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Reviving Function in CD4⁺ T Cells Adapted to Persistent Systemic Antigen

Magali Noval Rivas,* Kathleen Weatherly,* Marc Hazzan,* Benoit Vokaer,* Sarah Dreimer,* Florence Gaudray,* Michel Goldman,* Isabelle Salmon,* and Michel Y. Braun²*

In bone marrow-transplanted patients, chronic graft-versus-host disease is a complication that results from the persistent stimulation of recipient minor histocompatibility Ag (mHA)-specific T cells contained within the graft. In this study, we developed a mouse model where persistent stimulation of donor T cells by recipient’s mHA led to multiorgan T cell infiltration. Exposure to systemic mHA, however, deeply modified T cell function and chronically stimulated T cells developed a long-lasting state of unresponsiveness, or immune adaptation, characterized by their inability to mediate organ immune damages in vivo. However, analysis of the gene expression profile of adapted CD4⁺ T cells revealed the specific coexpression of genes known to promote differentiation and function of Th1 effector cells as well as genes coding for proteins that control T cell activity, such as cell surface-negative costimulatory molecules and regulatory cytokines. Strikingly, blockade of negative costimulation abolished T cell adaptation and stimulated strong IFN-γ production and severe multiorgan wasting disease. Negative costimulation was also shown to control lethal LPS-induced toxic shock in mice with adapted T cells, as well as the capacity of adapted T cells to reject skin graft. Our results demonstrate that negative costimulation is the molecular mechanism used by CD4⁺ T cells to adapt their activity in response to persistent antigenic stimulation. The effector function of CD4⁺ T cells that have adapted to chronic Ag presentation can be activated by stimuli strong enough to overcome regulatory signals delivered to the T cells by negative costimulation. The Journal of Immunology, 2009, 183: 4284–4291.

Minor histocompatibility Ags (mHA)³ are thought to be key molecules in the graft-versus-tumor effect after bone marrow stem cell transplantation (1). Recipient’s mHA are considered to be the molecular targets recognized by donor T cells contained within the graft that promote this effect. However, mHA expression is not an exclusive feature of transformed malignant cells and normal healthy tissues do express mHA. How this situation might impair the capacity of T cells to promote antileukemia immunity is unknown. T cells that are chronically stimulated by their cognate Ag are known to develop the capacity to tailor, or adapt, their activation threshold to the strength of ambient Ag presentation (2–4). Because of apparent functional dysfunctions such as lack of IL-2 secretion and poor proliferation upon subsequent antigenic challenge, T cell adaptation has very often been confused with T cell anergy. There are, however, major functional differences between anergized T cells and T cells adapted to persistent Ag. Contrary to anergized T cells, which can be induced by a single administration of a high dose of soluble peptide Ag or chemically modified Ag-pulsed APC, adapted CD4⁺ T cells require in vivo Ag persistence to maintain their unresponsive state and adaptation to Ag is reversible upon T cell removal from the Ag-bearing host (3). Second, adaptation cannot be reversed by IL-2 because adapted T cells do not express high-affinity IL-2R (3). Third, whereas anergic T cells have impaired activation of the Ras signaling pathway, adapted CD4⁺ T cells show normal Ras activation but disabled TCR proximal signals such as reduced Zap70 kinase activity (5). Importantly, despite impaired Ag-mediated signaling, adapted CD4⁺ T cells keep the capacity to develop effector function after stimulation by higher levels of TCR ligands (4). Thus, contrary to T cell anergy, T cell adaptation could allow for the persistence of T cells with self-specificities that are potentially useful against pathogens and malignancies.

In this study, we report a model where persistent stimulation by recipient mHA led to the extensive expansion of specific CD4⁺ T cells in lymphopenic recipients. However, the T cells showed a higher activation threshold and were unable to mediate in vivo immune tissue damages and to produce effector cytokines and proliferate following subsequent in vitro antigenic challenge. Adapted CD4⁺ T cells also expressed high cell surface levels of negative costimulatory molecules. Disruption of negative costimulation led to the dramatic increase in the number of mHA-specific CD4⁺ T cells and the development of a severe form of T cell-mediated immune disease. We also observed that the presence of adapted mHA-specific CD4⁺ T cells conditioned the intensity of LPS-induced septic shock in mHA-expressing recipients. Combining negative costimulation blockade along with a sublethal dose of LPS induced a fast lethal wasting disease in recipients with Ag-adapted T cells. Moreover, blockade of negative costimulation stimulated skin graft rejection by adapted T cells. Taken together, our results demonstrate that chronic exposure to Ag allows the survival of Ag-specific CD4⁺ T cells whose effector function can be revived by modulating positive/negative activation signals.

