Microenvironment and Immunology

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 deletional 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 adoptively 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. Cancer Res; 73(2); 605–16. ©2012 AACR.

Introduction

Adoptive 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 opportunities (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 naive 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

AlbGag 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–/– TCRGag transgenic 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 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 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.

Adoptive T-cell transfer

Gag-specific T-cells were isolated from spleens and lymph nodes of Rag–/– TCRGag donors. Whole-cell suspensions containing 3 × 10^6 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 × 10^6 FBL cells intraperitoneally 3 days before T-cell transfer. To provide an inflammatory environment, B6 recipients were infected with 3 × 10^7 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 Cytotox/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 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 FACScantio 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 × 10^7 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 × 10^6 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 with 0.5 to 1 × 10^6 Gag or Ova peptide-pulsed B6 target cells.
intravenously (as earlier) with FBL tumor transduced to express enhanced GFP (FBLGF6). Hair was shaved around the abdomen, and animals anesthetized (2.5% isoflurane, 0.25 L/min) and imaging using an IVIS Spectrum (Xenogen). Images were analyzed with Live Image v3.1 software (Caliper Live 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).

![Image of T-cell analysis](image-url)
but were still deleted in Alb:Gag recipients in which expression of the tumor antigen was shared in healthy self-tissues—recapturing 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 PDL1 (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 CD44/CD62L 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 CD44$^{hi}$ CD62L$^{lo}$ phenotype induced by self-antigen were nearly completely deleted by day 8, whereas CD44$^{lo}$CD62L$^{hi}$ cells activated by FBL persisted (Fig. 1F).

Expression of a CD44$^{hi}$CD62L$^{lo}$ profile has been associated with differentiation toward effector CTL (13, 33, 34), suggesting that proliferating T-cells in Alb:Gag mice, which remained CD62L$^{lo}$, 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$^+$ (CFSE$^{hi}$) and control (CFSE$^{lo}$) 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 Bim$^{WT}$ 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, 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-

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>live</sup> or CFSE<sup>high</sup>, respectively. Target cell frequency was assessed 20 hours later, and is shown as histograms with the percentage of total Thy1.1<sup>+</sup> cells inset above the indicated regions. B, the ratio of CFSE<sup>low</sup>:CFSE<sup>high</sup> target cells is graphed with the average ratio in unimmunized B6 recipients set to a value of 1.0 for comparison. Data in the graphs were pooled from 3 independent experiments each with triplicate mice per group. Error bars represent SEM with P-values between significantly different groups and nonstatistically significant (ns) groups indicated. C, total cells from recipient lymph nodes (LN) and spleen were stimulated overnight, and production of IFN-γ and TNF by Thy1.1<sup>+</sup> CD8<sup>+</sup> cells assessed 18 hours later by intracellular cytokine staining. Inset numbers within contour plots are the percentage of total Thy1.1<sup>+</sup> cells in each quadrant. Results are consistent with 3 separate experiments.

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 cancer immunotherapy (18, 20–22). We evaluated if persistence of adoptively transferred T-cells was influenced in Alb:Gag mice receiving blockade of PD1 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 report (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 PD1 and CTLA4 blockade, we examined surface expression of known negative regulatory receptors on T-cells after infusion into Alb:Gag hosts. Compared with naïve T-cells from B6 recipients, those remaining in Alb:Gag mice displayed elevated levels of both PD1 and CTLA4 (Fig. 3C), but PD1 expression was reduced following PD1/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 PD1/CTLA4 blockade (Fig. 3C). Thus, inhibition of negative signaling via blockade of PD1 and CTLA4 reduced expression of the regulatory receptors PD1 and LAG3 on adoptively transferred T-cells,

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. 5B). 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**

CTLA4 and PD-1 regulate deletion of adoptively transferred CD8<sup>+</sup> T-cells. The frequency of transferred T-cells in our system (Supplementary Fig. 5B). 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.
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.
Persisting T-cells in mice receiving either double- or triple-blockade displayed an effector-like CD44<sup>high</sup> CD62L<sup>low</sup> phenotype (Fig. 3D). This hint at a restored effector phenotype was borne out upon examination of <i>in vivo</i> killing and <i>ex vivo</i> 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 <i>in vivo</i> effector function in recipients with leukemia, Gag-specific CD8<sup>+</sup> 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
tumor-bearing recipients, peptide-pulsed CFSE-labeled target cells were infused, and target cell frequency assessed 20 hours later. As before, transferred Gag-reactive T-cells displayed enhanced killing of Gag target cells in triple-blockaded recipients when compared with those from untreated and even double-blockade–treated AlbGag mice (Fig. 4A). This increased lytic activity could have been due to either quantitative or qualitative differences (or both) in the T-cell response induced by triple-blockade treatment. Indeed, triple-blockade resulted in greater expansion of responding T-cells when transferred into tumor-bearing AlbGag recipients compared with double-blockade (Fig. 4B). Triple-blockade also induced a higher frequency of T-cells with the capacity to produce the effector cytokine IFN-γ (Fig. 4C), indicating that a more differentiated effector T-cell population had been generated. The lack of a robust TNF response by T-cells treated with either blockade regimen (Fig. 4A and Supplementary Fig. S6) may be attributed to fewer multifunctional T-cells being generated—a reported limitation of anti-CTLA4 therapy (43). Despite elevated effector activity, no autoimmunity was detected in blockaded-treated mice as assessed by liver enzymes in serum, which were indistinguishable from untreated mice (data not shown). These data show that attenuation of LAG3 signaling results in a larger and more functional peak response by transferred T-cells, suggesting this strategy may be more effective than double-blockade treatment in a cancer immunotherapy setting.

