundation for Science and Technology (POCI) 2010 Grant SFRH/BD/21982/2005. L.L.L. is an American Cancer Society Research Professor, and work was supported by National Institutes of Health Grant R37 AI066897.

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J. Immunol. 2007;178;605-612
http://www.jimmunol.org/cgi/content/full/178/1/605

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Th1 and Type 1 Cytotoxic T Cells Dominate Responses in T-bet Overexpression Transgenic Mice That Develop Contact Dermatitis[1]

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Contact dermatitis in humans and contact hypersensitivity (CHS) in animal models are delayed-type hypersensitivity reactions mediated by hapten-specific T cells. Recently, it has become clear that both CD4$^+$ Th1 and CD8$^+$ type 1 cytotoxic T (Tc1) cells can act as effectors in CHS reactions. T-bet has been demonstrated to play an important role in Th1 and Tc1 cell differentiation, but little is known about its contribution to CHS. In the present study, we used C57BL/6 mice transgenic (Tg) for T-bet to address this issue. These Tg mice, which overexpressed T-bet in their T lymphocytes, developed dermatitis characterized by swollen, flaky, and scaly skin in regions without body hair. Skin histology showed epidermal hyperkeratosis, neutrophil, and lymphocyte infiltration similar to that seen in contact dermatitis. T-bet overexpression in Tg mice led to elevated Th1 Ig (IgG2a) and decreased Th2 Ig (IgG1) production. Intracellular cytokine analyses demonstrated that IFN-γ was increased in both Th1 and Tc1 cells. Furthermore, Tg mice had hypersensitive responses to 2,4-dinitrofluorobenzene, which is used for CHS induction. These results suggest that the level of expression of T-bet might play an important role in the development of contact dermatitis and that these Tg mice should be a useful model for contact dermatitis. *The Journal of Immunology,* **2007, 178: 605–612.**

The Th1/Th2 paradigm proposed by Mosmann et al. (1) holds that CD4$^+$ T cells can be subdivided into two categories, namely Th1 and Th2 (2). These two polarized subsets can be identified on the basis of the cytokines they secrete (3). Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. More recently, a similar heterogeneity among CD8$^+$ T cytotoxic (Tc)[4] cells has also been recognized with the identification of Tc1 and Tc2 subpopulations (4, 5). IFN-γ is also one of the main cytokines produced by differentiated CD8$^+$ effector T cells and has been shown to have a fundamental role in CD8$^+$ T cell-mediated immunity (6). Lineage commitment of CD4$^+$ and CD8$^+$ T cells is transcriptionally regulated, often by the same factors that mediate T cell effector function.

T-bet is known as a Th1 lineage commitment transcription factor as a result of its transactivation of the Th1 cytokine IFN-γ (7). Recently, T-bet has also been shown to regulate cytolytic effector mechanisms of CD8$^+$ T cells (8). T-bet expression is rapidly induced in CD8$^+$ T cells by signaling through the TCR and the IFN-γR, and it functions downstream of STAT1 (6, 9, 10). In the context of Ag-specific activation, T-bet is required for the differentiation of naive CD8$^+$ T cells into effector CTLs.

Contact dermatitis is one of the most common skin diseases (11). Knowledge of the pathophysiology of contact dermatitis is derived chiefly from animal models in which the inflammation induced by hapten painting of the skin is referred to as contact hypersensitivity (CHS) (12). Contact dermatitis and CHS are delayed-type hypersensitivity reactions that are mediated by hapten-specific T cells (12). Skin sensitization resulting in contact dermatitis and CHS is dependent on the initiation of specific T lymphocyte responses (11, 13). Until recently it was believed that the most important cells in these responses were CD4$^+$ T lymphocytes. IL-2 and IFN-γ produced by Th1 cells are thought to play a preeminent role in the evolution of CHS (14, 15). Some investigations in mice found CHS to be associated with CD4$^+$ T lymphocyte function and to be compromised when such cells were deleted (15, 16). However, there is growing evidence that in many instances the predominant effector cell in CHS may be a CD8$^+$ T lymphocyte (13, 17, 18). Wang et al. (17) clearly demonstrated that the deletion of CD8$^+$ Tc1 cells had a more significant suppressive effect than the deletion of CD4$^+$ Th1 cells in CHS responses to 2,4-dinitrofluorobenzene (DNFB). According to these results, both CD4$^+$ Th1 and CD8$^+$ Tc1 cells are key players in CHS.

Although T-bet plays an important role in Th1 and Tc1 cell induction, little is known about its contribution to CHS. In the present study we used T-bet overexpression in T cell transgenic (Tg) mice to address this issue.

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Received for publication March 10, 2006. Accepted for publication October 12, 2006.

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[1] This work was supported by Grants-in-Aid for Young Scientists (B) and Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture and a grant of the Genome Network Project from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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[4] Abbreviations used in this paper: Tc, cytotoxic T; CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; LNC, lymph node cell; Tg, transgenic; WT mice, wild-type transgene-negative littermates.

Materials and Methods

Generation of T-bet Tg mice

A 2.5-kb, full-length cDNA encoding the murine T-bet protein was inserted into a VA CD2 transgene cassette containing the upstream gene regulatory region and locus control region of the human CD2 gene. The VA vector has been reported to direct expression of the inserted cDNA in all single-positive mature T lymphocytes of Tg mice, with expression being linearly proportional to the transgene copy number (19). This T-bet construct was injected into BDF1 fertilized eggs to generate Tg mice. T-bet Tg mice were inbred with C57BL/6 mice for four generations. Mice were maintained in specific pathogen-free conditions in a laboratory animal resource center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba (Ibaraki, Japan), and the study was approved by the Institutional Review Board of the university.

Southern hybridization analysis of genomic DNA

Southern hybridization was performed by using the Gene Images random prime labeling module system (Amersham Biosciences). High m.w. DNA was prepared from the tail of each mouse, and 15 μg of DNA was digested with *Apa*I and then subjected to electrophoresis on 1.0% agarose gels. After electrophoresis, the DNA was transferred to a Hybond-N^+ membrane. A fluorescence-labeled *Apa*I/*Kpn*I fragment (0.5 kb) of the *T-bet* cDNA was used as a probe. The transgene copy number was determined from the blot with a BAS 1500 Mac image analyzer.