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3 Abbreviations used in this paper: mHA, minor histocompatibility Ag; GVHD, graft-versus-host disease; PD-1, programmed death 1; PD-L1, PD-1 ligand 1.

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Materials and Methods

Animals

Female RAG2<sup>−/−</sup> Marylin mice, transgenic for a TCR (TCRAV1.1, TCRBV6) specific for the male H-Y peptide NAGFNSRANSRSSR presented by I-A<sup>d</sup>, have been previously described (6). RAG2<sup>−/−</sup> mice were obtained from The Jackson Laboratory. B, T, and NK cell-deficient lymphoprogenic mice were H-2<sup>b</sup> or H-2<sup>d</sup>, RAG2<sup>−/−</sup> IL-2Rγ<sup>−/−</sup> mice (provided by J. Di Santo, Pasteur Institute, Paris, France). B6 mice were from Charles River. All mice used in this study were between 8 and 10 wk old and were bred under specific pathogen-free conditions at the Institute for Medical Immunology (Gosselies, Belgium).

T cell adoptive transfer and induction of graft-versus-host disease (GVHD)

For adoptive transfer experiments, purified transgenic TCRBV6<sup>6</sup>CD4<sup>+</sup> T cells (see below) were injected i.p. (1 × 10<sup>6</sup> cells/recipient) into male and female recipients. In some experiments, female recipients were immunized i.p. with 10<sup>7</sup> irradiated (20 Gy) spleen cells from B6 male donors. 

T cell purification

Spleen cells were incubated with FITC-conjugated anti-mouse TCRBV6 Ab (BD Biosciences), then with anti-FITC magnetic beads (Miltenyi Biotech) and sorted by magnetic sorting according to the manufacturer’s protocol. T cell purity assessed by flow cytometry was >90%.

Flow cytometry

The following anti-mouse mAbs were purchased from BD Biosciences: PB-conjugated anti-CD4, FITC- or PE-conjugated anti-CD4, FITC-conjugated anti-CD64, VEGF-conjugated anti-ICOS, APC-conjugated anti-CD3, APC-conjugated anti-CD52, PE-conjugated anti-CD62L, PE-conjugated anti-CTLA-4, PE-conjugated anti-CD40L, PE-conjugated anti-programmed death 1 (PD-1), PE-conjugated anti-PD-1 IgG1 (BD Biosciences), PE-conjugated anti-CD80 Abs, APC-conjugated anti-mouse Foxp3 Ab was obtained from eBioscience. Streptavidin-PE was purchased from BD Biosciences. Immunostained cell samples were analyzed on a CyAn ADP LX 9 Color with Summit version 4.3 software (DakoCytomation).

T cell stimulation and cytokine production assays

Purified T cells (200 × 10<sup>4</sup> cells/well) were cultured with anti-CD3/CD28-coated microbeads for 17 h or with T cell-depleted male H-2<sup>b</sup> spleen cells at the indicated ratios for 3 days in 96-well plates. ELISA technique was used to measure the production of IL-2 and IFN-γ in culture supernatants according to the manufacturer’s protocol (R&D Systems). In some experiments, blocking anti-PD-L1 (clone 10F.9G2; BioXcell), anti-PD-1 (clone 43J; BioXcell), anti-CTLA-4 (clone UC10-4F10-11; BioXcell), or control Abs (clones LTF-2 and hamster Ig; BioXcell) were added to each reaction. Isotype control IgG2b (clone LTF-2; BioXcell) injections were also conducted. Blockade of CTLA-4 was achieved by injecting neutralizing anti-CTLA-4 Abs (clone UC10-4F10-11, 200 µg per i.p. injection every 3 days; BioXcell) or control Abs (hamster Ig; BioXcell).

In some experiments, mice were injected i.p. with 200 µg of LPS from Escherichia coli (serotype 011:B4, Sigma-Aldrich). Three hours after LPS injection, blood samples were collected and serum cytokine levels were assessed by ELISA (R&D Systems).

Skin grafting

Tail skin grafting was conducted as previously described (6). Grafts showing >50% necrosis were considered rejected.

