Checkpoint Blockade Immunotherapy Relies on T-bet but Not Eomes to Induce Effector Function in Tumor-Infiltrating CD8+ T Cells
Melissa M. Berrien-Elliott1, Jinyun Yuan1, Lauryn E. Swier1, Stephanie R. Jackson1, Collin L. Chen1, Maureen J. Donlin1,2, and Ryan M. Teague1,3

Abstract
Coinhibitory receptor blockade is a promising strategy to boost T-cell immunity against a variety of human cancers. However, many patients still do not benefit from this treatment, and responders often experience immune-related toxicities. These issues highlight the need for advanced mechanistic understanding to improve patient outcomes and uncover clinically relevant biomarkers of treatment efficacy. However, the T-cell–intrinsic signaling pathways engaged during checkpoint blockade treatment are not well defined, particularly for combination approaches. Using a murine model to study how effector CD8+ T-cell responses to tumors may be enhanced in a tolerizing environment, we identified a critical role for the T-box transcription factor T-bet. Combination blockade of CTLA-4, PD-1, and LAG-3 induced T-bet expression in responding tumor/self-reactive CD8+ T cells. Eradication of established leukemia using this immunotherapy regimen depended on T-bet induction, which was required for IFNγ production and cytotoxicity by tumor-infiltrating T cells, and for efficient trafficking to disseminated tumor sites. These data provide new insight into the success of checkpoint blockade for cancer immunotherapy, revealing T-bet as a key transcriptional regulator of tumor-reactive CD8+ T-cell effector differentiation under otherwise tolerizing conditions. Cancer Immunol Res. 3(2): 116–24. ©2014 AACR.

Introduction
CD8+ T cells are important for tumor surveillance and play a major role in controlling tumor growth (1, 2). However, mechanisms of immune evasion undermine T-cell activity and promote disease (3). The quality of CD8+ T-cell responses to cancer is often dictated by environmental cues received during priming and after encounter with tumor cells. These signals determine the amplitude of the CD8+ T-cell response, and can be either stimulatory or inhibitory in nature. Although costimulatory signals induce T-cell differentiation and cytokine production associated with effective immunity, coinhibitory signals subvert costimulation to limit T-bet have important and well-described roles in CD8+ T-cell activation, differentiation, and memory formation (14–17). These T-box–mediated operations are undeniably valuable for the generation of antitumor immunity, which has been directly demonstrated in mouse models of cancer (18). More importantly, the induction of T-bet in tumor-infiltrating lymphocytes (TIL) has been correlated with positive outcomes in patients with colorectal cancer (19). However, there is only sparse evidence that coinhibitory receptor pathways influence T-bet and Eomes expression in T cells (20, 21), leaving the exact relationship between these transcriptional regulators and checkpoint blockade immunotherapy largely undefined.

We recently demonstrated that combination antibody blockade of CTLA-4, PD-1, and LAG-3 improved IFNγ production and cytotoxicity by transferred tumor/self-reactive CD8+ T cells during cancer immunotherapy (8). These effector responses hint at a possible link to T-box transcription factor–mediated CD8+ T-cell differentiation. We now report that low expression of T-bet and Eomes in tumors during these strategies have been translated into clinical successes in human patients with cancer (11, 12). Although checkpoint blockade is reshaping the landscape of cancer immunotherapy, little is known about the CD8+ T-cell–intrinsic pathways required to achieve therapeutic benefits. Additional unknowns surround the cellular mechanisms that lead to adverse immune-related toxicities in patients treated with checkpoint blockade antibodies, which may intriguingly coincide with therapeutic efficacy (13).

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required for restored effector function. T-bet was predictably important for expression of known T-bet target genes, such as Ifng and Gzmb, but also for expression of other effector genes not previously associated with T-bet transcriptional regulation. Moreover, T-bet expression in transferred tumor/self-reactive T cells was necessary to achieve cure rates in more than 95% of leukemia-bearing mice treated with checkpoint blockade immunotherapy. These results provide a new understanding into the molecular mechanisms engaged during checkpoint blockade treatment that likely transform the immune response against cancer by facilitating direct cytolytic killing of tumors by infiltrating CD8⁺ T cells.