RT-PCR for transgene expression analysis

Total RNA was prepared from the thymus of 10 wk-old Tg mice or their wild-type transgene-negative littermates (WT mice) using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). First-strand cDNA was synthesized at 42°C for 50 min using the SuperScript II RNase H2 reverse-transcriptase kit (Invitrogen Life Technologies), and 1 μl of this 20 μl reaction mixture was used for the PCR. Amplified products were analyzed on 2% agarose gels. PCR primer sequences were as follows: T-bet, 5′-CGGTACCAGAGCGGCAAGT-3′ and 5′-AGCCCCCTTGTTGTTGGTG-3′; GATA-3, 5′-TCTCACTCTCGAG GCAGCATGA-3′ and 5′-GGTACCATCTCGCCGCCACAG-3′; GAPDH, 5′-CCCCTTCATTGACCTCAACTACATGG-3′ and 5′-GCCTGCTTCAC CACCTTCTTGATGTC-3′.

Western blot analysis

Thymocyte nuclear extracts were prepared from 10 wk-old Tg mice or WT. The extracts were size-fractionated on a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (FluoroTrans), and reacted with primary and secondary Abs. For detection of the T-bet protein, a goat anti-mouse T-bet (N-19; Santa Cruz Biochemicals) was used as the primary Ab and peroxidase-conjugated rabbit anti-goat IgG (Zymed Laboratories) was used as the secondary Ab. For normalization with respect to the amount of protein in each sample, anti-lamin B Ab (Santa Cruz Biochemicals) was used as a control.

Histopathological analysis

Organs were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.2) and embedded in paraffin. Sections (3 μm) were stained with H&E for histopathological examination by light microscopy.

Measurement of serum Ig

Total serum Ig was determined by ELISA as previously described (20). Briefly, Nunc immunoplates were coated with goat anti-mouse Ig (ICN Pharmaceuticals). The plates were kept at room temperature for 1 h and then washed with 0.1 M PBS. After washing, the plates were blocked with 0.5% BSA in PBS solution. Serial dilutions of test serum samples were applied and incubated at room temperature for 1 h. After washing with PBS, the plates were treated with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Sigma-Aldrich) at room temperature for 1 h. After additional washes, alkaline phosphatase substrate (Sigma-Aldrich) solution was added and allowed to develop. Absorption at 405 nm was measured with an immunoplate reader (BenchMark; Bio-Rad).

Culture medium, cytokines, and Abs

RPMI 1640 medium supplemented with 10% FCS, 2-ME (0.05 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES buffer (10 mM), and sodium pyruvate (1 mM) was used as culture medium. Recombinant mouse cytokines were IL-2 (Genzyme Techne), IL-4 (BD Pharmingen), and IL-12 (BD Pharmingen). Purified rat anti-mouse IL-4 (11B11), IL-12 (C17.8), CD3e (145–2C11), and CD28 (37.51) mAb, PE-conjugated anti-mouse IL-5 (TRFK5), and FITC-conjugated anti-mouse IFN-γ (XMG1.2) were purchased from BD Pharmingen.

Preparation of T Cells

$CD4^+$ and $CD8^+$ T cells were prepared from each mouse spleen and lymph nodes. $CD4^+$ and $CD8^+$ T cells were enriched by positive selection using a MACS system with anti-CD4 and anti-CD8 mAb (Miltenyi Biotec). In the spleen cell transfer experiment the cells (3×10^6 cells) were transferred i.v.

Stimulation of Tg $CD4^+CD8^+$ T cells for cytokine production

Primary stimulations of $CD4^+/CD8^+$ T cells (2.5×10^5 cells/well) were performed with cross-linked anti-CD3e (1 μg/ml) and anti-CD28 (10 μg/ml) plus IL-2 (10 ng/ml) in a total volume of 2 ml in 24-well plates. In addition, some cultures received cytokines (10 ng/ml IL-4 or 10 ng/ml IL-12) or mAb to block endogenous cytokines (10 μg/ml anti-IL-4 or 10 μg/ml anti-IL-12). T cells were expanded and maintained under constant culture conditions for 1 wk.

Flow cytometric analysis of intracellular IL-5 and IFN-γ synthesis

Cells were resuspended at 10^5 to 10^6 cells/ml and stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml). Two hours before cell harvesting, brefeldin A was added at 10 μg/ml using a stock solution of 1 mg/ml in ethanol (100%). Cells were harvested, washed, and resuspended in PBS with brefeldin A before the addition of an equal volume of 4% formaldehyde fixative (final concentration, 2%). After fixation for 20 min at room temperature, cells were stained for cytokines. For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin (Sigma-Aldrich), and all incubations were performed at room temperature. Cells were washed and preincubated for 10 min in PBS/BSA/saponin and then incubated with allophycocyanin-conjugated anti-mouse IL-5 (5 μg/ml) and anti-mouse IFN-γ (5 μg/ml) or isotype-matched control Abs (10 μg/ml) for 30 min. After 20 min, cells were washed twice with PBS/BSA/saponin and then washed with PBS/BSA without saponin to allow membrane closure. Samples were analyzed with a FACScalibur flow cytometer (BD Biosciences). Results were analyzed by using CellQuest software.

Induction of CHS

Induction of CHS was conducted using the methods described previously (21). Briefly, mice were sensitized to DNFB by painting the shaved abdomen with 50 μl of 0.5% DNFB in acetone/olive oil (4:1) and each footpad with 5 μl of the mixture on days 0 and 1. On day 5, mice were challenged with 20 μl of 0.3% DNFB on each side of the left ear. As a control, the right ear was painted with an identical amount of vehicle. The ear thickness was measured at 12, 24, 48, and 72 h after challenge at three locations. The ear swelling was calculated as $[(T - T_0)$ left ear$] - [(T - T_0)$ right ear], where T_0 and T represent the values of ear thickness before and after the challenge, respectively.

Results

Generation of Tg mouse lines overexpressing T-bet in T cells

To generate Tg mouse lines expressing high levels of T-bet specifically in T cells, the mouse *T-bet* cDNA was inserted into the VA vector (Fig. 1*A*). Genomic Southern blotting analysis was performed to confirm the integrity and copy number for each Tg mouse line. The length of the *Apa*I fragment containing the *T-bet* transgene was 1.2 kb, whereas the corresponding fragment for the endogenous *T-bet* gene was 4.0 kb (Fig. 1*A*). The transgene was detected in mice of Tg lines 710, 725, and 731 (Fig. 1*B*). In densitometric analyses, line 710 seemed to contain more than 12 copies of the transgene, whereas lines 725 and 731 contained approximately 12 and 8 copies, respectively. However, line 710 could not transmit the genes to the next generation, but the transgenes in both line 725 and line 731 were stably transmitted to progeny.

