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T-Bet Plays a Key Role in NK-Mediated Control of Melanoma Metastatic Disease

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Antitumor responses depend on type 1 immunity, which is severely impaired in mice deficient for the T-box expressed in T cells (T-bet) transcription factor. Both T-bet-deficient (T-bet−/−) NK and CTL show defective function, which can be overcome by strong stimuli due to the expression of eomesodermin, another member of the T-box family. The effective response from T-bet−/− mice to viral infection and tumor initiation corroborates with these findings. However, T-bet−/− animals fail to control cancer metastasis and are, therefore, highly susceptible to tumor spread. The mechanism of T-bet-dependent resistance to metastatic disease is not known. In this study, we show that T-bet plays a role in inhibiting cancer metastasis by regulating the longevity and function of NK cells. Our data demonstrate that the absence of a proper innate immune response driven by NK cells in T-bet−/− mice precludes the initiation of a potent adaptive response to tumors. Adoptive transfer of wild-type activated NK cells protects T-bet−/− animals after melanoma challenge showing that reconstitution of the NK compartment in these mice is sufficient to mediate a significant reduction in tumor burden. Transfer of T-bet−/− A-NK cells fails to do so, due to their reduced in vivo survival, inefficient lysis of cancer cells, and poor IFN-γ production. Taken together, these results show for the first time an irreplaceable role for T-bet in the NK-mediated cross-talk between innate and adaptive immune responses to metastatic disease. The Journal of Immunology, 2008, 180: 8004–8010.

Although most tumors are weak stimulators of immunity, antitumor immune-responses are physiologically relevant and can retard tumor growth in vivo (1–6). Immunologic protection reflects the combined impact of cells and cytokines belonging to the innate and adaptive immune systems. This includes NK cells and CTLs, as well as effector cytokines characteristic of type 1 immunity (7–11). In the latter case, factors such as IFN-γ can directly inhibit the growth of tumor cells as well as promote the production of other type 1 cytokines and differentiation of cytotoxic NK and T cells (11–15). This cytokine milieu determines the differentiation fate of activated T lymphocytes and their development into Th/T cytotoxic (Tc)1 or 2 cells (14, 16). Hence, factors that regulate type 1 immunity are likely to alter the quality of antitumor responses.

The T-bet transcription factor is essential for the expression of genes that guide type 1 immune differentiation in naive T cells and other cells of the immune system. Furthermore, expression of T-bet redirects T cell differentiation as Th2 and Tc2 cells transduced with T-bet produce IFN-γ and repress IL-4 and IL-5 expression (17). T-bet plays a context-specific role in differentiation of CTL and activation of NK cells (18–20). Whereas in vitro-stimulated T-bet−/− CD8+ T cells produce normal levels of IFN-γ, in vivo killing by Ag-specific T-bet−/− CTL is severely impaired (19). NK cells that develop in the absence of T-bet have an immature phenotype and are reduced in numbers due to increased apoptosis (20). However, these cells show only a marginal reduction in IFN-γ production and cytotoxicity in response to in vitro cytokine stimulation, respond normally to poly(I:C) stimulation in vivo and promote control of murine CMV (MCMV) viral replication (20, 21). Thus, despite some developmental and differentiation defects, T-bet−/− NK and CTL can respond in vivo, possibly due to expression of other factors, such as the T-box transcription factor eomesodermin (Eomes) that counterbalances T-bet deficiency. Moreover, T-bet−/− mice in the transgenic adenocarcinoma mouse prostate background develop tumors with the same rate and kinetics as control animals (22). Nonetheless, the incidence of metastatic disease and the size of secondary nodules are increased in the absence of T-bet (22). Consequently, the immune response to cancer may reflect T-bet-independent control of tumor initiation and T-bet-dependent response to tumor spread. The mechanism underlying the susceptibility of T-bet-deficient mice to tumor metastasis is not understood.
To investigate the mechanism through which T-bet expression modulates later steps in cancer establishment and growth, we used the B16F10 melanoma model (23, 24). Both innate and adaptive immune cells contribute to the control of B16F10 metastasis, allowing analysis of both arms of the immune system (25–28). In this study, we show that the in vivo NK response to tumor metastasis is highly dependent upon T-bet expression due to reduced cell survival and effector function. Although T-bet deficiency does not alter the protective effect of tumor vaccination, which depends on an adaptive immune response, T-bet-deficient nonimmune mice are extremely susceptible to metastatic spreading of B16F10 cells. We conclude that T-bet expression by NK cells is essential to protect against tumor metastasis and important for NK-mediated cross-talk between innate and adaptive immune systems in response to metastatic disease.