Materials and Methods

Mice

Alb:Gag and B6.129S7-Ragfltm1Axm/J (rag1⁻/⁻) transgenic mice on wild-type (WT) and B6.129S6-Tbx21tm12Cal/J (Tbx21⁻/⁻). B6.129S1(Cg)-Eomesfltm1Glm/J (Eomes⁻/⁻), or T-bet-Tg backgrounds have been described previously (8, 17). C57BL/6 (B6) and CD90.1 (Thy1.1, B6.P-L-Thy1(Cyt)) congenic mice were purchased from The Jackson Laboratory. T-bet-ZsGreen reporter mice were obtained our (Supplementary Fig S1). Expression of the gene encoding the lymphoid homing molecule L-selectin (Sell) was measured in complete high-glucose DMEM with 10% fetal bovine serum (Sigma). Flow cytometry, staining, and ex vivo stimulations were performed as previously described (8), and nuclear staining for transcription factors was performed according to the manufacturer's protocol (ebioscience). Antibodies used here are described in Supplementary Methods.

Adoptive T-cell transfer

Gag-specific T cells were isolated from spleens and lymph nodes (LN) of indicated rag1⁻/⁻ TCRGag donors. Cell suspensions containing 3 × 10⁶ Vα1-Vγ3 CD8⁺ were intravenously (i.v.) injected into sex- and (6–12-week-old) age-matched recipients. Mice treated with checkpoint blockade received 100 μg each of anti–CTLA-4 and anti–PD-1 and anti–LAG-3 (blockade) intraperitoneally (i.p.). In vivo killing assays were performed as previously described (8).

Immunotherapy assay

Disseminated FBL leukemia was established in Alb:Gag mice by i.v. injection with 5 × 10⁵ viable FBL tumor cells. One week later, tumor-bearing mice received blockade antibodies and adoptive transfers of 1 × 10⁶ Gag-reactive CD8⁺ T cells. Recipient survival was tracked out to 75 days with daily health monitoring.

Microarray

Naïve Gag-specific T cells were transferred into B6 mice with established FBL tumor (immune) or into Alb:Gag mice (tolerant).

Two days later, transferred T cells were sorted on the basis of CD8⁺ CD90.1⁺ CD69⁻ expression to >96% purity using a FACSAria III (BD Biosciences), and RNAs were isolated from sorted cells using the RNeasy Plus Mini Kit (Qiagen). Samples were hybridized to a GeneChip Mouse Genome 430 2.0 Array and scanned using a GeneChip scanner 3000 7G (Affymetrix). Results were obtained from three biologic replicates per condition. All data have been deposited in the Gene Expression Omnibus (GEO) with accession code GSE58722.

Real-time quantitative PCR

T cells were sorted to >95% purity and total RNA isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA synthesized using SuperScript III RT (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed with SYBR Select Master Mix (Life Technologies) on a 7500 Fast Real-Time PCR System (Applied Biosystems). β-Actin was used as the endogenous amplification control. Primer sequences are listed in Supplementary Methods.

Statistical analysis

The Kruskal–Wallis test was used for all cell-frequency comparisons. Survival data were analyzed with the log-rank test. P values of less than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 4.