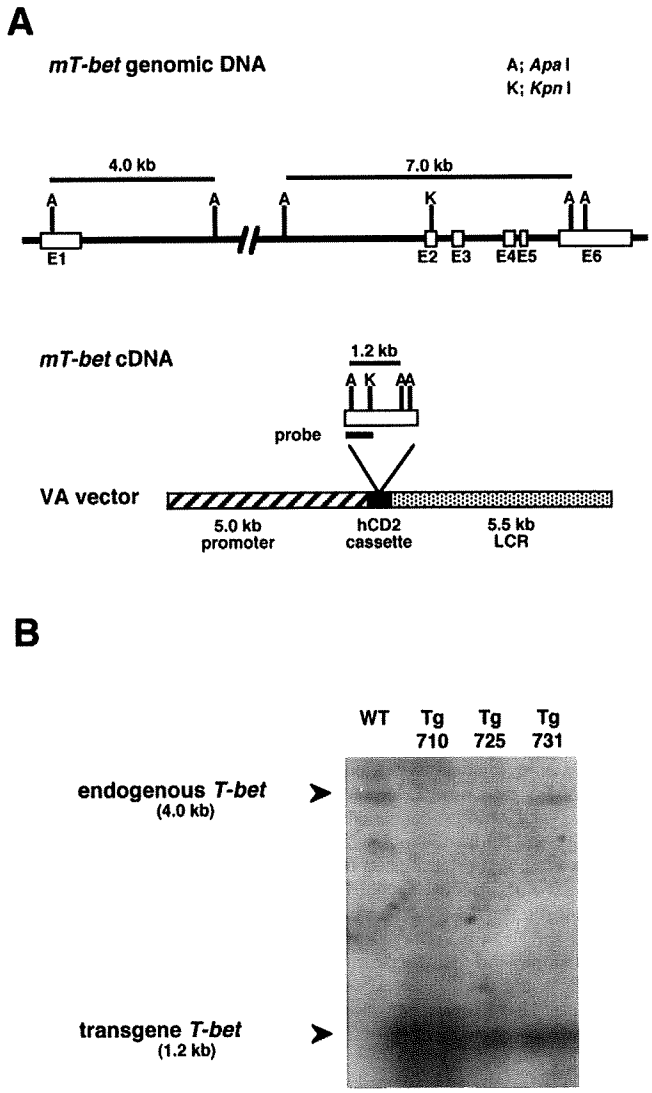

FIGURE 1. Generation of T-bet-overexpressing mice. *A*, Diagram showing the structures of the mouse *T-bet* (*mT-bet*) gene locus and the Tg construct. *T-bet* cDNA was inserted into a vector (VA vector) containing a human CD2 transgene cassette. The Southern blotting probe site, the restriction sites, and the predicted sizes of the endogenous gene and the transgene (with *Apa*I restriction sites) are indicated. E, Exon; LCR, locus control region. *B*, Southern blot analysis of the endogenous and Tg *T-bet* genes in Tg mice. The fragment with *Apa*I and *Kpn*I restriction sites in *mT-bet* cDNA in panel (*A*) was used as the probe. The 4.0-kb endogenous and 1.2-kb Tg genes are shown for Tg line 710 (Tg 710), Tg 725 (Tg 725), and Tg 731 (Tg 731) mice. The transgene copy numbers for Tg lines 710, 725, and 731 were over 12, 12, and 8 copies, respectively.

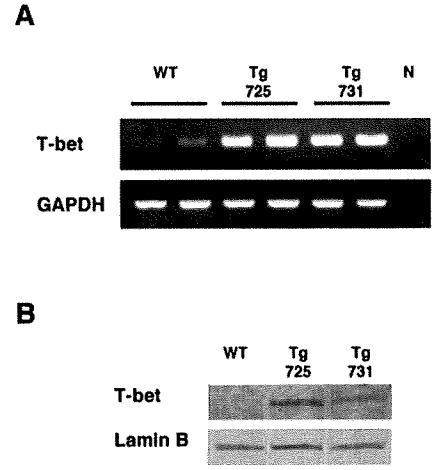

FIGURE 2. T-bet expression analysis by RT-PCR and Western blot in the thymus. *A*, RT-PCR analysis. In Tg line 725 (Tg 725) and Tg line 731 (Tg 731), T-bet gene expression was higher than that of WT. N, PCR without template as negative control. Two individual mice were used in each genotype. *B*, T-bet protein in nuclear extracts from thymocytes. T-bet protein was clearly identified by Western blotting in the extracts from Tg lines 725 and 731. In this analysis, however, the normal level of T-bet from WT thymocytes could not be detected.

Overexpression of T-bet in Tg mice

To confirm expression of the transgene, RT-PCR and immunoblot analyses were performed to monitor T-bet mRNA and protein levels in thymocytes from the two Tg lines (Fig. 2, *A* and *B*). Overexpression of T-bet mRNA and protein was detected in all Tg mice tested. The amount of T-bet protein in Tg line 725 cells was slightly higher than the amount in Tg line 731, indicating that the expression level of the protein was copy number dependent. The T-bet protein was not detected in WT mice in this analysis.

Higher ratio between IgG2a and IgG1 in T-bet Tg mice

To determine cytokine levels we first analyzed serum by the ELISA method, but all samples were below the level of detection (data not shown). Because Th1/Th2 cytokines contribute to control of Ig subtype production, we next analyzed serum IgG1 and IgG2a. Th1 cells support macrophage activation, delayed-typed hypersensitivity responses, and Ig isotype switching to IgG2a. In contrast, Th2 cells provide efficient help for B cell activation and class switching to IgG1 (22, 23). To confirm the Th1-dominant response in T-bet Tg mice, serum IgG levels were measured by ELISA (Table I). Tg line 731 mice had serum total IgG levels similar to those of WT mice (Tg line 731, 394.0 ± 37.6 mg/dl; WT, 340.3 ± 18.7 mg/dl), but Tg line 725 levels were significantly higher than those of WT mice (547.4 ± 108.9 mg/dl). Serum IgG1 levels of Tg mice (Tg line 731, 79.4 ± 7.4 mg/dl) tended to be lower than those of WT mice (174.0 ± 33.7 mg/dl) but, in contrast, IgG2a levels were higher (Tg line 725, 253.1 ± 77.9 mg/dl; WT, 90.7 ± 12.6 mg/dl). To confirm the promotion of the IgG2a class switch and repression of IgG1 Tg mice, IgG2a/IgG1 ratios were calculated. These were found to be significantly higher in Tg mice (Tg line 725, 2.93 ± 0.95; Tg line 731, 1.22 ± 0.13) than in WT mice (0.62 ± 0.08) ($p < 0.01$).