Materials and Methods

Mice

T-bet−/− mice generated in our laboratory were backcrossed 13 generations to the C57BL/6 background. C57BL/6 mice were purchased from The Jackson Laboratory and Rag2−/− mice from Taconic Farms. T-bet−/− Rag2−/− and Rag2−/− double knockout (DKO) animals were generated by intercrossing T-bet−/− and Rag2−/− mice. hCD2-reverse tetracycline (Tet)-controlled transcriptional activator (rtTA) T-bet−/− and Tet operon-responsive element (TRE)-T-bet−/− transgenic mice were generated by integrating the Tet-inducible expression system (Invitrogen) in T-bet−/− mice as described in Ref. 29. In brief, the rtTA linked to the hCD2 promoter (CD2TA construct) was injected into T-bet−/− fertilized eggs to generate CD2-rtTA T-bet−/− mice. TRE placed upstream of the mouse T-bet cDNA construct was injected likewise to generate TRE-T-bet T-bet−/− mice. CD2-rtTA T-bet−/− and TRE-T-bet T-bet−/− transgenic mice were intercrossed to generate CD2-rtTA TRE-T-bet T-bet−/− (T-bet−/− CD2-Cond) animals. T-bet expression was induced in vivo by feeding T-bet−/− CD2-Cond mice a short incubation in 0.02% EDTA in PBS. T cells were depleted by positive selection with anti-CD4 (L3T4) and anti-CD8 mAb (BioLegend). Single-cell suspensions stained with anti-NK1.1 mAb (BD Pharmingen) and cells retrieved divided by the ratio between the number of NK1.1+ CD5+ CD3− NK cells as analyzed by surface staining and FACS.

Cells

B16F10 and B16-GM-CSF cells were a gift from G. Dranoff (Harvard Medical School, Boston, MA). YAC-1 cells were purchased from American Type Culture Collection.

Tumor vaccination

Perform as described in Ref. 26. Briefly, B16-GM-CSF cells were collected and irradiated at 35 Gy, washed, and resuspended in PBS for s.c. administration of 0.5 × 106 per mouse, in 100 μl.

Generation of activated NK cells

A-LAK cells were generated as described in Ref. 30. In brief, splenocytes were harvested from age-matched wild-type (WT) and T-bet−/− mice. Cells were cultured in complete RPMI 10 supplemented with 10% FBS (Sigma-Aldrich) and 1000 U/ml of human rIL-2 (hr-IL-2; kindly provided by Chiron Corporation, Emeryville, CA). After 3 days in culture, floating cells were carefully removed. Fresh RPMI 10 media supplemented with 1000 U/ml hr-IL-2 was added and cells were cultured for an additional 4–5 days, when floating cells were removed and adherent cells harvested with a short incubation in 0.02% EDTA in PBS. T cells were depleted by positive selection with anti-CD4 (L3T4) and anti-CD8 (Ly-2) microbeads (Miltenyi Biotec). Remaining cells were 85–90% pure NK cells by flow cytometry.