Results and Discussion

To uncover the intrinsic mechanisms that dictate whether CD8⁺ T cells become tolerant or differentiate into effector cells after priming, we compared the gene expression profiles of T cells shortly after in vivo encounter with antigen (Gag) expressed in distinct contexts. Specifically, naïve Gag-specific CD8⁺ T cells were transferred into B6 mice with an established immunogenic Gag-positive FBL leukemia (immune), or into Alb:Gag mice that express the same Gag protein as a tolerizing self-antigen in healthy hepatocytes (tolerant). To be clear, T-cell tolerance in this model system is due to self-antigen encounter, regardless of the presence of FBL tumor (8, 23). Two days after transfer, genes encoding the negative regulatory receptors PD-1 (Pdcd1), CTLA-4, and LAG-3 were induced under immune and tolerizing conditions, but expression was uniquely high in tolerant T cells (Fig. 1A and Supplementary Fig. S1). Expression of the gene encoding the lymphoid homing molecule L-selectin (Sell) remained high under tolerizing conditions, whereas Cd44 expression was limited mostly to immunized T cells (Fig. 1A). These results confirmed our previous protein expression analysis (8), providing confidence that this transcriptional profile accurately reflects the biology of CD8⁺ T cells actively receiving tolerizing signals in vivo.

In contrast with immunized T cells, those within the tolerizing environment lacked expression of the T-box transcription factors T-bet (Tbx21) and Eomesodermin (Eomes), and the reported T-box target genes, Cxcr3 and Ifng (Fig. 1A). Because T-bet and Eomes are important for CD8⁺ T-cell differentiation into cytolytic effector cells (14–16), this failure to express these transcription factors may explain why T cells proliferate but do not acquire effector functions under tolerizing conditions. An intriguing extrapolation of this hypothesis is that simultaneous blockade of CTLA-4, PD-1, and LAG-3, which promotes effector T-cell differentiation and function under otherwise tolerizing conditions (8), does so by promoting T-box transcription factor activity and subsequent expression of effector target genes. Defining such
a relationship would provide novel mechanistic insight into the success of combination checkpoint blockade immunotherapy for patients with cancer (11, 12).

**Checkpoint blockade induces T-bet expression in tumor/self-reactive CD8+ T cells**

To determine whether checkpoint blockade influenced T-box transcription factor expression, T cells were again transferred into immunizing and tolerizing environments. An additional "blockade" condition was also examined in which tolerizing Alb:Gag recipients were treated with anti-CTLA-4/PD-1/LAG-3. Three days after transfer, Tbx21, Eomes, and the T-box target genes Ifng and Cxcr3 were all induced in immunized CD8+ T cells relative to those in naive T cells (Fig. 1B). These genes were not highly expressed in tolerant T cells, but checkpoint blockade treatment of Alb:Gag recipients resulted in increased expression of Tbx21, Ifng, and Cxcr3. Unexpectedly, Eomes was not affected by this same blockade treatment. The induction of T-bet, but not Eomes at this time point, was further confirmed by intracellular protein detection (Fig. 1C and D). Subsequent experiments using T-bet-deficient T cells (Tbx21−/−) showed that T-bet was
Checkpoint Blockade Drives T-bet-Mediated T-cell Responses

T-bet is required for T-cell effector function during checkpoint blockade immunotherapy