Increased synthesis of IFN-γ in T-bet Tg mice

From the above data, Tg line 725 mice had greater overexpression of T-bet than Tg line 731 mice and were therefore used in the following studies. To confirm the observed differences in

Table I. *Serum immunoglobulins for 30-week-old mice*[a]

Measurement	WT (n = 12)	Tg Line 725 (n = 8)	Tg Line 731 (n = 10)
IgG (mg/dl)	340.3 ± 18.7	547.4 ± 108.9[b]	394.0 ± 37.6
IgG1 (mg/dl)	174.0 ± 33.7	174.9 ± 89.0	79.4 ± 7.4[b]
IgG2a (mg/dl)	90.7 ± 12.6	253.1 ± 77.9[b]	97.1 ± 13.4
IgG2a/IgG1 ratio	0.62 ± 0.08	2.93 ± 0.95[c]	1.22 ± 0.13[c]

[a] Data are expressed as mean ± SEM.
[b] $p < 0.05$ versus WT.
[c] $p < 0.01$ versus WT.

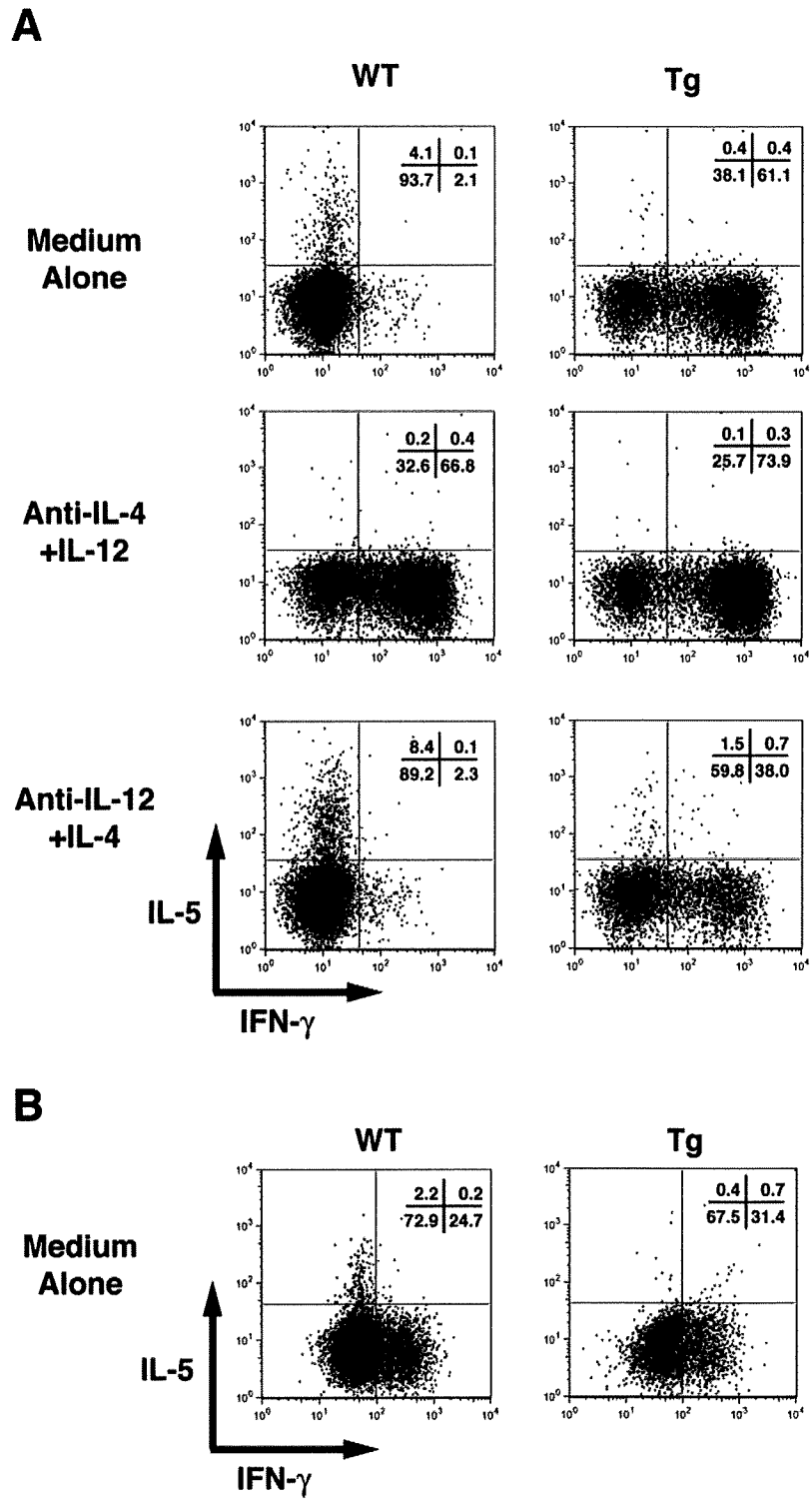

FIGURE 3. Intracellular cytokine analysis of $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells from each group. *A*, $CD4^+$ T cells from WT and Tg line 725 mice were cultured in the presence of medium alone, anti-IL-4 plus IL-12 (Th1 differentiation conditions), or anti-IL-12 plus IL-4 (Th2 differentiation conditions) and analyzed by flow cytometry for intracellular synthesis of IFN-γ and IL-5. The frequencies of IFN-γ-producing cells are shown on the *x*-axis and those of IL-5-producing cells on the *y*-axis. Intracellular synthesis of IFN-γ in Tg line 725 mice was increased under all conditions. *B*, $CD8^+$ T cells from WT and Tg line 725 mice were cultured in the presence of medium alone and analyzed by flow cytometry for intracellular synthesis of IFN-γ and IL-5. Results are representative of three independent experiments.

cytokine production at the single-cell level, we studied their intracellular synthesis by flow cytometry. $CD4^+$ T cells from Tg mice had higher levels of IFN-γ than WT mice either in medium alone or under conditions favoring Th1 differentiation (presence of anti-IL-4 Ab and IL-12) or Th2 differentiation (presence of anti-IL-12 Ab and IL-4) (Fig. 3*A*). Especially in the Th2 condition 38.0% of $CD4^+$ T cells from Tg mice produced IFN-γ, but only 2.3% from WT did so. In contrast to IFN-γ, the production of IL-5 in cells from Tg mice showed lower levels in medium and the Th2 condition. IL-4 production

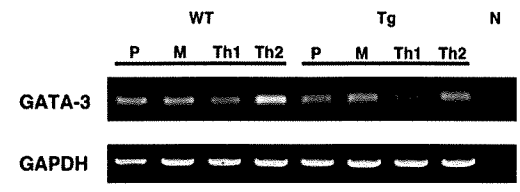

FIGURE 4. GATA-3 mRNA expression analysis by RT-PCR in intracellular cytokine production experiment. P, Prestimulation; M, medium-alone condition; Th1, Th1 differentiation conditions; Th2, Th2 differentiation conditions; N, PCR without template as negative control.