Competitive in vivo A-NK cell homing assay

Homing assays were performed as described (31). Briefly, WT and T-bet−/− A-NK cells generated as described above were labeled with 5(−and -6)−[(4-chloromethyl)benzoyl]aminato]tetramethylrhodamine (CMTMR) or CFSE (Invitrogen) and mixed at a 1:1 ratio. Ten to 15 × 106 mixed cells were injected i.v. into WT or T-bet−/− mice that had received 60,000 B16F10 melanoma cells i.v. 3 days prior. A total of 2 or 24 h after adoptive transfer of A-NK cells, 1 ml of blood, spleen, peripheral lymph nodes, mesenteric lymph nodes, liver, and lungs were harvested. Liver and lungs were treated for 30 min at 37°C with 0.5% collagenase type 2 (Worthington Biochemical). Single-cell suspensions stained with anti-NK1.1 mAb (BD Pharmingen) were analyzed by FACS. The homing index was calculated as the ratio between the number of NK1.1+CMTMR+ and NK1.1+CFSE− cells retrieved divided by the ratio of NK1.1+CMTMR+ and NK1.1+CFSE− cells in the input population.

In vitro cytotoxicity assay

Effector. A-NK cells were generated as described above and used as effector cells in a standard 4-h 51Cr release assay. Targets: 1 × 106 B16F10 cells were labeled with 200 μCi of 51Cr (PerkinElmer) for 2 h at 37°C, extensively washed and plated at 10,000 cells per well. Effector cells were plated at the E:T ratio described in the figure. Spontaneous release was <20% of maximum release. Percent specific lysis was calculated as 100 × (cpm test sample – cpm spontaneous release)/(cpm total release – cpm spontaneous release).

Flow cytometry

Annexin V, 7-AAD, fluorochrome-conjugated mAb against NK1.1 (PK136), IFN-γ, and others were purchased from BD Pharmingen and cells were stained as described (20). Intracellular staining for IFN-γ was performed using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s protocol. A-NK cells with 10 ng/ml IL-12 and 10 ng/ml IL-18 (R&D Systems). After staining, data were collected on a FACScalibur (BD Biosciences) and analyzed with CellQuest software (Tree Star).

Statistics

Data was analyzed using Microsoft Excel software and the two-tailed Student’s t test. The null hypothesis was rejected and differences were assumed to be significant at a value of p < 0.05.

Results

T-bet deficiency increases susceptibility to B16F10 melanoma

T-bet−/− animals can control the development of primary tumors in a murine prostate cancer model as well as WT littermates. However, T-bet deficiency is associated with increased metastatic disease in the same model (22). The selective susceptibility to metastasis but nonprimary tumor formation suggested that specific cell types may be responsible for this split phenotype. We chose the widely used B16F10 melanoma model of tumor metastasis to study homing and establishment of secondary tumor nodules, the later steps in the process of cancer spread. Both innate and adaptive immune cells constitute the anti-B16F10 response, and susceptibility of the host animals to melanoma is quantified by counting the number of pulmonary tumor nodules following i.v. injection of B16F10 cells. We first compared susceptibility of T-bet−/− and T-bet WT animals to B16F10 growth after i.v. injection at different days after inoculation. We observed a marked increase in the number of melanoma nodules in the lungs of T-bet−/− mice compared with WT littermate controls (Fig. 1, A and B). As early as 7 days after inoculation, over 1000 nodules could be seen on the surface of T-bet−/− lungs. Titration of the number of tumor cells transferred showed that 60,000 B16F10 cells injected i.v. were sufficient to generate over 160 nodules in the lungs of T-bet−/− mice 14 days after inoculation, at which point only ~2 nodules were detectable in WT counterparts (Fig. 1C). Histopathological analysis of the lungs of tumor-bearing WT and T-bet−/− animals confirmed the increase in frequency and also showed an increase in the size of melanoma nodules in this organ (data not shown). We conclude that T-bet−/− mice are highly susceptible to B16F10 melanoma, corroborating previous observations in the transgenic adenocarcinoma mouse prostate model of prostate cancer (22).
T-bet deficiency in the innate immune system severely compromises antitumor response