Previous reports have demonstrated the redundant roles of T-bet and Eomes in CD8\(^+\) T cells, showing that expression of either one is sufficient for IFN\(\gamma\) production, cytolytic function, and antitumor responses (15–18, 24). Thus, the loss of T-bet alone was not expected to grossly alter T-cell function here, but did so likely because Eomes was not adequately induced by checkpoint blockade within the tolerizing Alb:Gag environment (Fig. 1B and C). To specifically address the individual contributions of T-bet and Eomes with respect to cancer immunotherapy, WT, Tbx21\(^{−/−}\), or Eomes-deficient (Eomes\(^{−/−}\)) tumor/self-reactive CD8\(^+\) T cells were independently transferred into Alb:Gag recipients with disseminated FBL leukemia and treated with anti-CTLA-4/PD-1/LAG-3 (Fig. 2A). After 6 days, WT T cells were nearly undetectable in the absence of blockade treatment due to peripheral deletion of most tumor/self-reactive T cells at this time point (Fig. 2B). T cells deficient for Tbx21 or Eomes were similarly deleted in untreated Alb:Gag recipients (data not shown), preventing reliable assessment of T-cell function in untreated hosts. However, in recipients treated with checkpoint blockade, transferred T cells were detected in spleens regardless of genotype, with Tbx21\(^{−/−}\) T cells expanding to slightly higher numbers compared with WT and Eomes\(^{−/−}\) T cells (Fig. 2B). Despite this expansion, Tbx21\(^{−/−}\) T cells did not produce IFN\(\gamma\) in response to checkpoint blockade, whereas WT and Eomes\(^{−/−}\) T cells mounted similarly robust IFN\(\gamma\) responses under the same conditions (Fig. 2C and D). Likewise, Tbx21\(^{−/−}\) T cells again failed to express the chemokine receptor CXCR3 (Fig. 2C), a phenotype consistent with poor effector function and compromised tumor immunity (18, 25). Indeed, WT and Eomes\(^{−/−}\) T cells were capable of efficient in vivo cytolytic activity, but Tbx21\(^{−/−}\) T cells failed to demonstrate this same ability (Fig. 2E and F). These results imply that T-bet was required for checkpoint blockade to promote effector differentiation of responding tumor/self-reactive CD8\(^+\) T cells.
In a departure from our results, CD8\(^+\) T cells that lack both T-bet and Eomes have been shown to carry out essentially normal effector activity in a nontolerant tumor-vaccine model of murine melanoma (18). However, antitumor responses were still compromised, which was attributed to poor T-cell migration due to low expression of the chemokine receptor CXCR3. Likewise, our results correlate with low CXCR3 expression in the absence of T-bet (Figs. 1 and 2). To determine whether T-bet-dependent CXCR3 expression was required for T-cell trafficking to disseminated tumor sites, TILs were examined within leukemic foci that form in recipient livers (Fig. 3A). WT, Tbx21\(^{-/-}\), or Eomes\(^{-/-}\) T cells were transferred into Alb.Gag mice with disseminated FBL leukemia and treated with anti–CTLA-4/PD-1/LAG-3 antibodies. Six days later, the frequency of infused T cells within tumor foci was similar regardless of genotype (Fig. 3B). Tumor infiltration did not appear to rely on CXCR3, which was very low on Tbx21\(^{-/-}\) T cells compared with WT and Eomes\(^{-/-}\) T cells (Fig. 3C). However, taking into consideration the higher frequency of Tbx21\(^{-/-}\) T cells in the spleens of these same animals (Fig. 2B), a somewhat lower frequency of Tbx21\(^{-/-}\) TILs indicates at least a minor defect in migration (Fig. 3D). Separate studies using

![Figure 3.](image)

T-bet is required for TIL effector function and immunotherapy but not for tumor infiltration. FBL tumor-bearing Alb.Gag mice were infused with of WT, Tbx21\(^{-/-}\), or Eomes\(^{-/-}\) Gag-reactive CD8\(^+\) T cells and treated on days 1, 1, and 3 with combination checkpoint blockade. A, diagram of experimental setup for B–E, and example of tumor foci (inset box) on a representative liver at day 6. B, the frequency of transferred CD90.1\(^+\) CD8\(^+\) T cells among all TILs was assessed 6 days after T-cell infusion, and data pooled from three separate experiments and displayed graphically. C, IFN\(\gamma\) production by transferred T cells after overnight restimulation with Gag peptide and CXCR3 surface expression directly ex vivo were assessed. D, the ratio of CD90.1\(^+\) CD8\(^+\) T-cell frequency in TILs versus in spleens from the same recipients in Fig. 2B is shown. E, pooled data from three individual experiments showing the frequency of CD90.1\(^+\) CD8\(^+\) TILs producing IFN\(\gamma\). Circles within all graphs represent individual recipient mice and horizontal lines show the mean of each group with \(P\) values indicated for the bracketed groups; all error bars represent SEM. F, survival of tumor-bearing Alb.Gag recipients was assessed following treatment with WT T cells only (gray line), checkpoint blockade (anti–CTLA-4/PD-1/LAG-3) only (dashed gray line), or checkpoint blockade and adoptive transfer of WT T cells (black line), Cxcr3\(^{-/-}\) T cells (blue) or Tbx21\(^{-/-}\) T cells (red). The graph displays pooled data from five independent experiments, showing percentage survival (y-axis) over time in days (x-axis). The \(n\) values denote the number of total mice per treatment group and the \(P\) value is indicated for the bracketed groups.
T-bet expression by transferred T cells is required for checkpoint blockade immunotherapy of disseminated leukemia