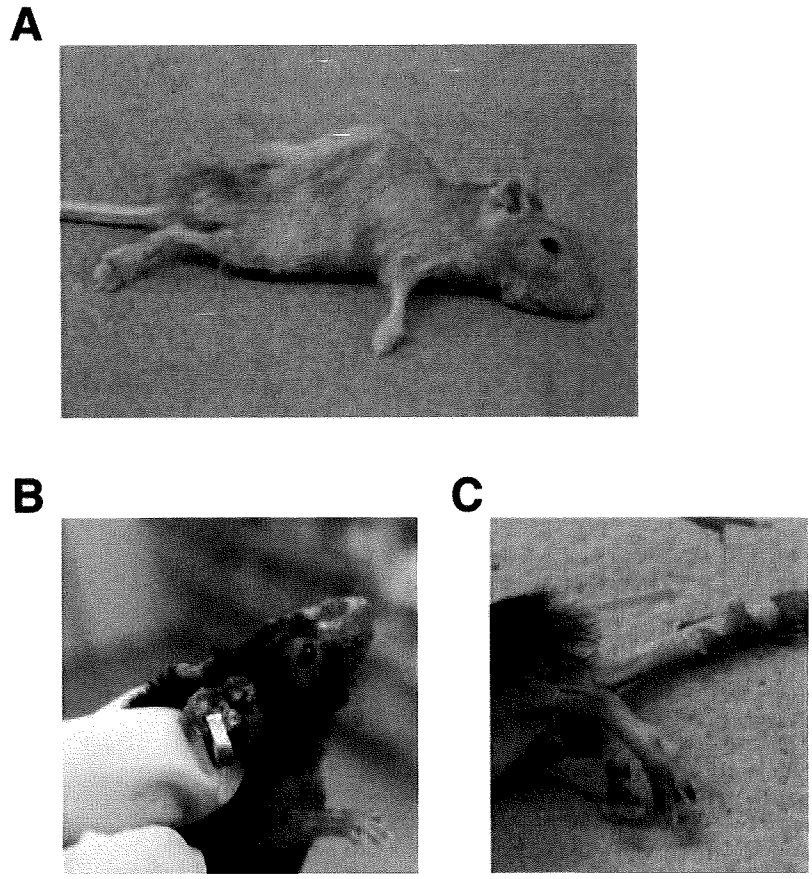

FIGURE 5. Tg mice develop dermatitis. In severe cases, individual Tg mice lost hair all over the body (*A*). In mild cases, the surface of the face, ear, foot, and tail showed redness and scaling (*B* and *C*).

analyses were also done. IL-4 production in cells from Tg and WT mice was similar and the percentage of positive cells was very low under all conditions (data not shown). These results demonstrate that T cells from T-bet Tg mice have a dominant Th1 differentiation pattern and suggest that overexpression of T-bet prevents Th2 differentiation. $CD8^+$ T cells from Tg mice also had higher levels of IFN-γ than WT mice in neutral condition, but not so markedly as that of $CD4^+$ T cells from Tg mice (Fig. 3*B*). We also determined the GATA-3 mRNA expression in the intracellular cytokine production analyses (Fig. 4). GATA-3 mRNA expression in Tg mice showed lower levels than those of wild mice, especially in a Th1 condition.

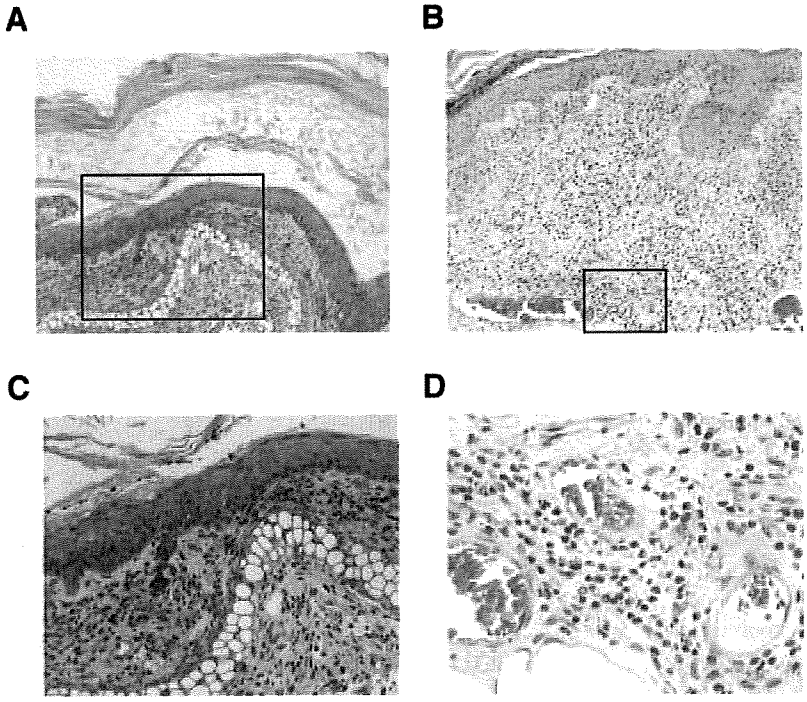

FIGURE 6. Histological appearance of the ear skin. *A* and *B*, In the histological analysis of ear skin, hyperkeratosis, acanthosis, broadening of the papillae, and infiltration of neutrophils lymphocytes, and melanophages are seen. *C and D*, At higher magnification of the squares from *A* and *B*, infiltration of mononuclear cells and neutrophils is observed. (H&E staining; magnification: ×100 (*A*), ×100 (*B*), ×200 (*C*), and ×400 (*D*).