The robust tumor growth observed as early as 7 days after melanoma inoculation into T-bet−/− mice suggested that the innate response to tumors might be compromised. FACS analysis of single-cell suspension of lungs 7 days after tumor injection showed no difference in the relative numbers of dendritic cells, macrophages, granulocytes, CD4+ cells, or CD8+ T cells between T-bet−/− and WT mice (data not shown). However, there was a 20-fold decrease in the proportions of NK cells and an absence of NKT cells in the lungs of T-bet−/− animals, a difference observed to a smaller extent in naïve T-bet−/− animals (5-fold fewer NK cells in T-bet−/−) but which was accentuated by melanoma growth (Fig. 2A and data not shown). Both NK and NKT cells contribute to protective antitumor responses. NK cells display potent ex vivo cytolytic activity against tumor cells while NKT cells produce large amounts of IFN-γ upon activation and show in vivo antitumor activity after stimulation with α-galactosyl ceramide (28, 32).

We crossed T-bet−/− animals to mice that lack Rag2 expression to define the role of the NK response to B16F10 in the absence of T cells and NKT cells, which are virtually absent in Rag2−/− mice. Rag2−/− animals rejected B16F10 melanoma cells more efficiently than WT animals, possibly due to their increased NK numbers (data not shown) and lack of T regulatory cell-mediated suppression of NK cells (33). T-bet−/− and Rag2−/− T-bet−/− DKO mice displayed the same enhanced numbers of tumor nodules in the lungs, demonstrating that the lack of T and NKT cells does not alter the defective T-bet−/− phenotype (Fig. 2B).

These results may reflect either the absence of a T cell contribution to tumor protection or failure to prime T cells in T-bet−/− mice. To address this question, we selectively expressed T-bet in T cells of T-bet−/− animals and assessed susceptibility to B16F10 challenge. To this end, we generated T-bet−/− Tet on transgenic mice where rTFA is constitutively expressed under the hCD2 promoter (T-bet−/− CD2-Cond). Upon Dox administration, rTFA binds to TRE and drives expression of Flag-tagged T-bet. In vivo treatment with Dox led to physiological levels of T-bet expression by T-bet−/− CD2-Cond T cells and restored the Th1 phenotype of T-bet−/− CD4+ T cells, which otherwise display a tendency toward Th2 differentiation upon stimulation (21). Activation of T-bet−/− CD2-Cond CD4+ T cells leads to expression of IL-2 and lack of IFN-γ. In the presence of Dox, expression of IL-2 is unimpaired while IFN-γ is up-regulated (data not shown). Expression of T-bet by NK cells in this system is small, reaching <40% of WT levels (data not shown). We then tested the susceptibility of T-bet−/− CD2-Cond mice to B16F10 tumor growth. WT, T-bet−/−, and T-bet−/− CD2-Cond mice received B16F10 cells 3–5 days after starting in vivo Dox treatment in food and water, and Dox administration was maintained throughout the experiment. Despite robust induction of T-bet expression (data not shown), the growth of pulmonary B16F10 nodules was not altered by Dox administration to T-bet−/− CD2-Cond animals compared with T-bet−/− mice that underwent the same regimen; both groups developed ~350 nodules 21 days after tumor challenge, compared...
with ~40 of WT controls (Fig. 2C). Therefore, expression of T-bet in the T cell compartment does not protect T-bet−/− animals from B16F10 melanoma growth.

The induction of T cell responses is a complex process that requires immune recognition and Ag presentation by cells of the innate immune system. Although the absence of T cells does not increase susceptibility to tumor growth and expression of T-bet in T cells fails to protect T-bet−/− mice, it is possible that T lymphocytes in T-bet−/− hosts are not properly activated rather than intrinsically defective. We used the B16-GM-CSF vaccination protocol, which stimulates a potent CTL response against subsequent B16F10 challenge in a dendritic cell (DC) and CD4+ T cell-dependent manner (26), to determine whether the lack of T cell response in tumor-bearing T-bet−/− animals was due to inefficient stimulation of T cells by members of the innate immune system. In agreement with previous reports, vaccination of WT mice with irradiated B16-GM-CSF cells before challenge with live B16F10 cells protected the animals to the extent that no tumor nodules were detected in the lungs of vaccinated mice. T-bet−/− mice were highly protected from B16F10 challenge upon vaccination, displaying a 10-fold reduction in tumor nodules when compared with mock-vaccinated animals (Fig. 2D). This result demonstrates that T-bet−/− lymphocytes are able to mount an effective anti-melanoma response if properly stimulated, and suggest that GM-CSF-induced DC activation overcomes T-bet deficiency.