Administration of anti–CTLA-4/PD-1/LAG-3 blockade antibodies engages endogenous immune cells and is sufficient to cure approximately 65% of Alb:Gag mice with an established and disseminated FBL leukemia (8). This approach 100% when checkpoint blockade is combined with the transfer of Gag-specific CD8+ T cells. Because T-bet was required for effecter differentiation of transferred tumor-reactive T cells (Fig. 2), we examined whether loss of T-bet affected the efficacy of adoptive T-cell immunotherapy. Consistent with our previous study, mice receiving WT T cells alone (no blockade) had a median survival of only 16 days due to induction of T-cell tolerance (Fig. 3F). The addition of combination checkpoint blockade overcame these tolerizing influences and resulted in a 96% survival rate 75 days after tumor inoculation. This was not the case for mice administered Tbx21−/− T cells with checkpoint blockade, which gained no survival benefit compared with those receiving checkpoint blockade alone. Despite a modest reduction in tumor infiltration by Tbx21−/− T cells expressing low levels of CCRX3 (Fig. 3D), adoptive transfer of Gag-specific Cxcr3−/− T cells with checkpoint blockade led to recipient survival rates nearly identical to WT-T-cell recipients (Fig. 3F). Together, these data affirm that the success of this combination checkpoint blockade immunotherapy relies on the induction of T-bet in responding tumor-reactive CD8+ T cells, and does not require expression of the T-bet target molecule CCRX3.

Induction of T-bet drives effector gene expression in tumor-reactive CD8+ T cells responding to checkpoint blockade

To define how T-bet influences responding CD8+ T cells during immunotherapy, gene expression was compared in WT and Tbx21−/− T cells cotransferred together into the same tumor-bearing Alb:Gag recipients treated with combination checkpoint blockade. Transferred T cells were sorted 6 days after infusion and subjected to gene expression analysis by qRT-PCR (Fig. 4A). Specific genes were chosen on the basis of uniquely low expression in tolerant CD8+ T cells as determined by prior gene array studies (Supplementary Fig. S1), indicating a possible link to the dysfunctional phenotype. In line with our data to this point, Tbx21, Ifng, and Cxcr3 were all induced with checkpoint blockade in WT but not Tbx21−/− T cells. Similarly, the well-described T-bet target gene Gzmb was also induced in a T-bet–dependent manner. Two other granzyme genes, Gzma and Gzmb, which have not been directly associated with T-bet transcriptional regulation, were nevertheless reliant on T-bet. In contrast, the reported T-bet target gene Fasl was induced independently of T-bet after checkpoint blockade, whereas Prf1 (Perforin) and the IL12 receptor genes (Il12rb1 and Il12rb2) were not induced at all (Fig. 4A).

Conceivably, any or all of the T-bet–dependent genes identified here have the potential to influence antitumor immunity. This is particularly true for granzymes and IFNγ, which have established roles in T-cell effector activity. For other genes like those encoding the chemokine CCL5 (RANTES) and its receptor CCR5, the contribution may be geared more toward T-cell migration, differentiation, or the recruitment of other effector cells rather than direct tumor killing. Indeed, increased motility of melanoma-specific T cells was recently attributed to CCL4-4 blockade (26). To support these gene expression results, we analyzed T-bet, Eomes, and CCR5 protein expression in transferred T cells from tumor-bearing Alb:Gag mice receiving checkpoint blockade. Here, mice received a cotransfer of either WT and Tbx21−/− T cells or WT and Eomes−/− T cells and analysis was performed 6 days later. Intracellular T-bet protein was expressed equivalently in both WT and Eomes−/− T cells in spleens and TILs, but not in Tbx21−/− T cells (Fig. 4B). Although expression of intracellular Eomes was detected in WT and Tbx21−/− T cells from the spleens, Eomes protein was essentially absent in CDS8+ TILs regardless of genotype (Fig. 4B, middle). These data further marginalize the possible contributions of Eomes in driving CD8+ T-cell effector mechanisms within the tumor. In contrast, CCR5 surface protein was markedly enriched on CD8+ T cells from tumors compared with the spleens. As in transcript analysis (Fig. 4A), CCR5 expression was reduced on Tbx21−/− TILs relative to WT TILs (Fig. 4B, right), indicating a potentially important role for T-bet–mediated CCR5 expression in CD8+ T-cell migration to tumors during checkpoint blockade immunotherapy. This is supported by limited Tbx21−/− T-cell infiltration into disseminated tumor sites (Fig. 3D). It is worth noting that induction of a gene or protein in a T-bet–dependent manner here does not necessarily predict its requirement during immunotherapy, and this is a good example of this. Clearly, further study is needed to fully discern the precise impact these genes have on responding T cells.

One of the complicating factors in promoting combination blockade therapies is identifying which components provide a given effect. To determine which blockade antibodies induced T-bet expression in responding tumor/self-reactive T cells, we used a sensitive fluorescent (ZsGreen) T-bet reporter system (22). Our previous work showed that double blockade of PD-1 and CTLA-4 produced only mild effecter T-cell responses, which could be accentuated with the addition of anti–LAG-3 (8). Given the importance of T-bet for such effector mechanisms, we anticipated that this triple antibody combination would induce more T-bet–expressing T cells relative to any double blockade combinations, and this was true when compared with anti–PD-1 and CTLA-4 (Fig. 4C and D). Unexpectedly though, the alternative combination of anti–PD-1 and LAG-3 induced equivalent T-bet expression as that induced by the triple blockade, suggesting the PD-1 and LAG-3 receptors may be most effective at limiting T-bet expression and effector responses in tolerant T cells. However, the number of persisting T cells was significantly higher in the triple blockade–treated group relative to any double combinations, indicative of a more effective overall immunotherapeutic strategy (Fig. 4E).

Several mechanisms have been proposed to explain exactly how checkpoint blockade antibodies boost T-cell immunity, including depletion of regulatory cells (27, 28) and attenuation of negative regulatory signaling (29). However, these and other mechanisms are not mutually exclusive, and may cooperate to achieve enhanced antitumor responses. The current study was not designed to identify these mechanisms, but rather to define T-cell–intrinsic pathways engaged during checkpoint blockade immunotherapy. To this end, our results support a requirement for T-bet in promoting blockade-induced CD8+ T-cell effector responses.
sufficient to eradicate disseminated and progressive leukemia. It is difficult to predict how closely the animal model examined here reflects the nuances of human checkpoint blockade immunotherapy. It makes sense though that boosted T-cell responses in patients may arise from increased T-bet expression. This could distinguish T-bet as a potential biomarker to indicate responsiveness to therapy. Such a sentinel molecule would allow clinicians to gauge whether individual patients are likely to benefit from...
continued treatment or those more at risk of treatment-related toxicities. It is also possible that checkpoint blockade induces T-cell expression in a variety of other cells, including CD4+ T cells, NK cells, or even innate lymphoid cells (ILC1) that could contribute to antitumor immunity, or to autoimmunity in human patients (30). Identifying which cells are engaged by different checkpoint blockade regimens could provide translational insight toward higher fidelity treatment, thereby leading to better outcomes in patients with cancer receiving immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The content of this article is solely the interpretation of the authors and does not necessarily represent the official views of the NIH.

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References


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