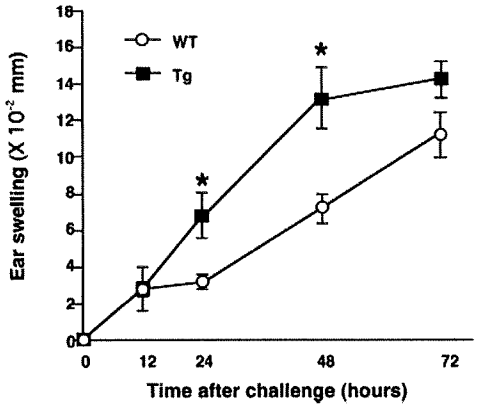

FIGURE 7. CHS reaction to DNFB in Tg and WT mice. The CHS response was determined by ear swelling at various times after hapten challenge. Time after challenge was 24 and 48 h; the ear swelling values in Tg mice (■, $n = 5$) were significantly higher than those in WT mice (○, $n = 5$). *, $p < 0.05$.

Development of contact dermatitis in T-bet Tg mice

During the initial analysis of the Tg cohorts we found that they developed dermatitis. At ~10 wk of age, ~28% of Tg line 725 (12 of 43) and 5% of Tg line 731 (3 of 61) mice spontaneously developed dermatitis characterized by swollen, flaky, and scaly skin in regions lacking body hair (e.g., tail or ears), which in some individuals progressed all over the body, together with alopecia (Fig. 5). Histological examination of the affected skin showed epidermal hyperkeratosis and neutrophil and lymphocyte infiltration similar to what is seen in contact dermatitis in humans (Fig. 6). To prove that the skin lesion was contact dermatitis although the Ag was not determined, we performed cell transfer experiment. We transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within one month. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone.

Augmentation of CHS reactions in Tg mice

To determine the role of T-bet overexpression in T cell subpopulations in CHS responses, 6-wk-old WT and Tg (Tg line 725) mice, which did not develop dermatitis, were sensitized with DNFB as described in *Materials and Methods*. Ear swelling responses to DNFB were significantly increased in Tg mice compared with WT mice (Fig. 7). The CHS response was significantly higher at 24 and 48 h after challenge. Histological analysis of hapten-treated WT and Tg ears showed characteristic features of CHS including dermal edema, mononuclear cell infiltration, and vascular enlargement (Fig. 8*B*). These histological changes were dramatically enhanced in hapten-treated Tg mouse ears (Fig. 8*B*).

Both $CD4^+$ and $CD8^+$ T cell lymph node cells (LNCs) produce significant amounts of IFN-γ in T-bet Tg mice

To determine IFN-γ production in skin-draining lymph nodes of Tg and WT mice, DNFB-primed LNCs were cultured under condition with medium alone. A significant increase of IFN-γ-producing cells was found among both $CD4^+$ and $CD8^+$ LNCs from DNFB-stimulated Tg mice (Fig. 9). In Tg mice, IFN-γ-producing cells were increased in $CD4^+$ LNCs even without DNFB stimulation. After stimulation, IFN-γ-producing cells increased further (Fig. 9*A*). Regarding $CD8^+$ LNCs, WT and Tg mice had the similar levels of IFN-γ-producing cells, but after DNFB stimulation the increase of IFN-γ-producing cells was greater in Tg mice (Fig. 9*B*). IL-10 production was also analyzed as a marker cytokine of

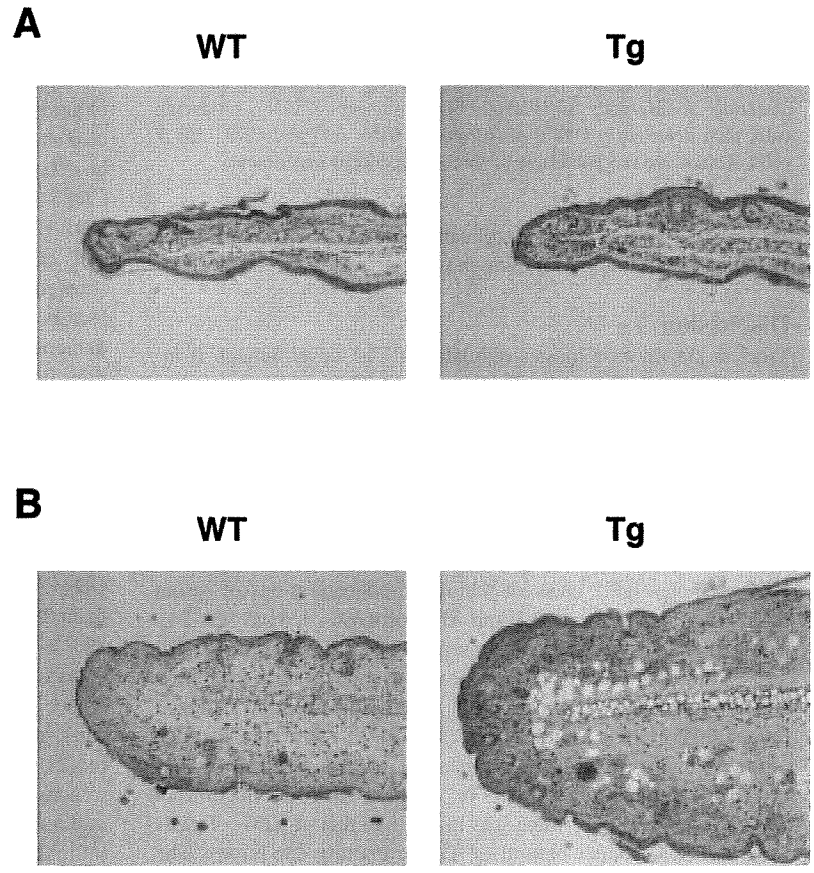

FIGURE 8. Representative results from histological analysis of CHS reactions. At 72 h after challenge, skin sections are shown from vehicle-treated mice (*A*) and hapten-treated mice (*B*) (H&E staining; magnification: ×100). *A*, Sections from vehicle-treated Tg mice exhibited normal histological features similar to those of vehicle-treated WT mice. *B*, Sections from hapten-treated WT and Tg mice displayed characteristic features of the CHS reaction including dermal edema, mononuclear cell infiltration, and vascular enlargement (*B*). The severity of the CHS reaction was greater in Tg mice.

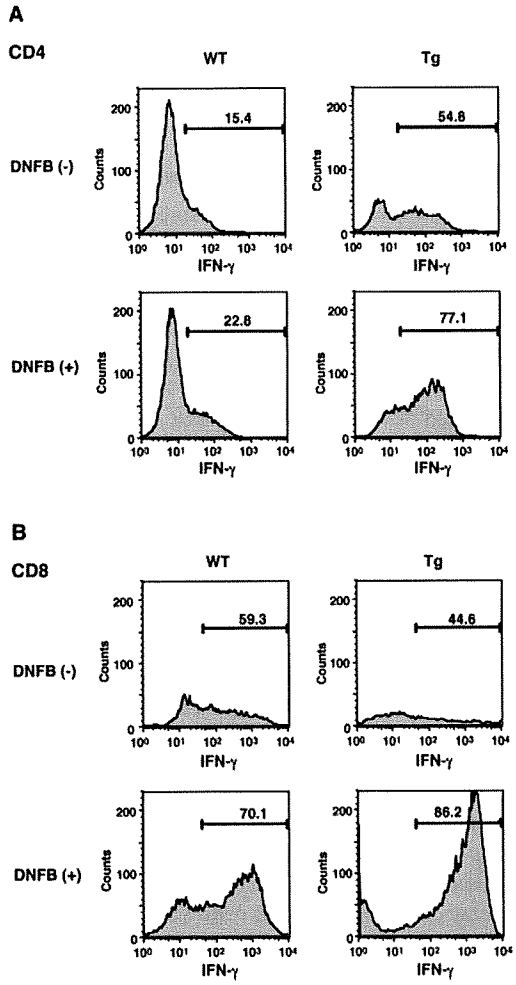

FIGURE 9. The number of IFN-γ-producing cells among $CD4^+$ and $CD8^+$ T cells was increased in CHS-sensitized Tg LNCs. LNCs from 72 h DNFB-sensitized or non-sensitized WT and Tg mice were cultured in medium alone and subjected to intracellular cytokine staining as described in *Materials and Methods*. Significantly higher numbers of IFN-γ-producing cells were detected in DNFB-sensitized Tg $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells. Results are representative of three independent experiments.

Th2 or Tc2. However, no significant differences between Tg and WT mice were found (data not shown).

Discussion

T-bet is known as a master regulator of Th1 development. It induces IFN-γ production and repression of Th2 cytokines in vitro (7). In this study, we generated T-bet-overexpressing mice characterized by Th1-dominant responses in vivo and in vitro. Thus, T-bet Tg mice showed Th1-dominant Ig production in vivo and Th1-dominant intracellular cytokine production in vitro. Because serum cytokines were below the level of detection, we analyzed serum Ig isotypes. IFN-γ promotes IgG2a class switching while IL-4 promotes IgG1 class switching, so concentrations of serum IgG2a and IgG1 reflect Th1 and Th2 responses in vivo (22–24). The higher IgG2a/IgG1 ratio present in the sera of Tg mice suggests that T-bet overexpression promotes IgG2a class switching and represses IgG1 class switching. At the same time, we analyzed intracellular cytokines and found that $CD4^+$ T cells from Tg mice produced higher levels of IFN-γ than WT mice under neutral, Th1- or Th2-promoting conditions. In contrast, IL-5 production in $CD4^+$ T cells from Tg mice was lower than that in $CD4^+$ T cells from WT mice under neutral or Th2-promoting conditions. The serological Ig findings are therefore consistent with data from $CD4^+$ T cell intracellular cytokine assays, emphasizing that these do indeed reflect the Th1/Th2 balance in vivo. T-bet has also been shown to regulate cytolytic effector mechanisms of $CD8^+$ T cells (8). Its expression is rapidly induced in $CD8^+$ T cells by signaling through the TCR and the IFN-γR, and it functions downstream of STAT1 (6, 9, 10). In the context of Ag-specific activation, T-bet is required for the differentiation of naive $CD8^+$ T cells into effector CTLs. In the present study, Tg mice also had a higher fraction of IFN-γ-producing $CD8^+$ T cells according to intracellular cytokine assays, but not as markedly as $CD4^+$ T cells. However, Tg mice showed higher response for IFN-γ-producing $CD8^+$ T cells in the CHS response. From the above results, we speculated that Tg mice not only had a Th1-dominant background but also a potential Tc1-dominant background.

In this study, spontaneous skin inflammation was observed in Tg mice, first occurring in regions lacking body hair such as the tail or ears, where the skin is easily in contact with external agents. Histological examination of affected skin showed epidermal hyperkeratosis, spongiosis, and neutrophil and lymphocyte infiltration. This is typical for contact dermatitis, a delayed-type hypersensitivity reaction. It is known that Th1 activity greatly contributes to the development of dermatitis (25, 26). It is shown here that a Th1-dominant response occurs in T-bet Tg mice. Tg mice expressing IFN-γ in the epidermis have also been investigated previously and found to have reddened skin, growth retardation, hair loss, and flaky skin lesions (25). Keratinocyte proliferation was increased and there was epidermal thickening with spongiosis and parakeratosis. The possible importance of several cytokines such as IL-2 (27), IL-6 (28), and IL-7 (29) in the development of contact dermatitis has also been investigated using Tg mouse models (30). In this study we used Tg mice overexpressing the T cell differentiation transcription factor T-bet, but not cytokine transgenes, to address contact dermatitis for the first time. The results from this study suggested that transcriptional regulation of T-bet might play an important role in contact dermatitis.

Contact allergens such as DNFB, oxazolone, and 2,4-dinitrochlorobenzene are used to induce CHS. In the present study we also showed that Tg mice responded significantly to DNFB. There have been major controversies on the respective roles of $CD4^+$ and $CD8^+$ T cells in the development of CHS inflammatory reactions (12). However, it has become clear that both $CD4^+$ and $CD8^+$ T cells can act as effector cells in this context (17). Wang et al. (17) showed that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells are crucial effectors in the CHS response to DNFB. Previous studies have also demonstrated that cytokines play important effector and regulatory roles in CHS responses. The type 1 cytokine IFN-γ promotes CHS, whereas type 2 cytokines down-regulate CHS responses (31–33). It has been demonstrated that type 1 cytokines can be produced by $CD8^+$ Tc1 cells as well as by $CD4^+$ Th1 cells in the same way that type 2 cytokines can be derived from Th2 and Tc2 cells (5, 34). Intracellular cytokine analyses showed that T-bet-overexpressing Tg mice had a large fraction of IFN-γ-positive cells within both $CD4^+$ and $CD8^+$ T cells. We also transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within 1 mo. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone. These results suggest that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells may be active as effector cells in CHS responses in this model. Furthermore, it is known that activated cells produce IFN-γ to activate skin resident cells, which produce cytokines and chemokines allowing the recruitment of the polymorphonuclear cell infiltrates characteristic of CHS. This effector phase of CHS takes

24 to 48 h in the mouse (12), coinciding closely with our results on 24- and 48-h DNFB hyperresponsiveness.