Combining adoptive T-cell transfer and antibody blockade enhances survival of mice with disseminated leukemia

To determine if enhanced T-cell persistence and effector function following blockade treatment could be translated into improved adoptive immunotherapy for cancer, AlbGag mice bearing an established and trackable FBLGFP leukemia received adoptive T-cell transfers with or without blockade therapy. A, anesthetized recipients treated with isotype control were imaged in triplicate on day 16 after tumor inoculation and compared with a no-tumor control. B, internal tissues from a representative isotype control-treated recipient were analyzed on day 16. C, corresponding recipients treated with double-blockade or triple-blockade were also imaged on day 16. Data are representative of 3 independent experiments. D, survival of leukemia-bearing (GFP-negative) AlbGag recipients given T-cell immunotherapy was assessed following treatment with isotype control (thick gray line), double-blockade (dashed line), or triple-blockade (black line) during the shaded time period. The graph displays pooled data from 4 separate experiments, showing percentage survival (y-axis) over a period of 100 days (x-axis) with n values depicting the number of total mice per treatment group and P values indicated for the bracketed groups. The importance of both the qualitative and quantitative responses induced by triple-blockade was further evident in the survival curve for mice receiving T-cell transfer and double-blockade. While the majority of these mice survived, those that died mostly did so before day 35, whereas, mice receiving T-cell transfer and triple-blockade all survived this early phase (Fig. 5D). This is consistent with the idea that the advantage of triple-blockade (i.e., the addition of LAG3 blockade) was the induction of early antitumor T-cell activity such as a larger and more functional peak response, as suggested by the results in Fig. 4.

One of the unexpected outcomes of triple-blockade therapy was the ability to provide a survival benefit to AlbGag 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<sup>+</sup> and CD8<sup>+</sup> 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 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<sup>+</sup> 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 PD-L1 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 combinations are 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.

References

www.aacrjournals.org Cancer Res; 73(2) January 15, 2013 615

Overcoming Negative Regulation of T-Cells for Immunotherapy

Authors’ Contributions
Conception and design: M.M. Berrien-Elliott, H. Yagita, R.J. DiPaolo, R.M. Teague
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Berrien-Elliott, S.R. Jackson, J.M. Meyer, C.J. Rouskey, T.-L.M. Nguyen, R.M. Teague
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): M.M. Berrien-Elliott, C.J. Rouskey, P.D. Greenberg, R.J. DiPaolo, R.M. Teague
Writing, review, and/or revision of the manuscript: M.M. Berrien-Elliott, S.R. Jackson, H. Yagita, P.D. Greenberg, R.M. Teague
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Meyer, H. Yagita, R.M. Teague
Study supervision: H. Yagita, R.M. Teague

Acknowledgments
The authors thank Joy Edick and Sherr Koehm in the Saint Louis University School of Medicine flow cytometry core facility for assistance and discussion with experimental techniques, data collection, and expert analysis.

Grant Support
R.M. Teague is supported by grant R01AI087784 from the U.S. NIH/National Institute of Allergy and Infectious Disease, and by a Cancer Research Institute Investigator Award. P.D. Greenberg is supported by grant CA133084 from the U.S. NIH/National Cancer Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 31, 2012; revised October 15, 2012; accepted November 10, 2012; published OnlineFirst November 27, 2012.

Published OnlineFirst November 27, 2012; DOI: 10.1158/0008-5472.CAN-12-2179
29. Ohlen C, Kalos M, Hong DJ, Shur AC, Greenberg PD. Expression of a tolerizing tumor antigen in peripheral tissue does not preclude recovery of high-affinity CD8+ T-cells or CTL immunotherapy of tumors expressing the antigen. J Immunol 2001;166:2863−70.
Durable Adoptive Immunotherapy for Leukemia Produced by Manipulation of Multiple Regulatory Pathways of CD8+ T-Cell Tolerance

Melissa M. Berrien-Elliott, Stephanie R. Jackson, Jennifer M. Meyer, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-2179

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/11/27/0008-5472.CAN-12-2179.DC1

Cited articles  This article cites 50 articles, 28 of which you can access for free at: http://cancerres.aacrjournals.org/content/73/2/605.full#ref-list-1

Citing articles  This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/73/2/605.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.