**Transfer of WT A-NK cells restores tumor immunity to T-bet−/− animals**

We tested the capacity of NK cells to protect T-bet−/− animals by transferring WT NK cells to these mice after tumor challenge and assessing tumor growth in the lungs. We generated activated NK cells in vitro by collecting the adherent portion of lymphokine-activated killer (A-LAK) cell cultures. A-LAK cells transferred into tumor-bearing hosts infiltrate pulmonary melanoma nodules and promote tumor immunity both in mice and humans (34–36). A-LAK cells from WT and T-bet−/− splenocytes were enriched for NK cells (A-NK) and adoptively transferred into T-bet−/− animals shortly after tumor challenge. Similar numbers of A-NK cells were recovered from both T-bet−/− and WT cultures, as expected from previous studies of T-bet−/− NK cells (data not shown and Ref. 20). Transfer of WT A-NK cells early after melanoma challenge protected T-bet−/− mice, as judged by a significant delay in pulmonary tumor growth and reduction in size and numbers of melanoma nodules compared with T-bet−/− mice that received PBS only (Fig. 3). Moreover, transfer of T-bet−/− A-NK cells did not protect T-bet−/− recipients (Fig. 3). These observations indicate that reconstitution of the NK compartment of T-bet−/− mice with WT NK cells delays B16F10 tumor growth, suggesting that T-bet−/− NK cells have intrinsic defects that render them unable to control pulmonary tumor metastasis.

**T-bet deficiency does not impair A-NK cell recruitment to the lungs but reduces A-NK in vivo survival**

The failure of T-bet−/− A-NK cells to protect mice after B16F10 challenge may be due to the inability of these cells to migrate into relevant organs or, following successful organ infiltration, to impaired effector function. Because T-bet−/− animals can develop chronic lung inflammation (37), we asked whether migration of A-NK cells was altered in T-bet−/− hosts compared with their WT counterparts. After labeling WT and T-bet−/− A-NK cells with different fluorescent dyes, a 1:1 mixture of these cells was adoptively transferred into WT or T-bet−/− mice and FACS analysis was performed. No difference in the recovery ratio of WT and T-bet−/− A-NK cells was observed 2 h after injection, demonstrating that 1) homing of T-bet−/− and WT A-NK cells is not distinguishable; and 2) WT and T-bet−/− hosts support A-NK cell homing equally well (Fig. 4A). Analyses by confocal microscopy of WT tumor-bearing lungs 2 h after A-NK cell transfer were consistent with these conclusions (data not shown), and similar number/distribution of transferred NK cells were noted independently of the reagent used to label WT and T-bet−/− A-NK cells (data not shown).

These observations suggest that T-bet−/− A-NK cells have normal migration patterns and that tumor-bearing T-bet−/− hosts are as efficient as tumor-bearing WT mice in promoting A-NK recruitment to lymphoid and nonlymphoid organs. We then asked
The transcription factor T-bet is a key transcriptional regulator of type 1 immunity that promotes differentiation of Th1 and Tc1 cells, B cell class switch, and the expression of type 1 cytokines and cytokine receptors (17, 19, 21, 38–40). Whereas DCs and NKT cells depend on T-bet expression to sustain type 1 responses (20, 38, 41), the role of T-bet in NK and CTLs can be shared or supplemented by Eomes, another member of the T-box family of transcription factors (18, 42). As a result, stimuli that induce Eomes in NK and CTL may compensate for maturation issues due to T-bet deficiency and contribute to resistance to MCMV infection and response to poly(I:C) stimulation in T-bet-deficient mice (20, 21). In the case of antitumor responses, T-bet is essential for the control of tumor metastasis, but the mechanism of T-bet-dependent resistance is not understood (22).

In this study, we have used B16F10 i.v. challenge, an established murine model of cancer metastatic disease, to isolate events relevant to tumor metastasis. The already reduced number of lung-infiltrating NK and NKT cells in T-bet−/− mice fails to increase after tumor challenge, leading to a 20-fold reduction of NK cells and no NKT cells infiltrating tumor-bearing tissue (20). In the context of tumors, NK and DCs form an axis that can promote cancer cell death, subsequent presentation of tumor Ags to T cells and may lead to helper-independent activation of CTLs (43). Our data demonstrate that although in vivo expression of T-bet in T cells does not protect T-bet−/− mice, prophylactic vaccination with tumor cells engineered to express GM-CSF leads to a significant decrease in metastatic disease. These results are consistent with previous reports that T-bet−/− CTLs and NKT cells can respond efficiently to stimuli that induce Eomes activation and suggest that T-bet−/− DCs are successful in inducing an adaptive response when stimulated with GM-CSF (44). However, these data also demonstrate that in T-bet−/− naive hosts, the response initiated by B16F10 tumor growth does not induce such potent activation. We rule out tumor-induced natural regulatory T cell-mediated suppression of antitumor responses as well as the role of NKT cells by showing that T-bet−/− Rag2−/− doubly deficient mice (DKO) are as susceptible as T-bet−/−Rag2−/− animals to B16F10 melanoma (33, 45–48). These results also show that the lack of T and NKT cells does not increase susceptibility of T-bet−/− to B16F10, suggesting that T-bet deficiency in innate immune cells disables both innate and adaptive immune responses.

NK cells are an important component of the innate immune system and especially implicated in antitumor immunity. These cells can hinder tumor progression directly by perforin/granzyme-mediated cytolytic activity and production of the inflammatory cytokine IFN-γ, which independently inhibit tumor survival and growth or indirectly by activating and providing tumor Ags to professional APCs, which then induce an adaptive response to the tumor (4, 12–14, 49–53). Their ability to sense small changes in cellular homeostasis and quickly respond confers a great potential to recognize and eliminate transformed cells (50). We show that these characteristics depend on T-bet expression because transfer of T-bet−/− but not T-bet−/− activated NK cells before or after B16F10 inoculation conferred protection to T-bet−/− hosts. The altered maturation of T-bet−/− NK cells is not responsible for the global impairment of effector function because T-bet−/− mice efficiently control MCMV virus titers and T-bet−/− NK cells respond to poly(I:C) stimulation (20, 21). Further analysis showed that decreased IFN-γ production and modest lytic activity of T-bet−/− NK cells upon IL-2 stimulation may be accentuated by their reduced viability in vitro and in vivo. These results demonstrate that T-bet-dependent NK cell longevity and function is essential for
inhibition of metastatic development. In the absence of T-bet, NK cells do not mediate the crosstalk between innate and adaptive immunity, rendering the host highly susceptible to tumor spread. These observations open the possibility that T-bet polymorphisms may correlate with increased clinical metastatic disease as well as they do with asthma incidence (54), and suggests that therapies which augment T-bet expression in NK cells may ameliorate control of tumor spread.

Acknowledgments

We thank Dr. Diefenbach for assistance with confocal microscopy, Dorothy Hu for assistance with histology, Dr. Kim and Dr. Nolting for critical reading of this manuscript, Alison Angel for assistance with manuscript and figure editing, and Landy Kangalo, Jacobo Ramirez, Antonia Garcia, and Dina Laznik for technical assistance. M.B.F.W. thanks F.F.W., manuscript and figure editing, and Landy Kangalo, Jacobo Ramirez, Antonia Garcia, and Dina Laznik for technical assistance. M.B.F.W. for unconditional support.

Disclosures

L.H.G. holds equity in and is on the Corporate Board of Directors of the Bristol-Myers Squibb Pharmaceutical Company.

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