In contrast to T-bet, GATA-3 is known as a Th2 lineage commitment transcription factor (35, 36). We have previously shown that GATA-3 overexpression in Th1-dominant autoimmune disease can diminish autoimmune nephritis (20, 37) and, therefore, that therapy to regulate expression levels of transcriptional factors may be useful to control unbalanced Th1/Th2 activity in many diseases. The results of the present study also suggest that to control Th1 and Tc1 reactions via down-regulation of T-bet expression might be useful for alleviating contact dermatitis.

In conclusion, we have generated Th1- and Tc1-dominant mice that developed spontaneous skin inflammation very similar to contact dermatitis. These mice should be useful for revealing a link between some immune diseases and Th1/Th2 and Tc1/Tc2 dysbalance and may offer a valuable murine model of contact dermatitis.

Acknowledgment

We express our sincere thanks to Laurie H. Glimcher for providing us with mouse T-bet cDNA.

Disclosures

The authors have no financial conflict of interest.

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Exp. Anim. 58(2), 159–168, 2009

—Original—

T Cell Receptor Repertoire in BALB/c Mice Varies According to Tissue Type, Sex, Age, and Hydrocortisone Treatment

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Abstract: Diversity in T cell recognition of antigens is determined by diverse usage of T cell receptor (TCR) repertoire. TCR repertoire analysis provides fundamental information for understanding T cell immune responses in the pathogenesis of various diseases. In the present study, we examined the TCR repertoire in various tissues in normal BALB/c mice. The TCR α chain variable region repertoires were consistent among the spleen, lymph nodes, and the thymus. The TCR β chain variable region (TCRBV) repertoires were consistent between the spleen and lymph nodes, but different in the thymus. The TCR repertoires also differed in the lungs and the intestinal tract. The TCR repertoires were consistent between male and female mice, except for TCRBV15-1. TCR repertoire was almost similar in 3- and 7-week-old mice, except for TCRBV1-1, 8-3, and 14-1. The present findings suggest that the TCR repertoire of mice varies according to tissue type, sex and age. Additional analysis of the TCR repertoire, i.e., the effect of hydrocortisone (HC), was carried out. After the HC treatment, although the thymic T cells decreased to one-tenth, only a small fraction of $CD4^+CD8^+$ T cells survived the treatment. Furthermore, the percentages of thymic T cells bearing TCRBV3-1, 5-1, 5-2, and 16-1 substantially decreased, but the percentage of cells bearing TCRBV12-1 did not decrease. The present findings suggest that the HC susceptibility of immature thymic T cells is different between TCR families.

Key words: FACS, hydrocortisone, real-time PCR, repertoire, T cell receptor

(Received 26 September 2008 / Accepted 25 December 2008)

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Introduction

Diversity in antigen recognition is established by TCR α and β gene rearrangements through several stages of differentiation and maturation in the thymus [7, 8, 12, 18]. In addition to rearrangements of the TCR α and β genes comprising variable (V), diversity (D), and joining (J) loci, random nucleotide insertions (N regions) occur in the D-J region, leading to the formation of enormously diverse TCR repertoire [4]. After completion of TCR gene rearrangement in the cortex of the thymus, T cells migrate to the medulla. Through positive and negative selection, only clones with moderate affinities for major histocompatibility complex (MHC) molecules are selected. The selected T cells then emigrate from the thymus to the peripheral lymphatic tissues. The peripheral T cells recognize peptide fragments presented by MHC molecules via their TCRs, subsequently becoming activated, and produce various cytokines and proliferate.

T cells play important roles in recovery from infectious diseases [6, 23] as well as in the pathogenesis of allergic and autoimmune diseases [1, 17, 22, 27]. Analysis of TCR repertoires provides important information for understanding immunopathological mechanisms. As a base for TCR repertoire analyses in diseases, it is necessary to determine the TCR repertoires in the tissues of healthy individuals.

The effects of HC administration on T cells in the thymus have been described. Although HC treatment causes massive deletion of immature thymocytes, the biological significance of the effect of HC on thymocyte differentiation still remains unsolved. The HC-resistant thymocyte population, considered to be equivalent to medullary thymocytes, has similar phenotypes and functions to those of mature T cells [3, 10, 11]. On the other hand, it is known that the thymic cortex $CD62^+$ T cell shows HC resistance [20]. Generally, the HC affects various responses *in vivo* such as blood pressure, glucose elevation, anti-inflammatory action, and immunosuppression. However, there is no detailed report of the TCR repertoire of the HC response in the thymus.

In the present study, we analyzed the relative expression levels of CD3, a T cell marker, and usage of TCR repertoires in various tissues in BALB/c mice. We also compared the TCR repertoires between male and female mice, and between 3-week-old (sexual immature group) and 7-week-old (sexual mature group) mice. Furthermore, we examined the TCR repertoire in BALB/c mice after HC administration, and compared the TCR repertoire of the thymus, with those of the peripheral lymphatic organ, the spleen. To analyze the TCR repertoires, we used adaptor-ligation-mediated PCR (AL-PCR) and microplate hybridization assay (MHA) methods [6, 13, 23, 28]. Generally, other previous methods of TCR analysis were using many TCRV specific probes in independent PCRs. However, precise quantification was difficult, because amplification efficiencies differ among individual primers. The AL-PCR ligates universal primer to the variable region 5' end of the TCR gene and is able to amplify all TCR genes in one PCR. Moreover, the MHA has V/C values which have coherent strict color development intensities for all TCR genes determined by the ratio of the hybridization intensity of each TCR variable region-specific probe to that of the corresponding TCR constant region-specific probe. The analysis of the relative abundances of the TCRs are related to the MHA, the mRNA of the TCR gene, and the FACS analysis using monoclonal antibodies for each TCR [28]. Therefore, TCR repertoire analysis using the AL-PCR and MHA methods enables us to amplify and measure all the variable regions of the rearranged TCR genes without any artificial skewing.

Materials and Methods

Animals

Specific pathogen free (SPF) BALB/cAnCrj ($H\text{-}2^d$) mice were purchased from Charles River Japan Inc. (Tokyo, Japan). The mice were housed under barrier conditions with a temperature of 23 ± 3°C, relative humidity of 55 ± 15%, and a 12-h light/dark cycle. Irradiated commercial laboratory mouse chow (CRF-1, ORIENTAL YEAST Co, Ltd., Japan) and autoclaved water were provided *ad libitum*. Animal experiments were performed according to the Guidelines of the Committee of Animal Experiments of Sagamihara National Hospital Clinical Research Center for Allergy and Rheumatology.