Histology

Histology was performed on tissue sections obtained from different organs and stained with H&E after paraffin embedding.

Statistical analysis

The log-rank test was used to assess survival in adoptive transfer recipients. For cytokine secretion and cell proliferation, the Mann-Whitney U non-parametric test was used. For cell counts, an unpaired t test was used to determine the degree of significance.

Results

H-Y-specific CD4<sup>+</sup> T cells develop a state of functional unresponsiveness in nonirradiated lymphoprogenic male recipients

The injection of I-A<sup>d</sup>-restricted H-Y-specific monoclonal T cells in lymphoprogenic H-2<sup>b</sup> male recipients promoted the development of a mild GVHD (less than grade 1). However, transgenic T cells were able to expand and could be detected in many organs (Fig. 1 and FIGURE 1. TCR-transgenic anti-male CD4<sup>+</sup> T cells undergo Ag-driven expansion in lymphoprogenic male recipients. TCR-transgenic anti-male CD4<sup>+</sup> T cells were transferred into B, T, and NK-deficient or male or female recipients. A, Percentage of transgenic TCRBV6 CD4<sup>+</sup> T cells in nonirradiated female and male recipients 28 days after T cell injection as determined by flow cytometry analysis. B, Number of transgenic TCRBV6 CD4<sup>+</sup> T cells in the spleen of nonirradiated male or female H-2<sup>b</sup> or H-2<sup>d</sup> recipients. Some female H-2<sup>b</sup> recipients were immunized on day 1 after T cell transfer with irradiated male B6 spleen cells. *, p < 0.01 and **, p < 0.001. injected i.p. every 3 days. Isotype control IgG2b (clone LTF-2; BioXcell) injections were also conducted. Blockade of CTLA-4 was achieved by injecting neutralizing anti-CTLA-4 Abs (clone UC10-4F10-11, 200 µg per i.p. injection every 3 days; BioXcell) or control Abs (hamster Ig; BioXcell).

In some experiments, mice were injected i.p. with 200 µg of LPS from Escherichia coli (serotype 011:B4, Sigma-Aldrich). Three hours after LPS injection, blood samples were collected and serum cytokine levels were assessed by ELISA (R&D Systems).
supplemental Fig. S1). This expansion was Ag driven because transfer into H-2b male recipients that did not express the correct MHC molecules for peptide presentation to the T cells, or into immunodeficient H-2b females, did not lead to T cell extensive expansion (Fig. 1). Moreover, T cell expansion required Ag persistence as immunization of T cells 1 day after transfer into immunodeficient female recipients led to poor T cell expansion (Fig. 1). It has been proposed that persistent Ag exposure could induce a state of specific unresponsiveness in Ag-specific T cells often referred to as T cell Ag adaptation or desensitization (2–4). Thus, it was also possible that failure to induce severe GVHD might have resulted from T cell adaptation to persistent mHA. In vitro stimulation with anti-CD3/CD28 Abs did not induce proliferation and IL-2 production in transgenic T cells isolated 28 days after adoptive transfer into male recipients (supplemental Fig. S2). Thus, T cells that had expanded under persistent antigenic stimulation in lymphopenic hosts appeared to have developed a state of functional unresponsiveness similar to that previously described as T cell adaptation (3, 4). Whereas unresponsive T cells did not produce IL-2, they secreted IFN-γ in response to anti-CD3 stimulation (see supplemental Fig. S2). These observations confirmed those previously reported by Schwartz and colleagues (7) for T cells subjected to immune adaptation in T cell-depleted hosts. In our hands, adapted T cells did not secrete IL-17a after anti-CD3 stimulation (supplemental Fig. S2).

Ag-adapted CD4+ T cells develop a Th1 phenotype and constitutively express inhibitory molecules that regulate the activity of Th1 cells

We further investigated how persistent in vivo exposure to Ag could influence the phenotype and function of T cells by comparing ex vivo the gene expression profile of naive and Ag-adapted T cells. As presented in Fig. 2, Ag-adapted T cells expressed higher mRNA levels of many Th1-associated proteins, including IFN-γ (8), T-bet (9), Eomes (10), IL-12Rβ1 (11, 12), IL-12Rβ2 (11, 12), CXCR3 (13), CCR5 (14), CD95L (14), and TWIST1 (15). This observation confirmed the idea that stimulation by systemic Ag favored the differentiation of Th1 CD4+ T cells. