Durable Adoptive Immunotherapy for Leukemia Produced by Manipulation of Multiple Regulatory Pathways of CD8+ T-Cell Tolerance


Abstract
Tolerizing mechanisms within the host and tumor microenvironment inhibit T-cell effector functions that can control cancer. These mechanisms blunt adoptive immunotherapy with infused T-cells due to a complex array of signals that determine T-cell tolerance, survival, or deletion. Ligation of the negative regulatory receptors CTLA4, PD-1 (PDCD1), or LAG3 on T-cells normally hinders their response to antigen through nonredundant biochemical processes that interfere with stimulatory pathways. In this study, we used an established mouse model of T-cell tolerance to define the roles of these inhibitory receptors in regulating CD8+ T-cell tolerance during adoptive immunotherapy to treat leukemia. Blocking CTLA4 and PD-1 in vivo combined to promote survival of transferred T-cells despite powerful deleterious signals that mediate Bim (BCL2L11)-dependent apoptosis. However, this dual blockade was not optimal for stimulating effector function by responding T-cells, which required the additional blockade of LAG3 to induce full expansion and allow the acquisition of robust cytolytic activity. Thus, the cooperation of multiple distinct regulatory pathways was needed for the survival and effector differentiation of adaptively transferred tumor-reactive CD8+ T-cells. Our work defines the immune escape pathways in which simultaneous blockade could yield durable immunotherapeutic responses that can eradicate disseminated leukemia.

Introduction
Adaptive immunotherapy relies on the transfer of tumor-reactive T-cells into patients with cancer as a means to provide specific and potent antitumor immunity. One of the primary challenges to this approach is maintenance of a sufficiently large population of tumor-specific T-cells, which encounter numerous immunosuppressive influences either through contact with tolerizing self-tissue or by mechanisms of immune evasion within the tumor microenvironment. As a result, transferred T-cells are often deleted in the periphery, limiting therapeutic benefits (1–6). There is little consensus about the molecular pathways involved in determining whether responding T-cells will be activated, deleted, or rendered tolerant. Signals provided by costimulation, inflammatory mediators, and negative regulatory molecules during priming are clearly important (7–11), but the goal of identifying and exploiting these pathways to provide durable T-cell immunotherapy for cancer has been elusive.

Strategies to maximize T-cell activation and effector function before adoptive transfer have not reliably translated into the generation of T-cells optimized for immunotherapy (6, 12, 13), and deletion after infusion remains a major unresolved issue for success. It is likely that providing proactivation signals alone is not sufficient but alternatively or simultaneously blocking negative signaling pathways may provide the correct dialogue necessary to communicate complex survival and differentiation instructions to transferred T-cells. Indeed, attenuation of negative signaling through regulatory receptors has generated recent enthusiasm for clinical applications. For example, ligation of cytotoxic T lymphocyte–associated antigen-4 (CTLA4) on T-cells normally serves to dampen immune responses by interfering with both T-cell receptor (TCR) signaling and the costimulation pathway downstream of CD28 (14, 15). Clinicians have successfully exploited this by preventing CTLA4 ligation with blockade antibodies during vaccination to fortify T-cell responses against cancer (16–19). Additional reports suggest these responses may be further enhanced by blockade of other nonredundant regulatory pathways such as programmed death-1 (PD-1) or lymphocyte activation gene-3 (LAG3; refs. 20–24). Despite these advances, the specific mechanisms by which these pathways regulate T-cell function and the identity of the biologic processes being regulated, are still unclear.
While combination approaches have proven effective at bolstering T-cell proliferation and effector function during priming and tumor vaccination, how these regulatory pathways specifically influence survival and function of transferred CD8+ T-cells encountering intense tolerizing signals during cancer immunotherapy has not been described. This study examines the fate of adoptively transferred tumor/self-reactive CD8+ T-cells derived from a naïve population, as increasing evidence suggests the proliferative and differentiation potential of these cells may be greater than more activated ones (25). Previously believed unfeasible for human immunotherapy, recent technologies using genetic manipulation to induce expression of tumor-specific TCR have established naïve T-cells as viable therapeutic reagents (3, 5, 26–28). Here, we report that rapid Bim-mediated deletion of adoptively transferred CD8+ T-cells following recognition of a shared tumor/self-antigen is ameliorated by coadministration of PDL1- and CTLA4-blockade antibodies. This treatment reduced surface expression of the inhibitory receptors PD-1 and LAG3 on responding T-cells, and further blockade of LAG3 bolstered the expansion and effector differentiation in surviving cells, translating into effective and enduring immunotherapy for established and disseminated leukemia.

Materials and Methods

Mice

AllnAg and Rag+/– TCRGag transgenic mice have been previously described (4, 29, 30). C57BL/6 (B6), Bim+/–, and B6-Thy1.1 congenic mice were purchased from The Jackson Laboratory and crossed with our Rag+/– mice. All mice were maintained under specific pathogen-free conditions and used in accordance with our animal protocol approved by the Animal Care Committee of the Department of Comparative Medicine, Saint Louis University School of Medicine (St. Louis, MO).

Cell lines, peptides, and antibodies

FBL is a Friend virus-induced erythroleukemia of B6 origin, and were obtained from Dr. Philip Greenberg (University of Washington, Seattle, WA). To our knowledge, these tumor cells have not been commercially tested or authenticated. FBL expresses an immunogenic virus-derived H-2b-restricted Gag epitope as previously described (4, 29). Gag peptide (CCLCTLTVFL) and control ovalbumin peptide (SIINFEKL) were purchased from Pi Proteomics. Cells were washed in PBS, and cell culture was conducted in complete RPMI-1640 with 10% FBS (Sigma). Fluorochrome-conjugated antibodies to CD8 (53–67), CD4 (G07-58), CD19 (R4-33), CD45.1 (A20–30), and Gr1 (RB6–8C5) were purchased from BD Biosciences. Fluorochrome-conjugated antibodies to CD44 (IM7), CD62L (MEL-14), Th1 (OX-7), IFN-γ (XMG12), TNF-α (MP6-XT22), active caspase-3 (C92-605), and Vt3-TCR (RB3-16), were purchased from BD Biosciences. 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen. The anti-LAG3 hybridoma cell line (9B77W) was a gift from Dr. Dario Vignali (St. Jude Children’s Research Hospital, Memphis, TN). Blocking antibodies to PDL1 (10F.9G2), PDL-1 (RPM1-14), CTLA4 (9D9), and isotype controls for mouse IgG2b (MPC-11) and rat IgG2b (LTF-2) were purchased from BioXCell. Human recombinant interleukin (IL)-2 was a gift from Dr. Daniel Hoft (Saint Louis University School of Medicine).

Adaptive T-cell transfer

Gag-specific T-cells were isolated from spleens and lymph nodes of Rag+/– TCRGag donors. Whole-cell suspensions containing 3 x 106 Vx3-TCR+ CD8+ cells were intravenously injected into sex and age (6–12 week) matched recipients. In some experiments, transferred cells were labeled with 2 μg/mL CFSE before infusion. To provide an immunogenic environment, B6 recipients were given 5 x 106 FBL cells intraperitoneally 3 days before T-cell transfer. To provide an inflammatory environment, B6 recipients were infected with 3 x 107 cfu Listeria monocytogenes (ΔActA) intraperitoneally 1 day before T-cell transfer. Recipient spleens, peripheral lymph nodes, and livers were harvested for analysis at various time points. Tissues were homogenized into single-cell suspensions before analysis. Lymphocytes were isolated from liver by Ficoll-Paque (GE Health) gradient centrifugation.

Flow cytometry

Single cell suspensions were stained for extracellular markers at 4°C for 30 minutes. For intracellular staining, cells were fixed and permeabilized in Cytofix/Cytoperm buffer (BD Biosciences), and proteins stained in Perm/Wash buffer (BD Biosciences) for 30 minutes at 4°C according to the manufacturer’s protocol. Staining with Annexin V (BD Biosciences) and for intracellular active-caspase-3 was conducted after a 3-hour incubation at 37°C. Ex vivo cytokine production was assessed following overnight stimulation with 5 μg/mL Gag or Ova peptide in the presence of GolgiPlug (BD Biosciences). All flow cytometry was conducted using either an LSR II or FACScanto II (BD Biosciences), and resulting data analyzed using Flowjo software (Tree Star).

In vivo killing assay

Recipient mice received adoptive T-cell transfers, as described earlier. Three days after T-cell transfer, B6 splenocytes (targets) were harvested and pulsed with 10 μg Gag or control Ova peptide. Peptide-pulsed B6 target cells were differentially labeled with 0.7 or 2.1 μg/mL CFSE, respectively, and injected into recipient mice intravenously at a 1:1 ratio. Approximately 20 hours later, the frequency of CFSEhigh versus CFSELow targets from recipient spleens and lymph nodes was assessed by flow cytometry.

Immunotherapy assay

On day 0, disseminated FBL leukemia was established in Alb:Gag mice by intravenous injection with 1 x 107 viable FBL tumor cells. On day 6, tumor-bearing mice received 200 μg isotype control antibody, or 100 μg each anti-CTLA-4 and anti-PD-1 (double-blockade), or 100 μg each anti-CTLA-4, anti-PD-1, and anti-LAG3 (triple-blockade) intraperitoneally. On day 7, recipients received adoptive transfers of 3 x 106 Gag-reactive CD8+ T-cells by intravenous injection. Recipients were then given 5 subsequent blockade injections on days 8, 10, 13, 16, and 19. For in vivo tumor imaging, mice were inoculated...
intravenously (as earlier) with FBL tumor transduced to express enhanced GFP (FBLGFP). Hair was shaved around the abdomen, and animals anesthetized (2.5% isoflurane, 0.25 L/min) and imaged using an IVIS Spectrum (Xenogen). Images were analyzed with Live Image v3.1 software (Caliper Life Sciences). Recipient survival was tracked out to 100 days with daily health monitoring, and mice killed upon detection of tumor-induced ascites or becoming moribund.

Statistical analysis

The Kruskal–Wallis test was used for statistical comparison (GraphPad Prism 4) of total cell numbers between different treatment groups. A one-way ANOVA was used for statistical comparison of cell frequencies between multiple treatment groups. Survival data were analyzed with the log-rank test. $P$ values of less than 0.05 were considered statistically significant.

Results

Suboptimal activation of transferred CD8$^+$ T-cells precedes peripheral deletion

To examine deletion and induction of tolerance in T-cells during cancer immunotherapy, we used the well-characterized AlbGag mouse model in which a leukemia virus-derived Gag protein is expressed as a model self-antigen in healthy hepatocytes (29). The same Gag protein is also expressed as a bona fide tumor antigen in murine FBL leukemia. Here, Gag-specific CD8$^+$ T-cells (Thy1.1$^+$) transferred into AlbGag mice were rapidly deleted within 8 days due to encounter with tolerizing self-antigen, but were readily detectable in B6 mice in which Gag is not expressed (Fig. 1A). Recognition of Gag-antigen in the context of immunogenic FBL leukemia induced expansion of transferred tumor-reactive T-cells in B6 recipients (Fig. 1A).

Figure 1. Suboptimal activation precedes deletion of transferred T-cells. A, Gag-reactive T-cells (Thy1.1$^+$ CD8$^+$) were transferred into B6 mice, B6 mice with FBL tumor (B6 + FBL), Alb:Gag mice, and Alb:Gag mice with FBL tumor (Alb:Gag + FBL). The frequency of transferred cells in spleens 8 days later was assessed. B, liver tumor foci were harvested and the frequency of infiltrating T-cells assessed, with inset numbers representing the percentage of all cells within the designated region. Data are representative of 3 separate experiments. C, Gag-reactive T-cells were CFSE-labeled and transferred into B6 mice, B6 + FBL, or Alb:Gag mice. The frequency of transferred cells in lymph nodes (LN), spleen, and liver was assessed 3 days later, and inset numbers are the percentage of all cells within the designated regions. D, CFSE dilution by transferred Thy1.1-gated cells was compared by overlaying histograms from B6 (gray), B6 + FBL (blue), and Alb:Gag (red) recipients. E, CFSE intensity versus expression of CD44 or CD62L on transferred cells was directly compared by overlaying dot plots from B6 + FBL (blue) and Alb:Gag (red) recipient spleens 3 days after transfer, and data are representative of 5 independent experiments. F, total numbers of Thy1.1$^+$ CD8$^+$ cells from spleen (•) and lymph nodes (○) 3 and 8 days after transfer into the indicated recipients. Graphs display pooled data from 4 independent experiments on day 3, and 3 experiments on day 8.
but were still deleted in Alb:Gag recipients in which expression of the tumor antigen was shared in healthy self-tissues—recaptulating one of the major challenges to clinical immunotherapy. Predictably, transfer of Gag-reactive CD8+ T-cells alone into FBL-bearing Alb:Gag recipients was not sufficient to control disseminated leukemia, as these recipients displayed many large tumor foci in the liver 8 days after T-cell transfer, compared with only a few small foci seen in B6 recipients (Fig. 1B). Examination of tumor-infiltrating lymphocytes (TIL) within these foci revealed equivalent frequencies of total CD3+ CD8+ T-cells between B6 and Alb:Gag hosts but the frequency of transferred Thy1.1+ CD8+ T-cells in Alb:Gag mice was markedly reduced, likely reflecting the peripheral deletion of these tumor/self-reactive cells.

To define the mechanisms regulating peripheral T-cell deletion observed here, we evaluated early responses by Gag-reactive CD8+ T-cells following transfer into Alb:Gag hosts. FBL tumor cells have been defined as immunogenic for Gag-reactive CD8+ T-cells (29, 31, 32), and express MHC-I, the costimulatory molecules B7-1 and ICOSL, but also the inhibitory ligand PD-L1 (Supplementary Fig. S1). Therefore, to isolate only the deletion signals provided by encounter with self-antigen, no tumor was established in Alb:Gag recipients for these experiments. Instead, responses were compared following transfer into either normal B6 recipients (B6; no antigen), B6 recipients bearing Gag+ FBL tumor (B6 + FBL; immunizing antigen), or Alb:Gag recipients expressing Gag only as a self-protein in the liver (Alb:Gag; tolerizing antigen). Three days after transfer, T-cells that encountered either immunizing (FBL) or tolerizing (Alb:Gag) Gag-antigen had expanded in frequency (Fig. 1C) and undergone many rounds of cell division throughout multiple tissues (Fig. 1D), but the phenotype of these proliferating cells differed between the 2 antigenic conditions. Splenic T-cells that proliferated upon encounter with tumor-associated Gag-antigen showed elevated levels of the activation marker CD44 as early as 1 division and remained high, whereas T-cells responding to Gag self-antigen displayed a CD44 low to intermediate phenotype despite multiple divisions (Fig. 1E). Stimulation with FBL also induced downregulation of the lymphoid homing molecule L-selectin (CD62L), which was lowest after several divisions. In stark contrast, CD62L expression remained high on splenic T-cells responding to Gag self-antigen throughout all divisions. These phenotypic differences revealed a CD44hi/CD62Llo molecular profile that distinguished otherwise identical T-cells that had encountered the same Gag-antigen presented in different contexts. Similar profiles were also observed on T-cells from lymph nodes and livers of these same recipients (data not shown). This unique CD44/CD62L expression profile was likely a consequence of aberrant activation signals that ultimately decide the fate of responding T-cells, as those cells displaying a CD44hi/CD62Llo phenotype induced by self-antigen were nearly completely deleted by day 8, whereas CD44hi/CD62Lhi cells activated by FBL persisted (Fig. 1F).

Expression of a CD44hi/CD62Llo profile has been associated with differentiation toward effector CTL (13, 33, 34), suggesting that proliferating T-cells in Alb:Gag mice, which remained CD62Lhi, had not differentiated to CTL. To examine lytic potential in vivo, B6, FBL-bearing B6, and Alb:Gag recipients were given an adoptive transfer of Gag-specific CD8+ T-cells followed 3 days later by a second infusion of CFSE-labeled target cells. Tissues were removed 20 hours later to assess the frequency of Gag+ (CFSLhi) and control (CFSthi) target cells remaining. T-cells transferred into B6 hosts (no antigen) remained naïve and were not induced to become CTL, resulting in an equivalent frequency of both target cell populations (Fig. 2A). Effector function was readily observed by T-cells activated in FBL-bearing B6 recipients, which specifically destroyed Gag-positive target cells in vivo (Fig. 2A and B) and produced the effector cytokines IFN-γ and TNF upon restimulation (Fig. 2C). In contrast, T-cells transferred into Alb:Gag hosts failed to acquire effector function despite robust early expansion in response to peripheral self-antigen. Thus, the failure of transferred T-cells to control leukemia following encounter with shared tumor/self-antigen likely involves T-cell deletion and dysfunctional effector mechanisms.

**CD8+ T-cell survival and effector function are regulated by distinct mechanisms**

Elimination of self-reactive CD8+ T-cells often relies on the proapoptotic molecule Bim, particularly when T-cells are activated in the liver (35–37). Here, deletion of responding T-cells in Alb:Gag recipients coincided with elevated AnnexinV staining and active caspase-3 expression, indicating death by apoptosis (Supplementary Fig. S2). This apoptosis was indeed dependent on Bim expression, as Bim−/− Gag-reactive T-cells persisted in Alb:Gag recipients, whereas cotransferred BimWT Gag-reactive T-cells were eliminated (Supplementary Fig. S3). Survival of Bim−/− Gag-specific T-cells in Alb:Gag recipients provided a novel tool to determine if the failure of transferred T-cells to differentiate into CTL was simply a consequence of cells actively undergoing apoptosis, or instead if T-cell deletion and effector dysfunction were distinctly regulated. Here, Bim−/− T-cells displayed IFN-γ and TNF production if primed under immunizing conditions in FBL-bearing recipients, but not by self-antigen in Alb:Gag mice (Supplementary Fig. S3), suggesting the failure to acquire effector function was independent of apoptosis. Therefore, strategies to overcome T-cell tolerance during immunotherapy will likely require identification of the operative pathways that maintain both effector function and survival.

While Bim-mediated apoptosis is a known mechanism for T-cell deletion, the array of possible intrinsic and extrinsic signals that initiate this apoptotic pathway is broad (38, 39), and remains enigmatic in the setting of adoptive T-cell immunotherapy. The outcome of a T-cell response is influenced greatly by signals received from antigen-presenting cells (APC) engaged during priming, which often provide cytokines and costimulation that induce appropriate activation and differentiation (40). Healthy hepatocytes in Alb:Gag mice likely fail to provide such signals, contributing to T-cell tolerance and deletion (4, 36). However, the requirement for activated APC can be by-passed by providing inflammatory signals, leading to T-cell activation and memory formation even in the absence of some costimulatory molecules (41, 42). To examine if peripheral T-cell deletion could be prevented under inflammatory conditions, T-cells were transferred into Alb:Gag recipients that were treated with the costimulatory molecules B7-1 and ICOSL, but also the inhibitory ligand PD-L1 (Supplementary Fig. S1). Therefore, to isolate only the deletion signals provided by encounter with self-antigen, no tumor was established in Alb:Gag recipients for these experiments. Instead, responses were compared following transfer into either normal B6 recipients (B6; no antigen), B6 recipients bearing Gag+ FBL tumor (B6 + FBL; immunizing antigen), or Alb:Gag recipients expressing Gag only as a self-protein in the liver (Alb:Gag; tolerizing antigen). Three days after transfer, T-cells that encountered either immunizing (FBL) or tolerizing (Alb:Gag) Gag-antigen had expanded in frequency (Fig. 1C) and undergone many rounds of cell division throughout multiple tissues (Fig. 1D), but the phenotype of these proliferating cells differed between the 2 antigenic conditions. Splenic T-cells that proliferated upon encounter with tumor-associated Gag-antigen showed elevated levels of the activation marker CD44 as early as 1 division and remained high, whereas T-cells responding to Gag self-antigen displayed a CD44 low to intermediate phenotype despite multiple divisions (Fig. 1E). Stimulation with FBL also induced downregulation of the lymphoid homing molecule L-selectin (CD62L), which was lowest after several divisions. In stark contrast, CD62L expression remained high on splenic T-cells responding to Gag self-antigen throughout all divisions. These phenotypic differences revealed a CD44hi/CD62Llo molecular profile that distinguished otherwise identical T-cells that had encountered the same Gag-antigen presented in different contexts. Similar profiles were also observed on T-cells from lymph nodes and livers of these same recipients (data not shown). This unique CD44/CD62L expression profile was likely a consequence of aberrant activation signals that ultimately decide the fate of responding T-cells, as those cells displaying a CD44hi/CD62Llo phenotype induced by self-antigen were nearly completely deleted by day 8, whereas CD44hi/CD62Lhi cells activated by FBL persisted (Fig. 1F).

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Overcoming Negative Regulation of T-Cells for Immunotherapy

The inability to alter the fate of T-cells being deleted in Alb:Gag hosts by providing a disseminated inflammatory environment or systemic IL-2 during priming highlights the potent nature of deletional signals received during encounter with self-antigen. An alternative mechanism may involve signals received through ligation of negative regulatory receptors at the sight of antigen encounter. The inhibitory receptors PD-1 and CTLA4 are reported to play roles in T-cell tolerance (8, 9, 21, 39), and inhibition of these pathways by antibody blockade has proven effective at boosting T-cell responses for the benefit of cancer immunotherapy (18, 20–22). We evaluated if persistence of adoptively transferred T-cells was influenced in Alb:Gag mice receiving blockade of PDL1 and CTLA4. Treatment with either antibody alone resulted in a modest increase in the frequency of transferred T-cells persisting out to day 8 compared with isotype control (Fig. 3A). Persistence was markedly enhanced in mice treated with both antibodies simultaneously (double-blockade), producing a significant increase in the total number of adoptively transferred T-cells in Alb:Gag recipients (Fig. 3B). Consistent with a previous reports (21), blockade treatment induced modest and relatively uniform expansion of endogenous CD4<sup>+</sup>, CD8<sup>+</sup>, and Foxp3<sup>+</sup> T-cell populations (data not shown).

To identify potential mechanisms that influence survival of transferred T-cells as a result of PDLI and CTLA4 blockade, we examined surface expression of known negative regulatory receptors on T-cells after infusion into Alb:Gag hosts. Compared with naive T-cells from B6 recipients, those remaining in Alb:Gag mice displayed elevated levels of both PD-1 and CTLA4 (Fig. 3C), but PD-1 expression was reduced following PDL1/CTLA4 blockade. CTLA4 expression also seemed reduced after blockade, but the CTLA4 antibody used during in vivo blockade seemed to interfere with staining by the anti-CTLA4 used for flow cytometry, limiting reliable assessment of CTLA4 protein expression after blockade. However, expression of another regulatory receptor, LAG3, was also elevated on T-cells in Alb:Gag recipients, and was reduced to levels seen on naïve T-cells when mice were treated with PDL1/CTLA4 blockade (Fig. 3C). Thus, inhibition of negative signaling via blockade of PDLI and CTLA4 reduced expression of the regulatory receptors PD-1 and LAG3 on adoptively transferred T-cells.

Figure 2. Self-antigen fails to induce CTL differentiation. Gag-reactive T-cells were transferred into B6 mice, B6+FBL, or Alb:Gag mice. A, 3 days after transfer, recipients were infused with a 1:1 ratio of Gag and control peptide-pulsed target cells differentially stained CFSE<sup>low</sup> or CFSE<sup>high</sup>, respectively. Target cell frequency was assessed 20 hours later. Compared with uninfected Alb:Gag hosts, the frequency of transferred T-cells in infected Alb:Gag mice was nearly double at day 3, suggesting the inflammatory environ-

ment had a rather dramatic impact on early priming or expansion (Supplementary Fig. S4A). Despite this effect, systemic inflammation was not sufficient to overcome deletion of these T-cells, which were nearly undetectable in Alb:Gag hosts by day 8.

The benefit of IL-2 during clinical adoptive immunotherapy has been well documented (5, 32). However, daily administration of high-dose IL-2 also had no significant impact on survival of transferred T-cells in our system (Supplementary Fig. S5B). These results indicate that neither inflammatory signals nor prosurvival signals from IL-2 are sufficient to promote persistence of adoptively transferred tumor/self-reactive T-cells in this tolerizing environment, thus a lack of such signals during priming is not a likely mechanism of peripheral deletion.

### CTLA4 and PD-1 regulate deletion of adoptively transferred CD8<sup>+</sup> T-cells

Conditions, Gag-specific T-cells were transferred into Alb:Gag mice infected with attenuated (ActA-deficient) *Listeria monocytogenes*, and the persistence of these cells assessed 3 and 8 days later. Compared with uninfected Alb:Gag hosts, the frequency of transferred T-cells in infected Alb:Gag mice was nearly double at day 3, suggesting the inflammatory environ-

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potentially providing these cells with an intrinsic survival benefit by limiting the capacity to receive subsequent inhibitory signals.

**Inclusion of LAG3 blockade promotes expansion and differentiation of adoptively transferred T-cells**

Elevated LAG3 expression on T-cells undergoing deletion and tolerance induction suggests a potential role for LAG3 signaling during these processes, and implies that blockade of this pathway may influence the durability of adoptively transferred T-cells. Although LAG3 blockade had no detectable effect alone (data not shown), the addition of anti-LAG3 with anti-PDL1/CTLA4 (triple-blockade) modestly increased the frequency of transferred T-cells persisting in Alb:Gag recipients at day 8 relative to those receiving double-blockade (Fig. 3D). However, no statistically significant increase in the total number of transferred T-cells between double- and triple-blockade treated Alb:Gag recipient groups was observed (Fig. 3E), suggesting the LAG3 pathway has little influence on deletion or survival of adoptively transferred
T-cells compared with the PD-1 and CTLA4 pathways. Likewise, both double- and triple-blockade regimens resulted in markedly decreased apoptosis as measured by AnnexinV staining and active caspase-3 expression when compared with untreated Alb:Gag mice, but differences between double- and triple-blockade groups with regard to these apoptotic indicators were unremarkable (Supplementary Fig. S5).

While LAG3 did not seem to directly impact the persistence of adoptively transferred CD8+ T-cells, increased expression of LAG3 on T-cells responding to tolerizing signals in Alb:Gag recipients may indicate a role for LAG3 signaling in suppression of CTL activity. Relative to the few T-cells remaining in untreated Alb:Gag mice, a large percentage of persisting T-cells in mice receiving either double- or triple-blockade displayed an effector-like CD44high CD62Llow phenotype (Fig. 3D). This hint at a restored effector phenotype was borne out upon examination of in vivo killing and ex vivo IFN-γ production, both of which were restored in T-cells transferred into blockade treated Alb:Gag recipients, particularly when blockade of LAG3 was included (Supplementary Fig. S6).

To directly assess how these different blockade treatments impacted in vivo effector function in recipients with leukemia, Gag-specific CD8+ T-cells were transferred into B6 and Alb:Gag mice with 7 day established FBL, and blockade antibodies provided as indicated. Here, and in all subsequent experiments, anti-PD-1 was used rather than anti-PDL1 to avoid confounding results that could arise from antibody binding directly to the established FBL tumor. Five days after T-cell transfer into
One of the unexpected outcomes of triple-blockade therapy was the ability to provide a survival benefit to Alb:Gag mice that did not receive adoptive T-cell transfer (Fig. 5D), showing...
the capacity to boost a previously ineffective endogenous immune response against this established leukemia. It should be noted that Alb:Gag mice do not have peripheral Gag-reactive T-cells due to central deletion in the thymus (29), thus these responses were likely to minor tumor antigens and possibly antigen independent. By selectively depleting specific endogenous immune cell populations before blockade treatment (Fig. 6A), both CD4+ and CD8+ T-cells were shown to be required for this response (Fig. 6B). While depletion of natural killer (NK) cells produced a survival curve that failed to meet our rigid threshold for statistical difference compared with nondepleted recipients, a much lower proportion of NK-depleted mice survived beyond 60 days (30% compared with 70%). This indicates that triple-blockade may also elicit NK cells along with T-cells to combat leukemia, warranting further investigation.

To examine the ultimate fate of adoptively transferred T-cells in blockade-treated Alb:Gag mice that had overcome leukemia, T-cells were examined in long-lived recipients from Fig. 5D at more than 100 days after transfer. Mice that had received T-cells and either blockade regimen had a small but detectable population of Thy1.1+ transferred T-cells compared with background levels in recipients receiving only triple-blockade treatment alone (Fig. 7A). These persisting tumor-reactive T-cells were still capable of producing IFN-γ and even TNF upon ex vivo restimulation with tumor-derived Gag peptide (Fig. 7B), and underwent robust proliferation and expansion following transfer into secondary B6 recipients bearing a disseminated FBL leukemia (Fig. 7C). Thus, transferred T-cells that persisted in surviving Alb:Gag recipients following blockade treatment maintained effector function and reactivity to FBL leukemia long after treatment had ceased. These data show that intervention of these separate negative regulatory pathways provides clear and distinct therapeutic advantages during adoptive T-cell immunotherapy by combining to enhance expansion, effector function and long-term survival of tumor-reactive T-cells despite a highly tolerizing in vivo environment.

Discussion

Overcoming cancer in patients using adoptive immunotherapy requires the activity of large numbers of tumor-reactive T-cells. However, transferred T-cells are often deleted following recognition of tumor antigens shared in normal healthy tissues through mechanisms that are not completely understood, representing a substantial obstacle to the anticipated clinical benefit of adoptive immunotherapy. Despite technologies allowing manipulation and even customization of T-cells before infusion into patients (3), providing adequate survival signals to T-cells after transfer has been challenging. Some success has been achieved by coadministration of the
prosurvival cytokine IL-2, but results have been limited for most patients (6, 44). IL-2 had no impact on the survival of transferred T-cells in our system, nor did the provision of inflammatory mediators. These and other efforts to prevent T-cell deletion suggest that positive survival signals alone in the presence of potentially numerous negative regulatory influences are not sufficient to promote long term persistence or efficacy of adoptively transferred T-cells. Instead, inhibiting negative signaling pathways may provide the appropriate survival and differentiation instructions. Indeed, despite potent signals leading to Bim-mediated apoptosis in vivo, our results showed that blocking ligation of PD-1 and CTLA4 prevented deletion of adoptively transferred T-cells. Additional blockade of LAG3 had only a modest impact on T-cell survival, but was indispensable for generation of robust effector functions, revealing the nonredundant nature of these regulatory pathways and their cooperative potential for immunotherapy.

The ability of antibody blockade to “release the brakes” on T-cell responses against cancer has been clinically successful for anti-CTLA4 (ipilimumab), and generated enthusiasm for exploring blockade of other regulatory receptors such as PD-1 and PDL1 in patients (45–48). Comparatively, the immunobiology of LAG3 is less certain, but LAG3 expression on T-cells has now been linked to compromised antitumor immune responses in patients with ovarian cancer (23). So far though, blockade antibodies have only been tested clinically as monotherapies. Our data strongly support the idea that combination blockade is likely to yield the most dramatic antitumor responses, but caution for such approaches should be stressed as some immune-related toxicity has been reported for these immune modulating drugs (46–48).

Previous reports have shown how regulatory receptor blockade can influence T-cell proliferation and antiviral activity. However, a role for these negative regulatory pathways during deletion and differentiation of T-cells following adoptive cellular therapy for cancer has not been reported. Here, the induction of apoptosis and subsequent deletion of transferred tumor/self-reactive T-cells was prevented and effector function rescued even within the tolerizing environment by disrupting negative costimulatory signaling. Recent studies have suggested that tolerant CD8+ T-cells, while susceptible to transient rescue, ultimately regain a tolerant phenotype due to epigenetic factors the regulate permanent gene expression profiles associated with tolerance (49). Here, blockade treatment resulted in long-lived and fully responsive tumor/self-reactive T-cells within the tolerizing environment, suggesting that attenuation of negative costimulatory signaling early during priming prevented such epigenetic changes from being established. This result may also indicate that by blocking negative signaling during priming, responding T-cells are...
actually stably prevented from being tolerized, possibly by establishing alternative epigenetic changes associated with maintenance of function, but this has yet to be shown experimentally.

Our work shows that the biologic outcomes dictated by different negative receptor pathways are both distinct and complimentary. Inhibition of the PD-1/CTLA4 pathways was far more effective than either antibody individually at preventing Bim-dependent T-cell deletion, whereas additional inhibition of the LAG3 pathway was essential for inducing vigorous expansion and effector function in surviving T-cells and for optimal immunotherapy of disseminated leukemia. This is in concert with studies of exhausted T-cells during chronic viral infections in which the severity of the exhausted phenotype was directly related to the number and type of regulatory receptors expressed on virus-specific CD8+ T-cells (50). Therefore, it is likely that adoptive T-cell transfer and antibody blockade will prove most effective when multiple regulatory pathways are targeted and potentially used in combination with existing chemo or radiotherapy techniques, as these approaches are not mutually exclusive and together may yield synergistic cancer treatments in the future.

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

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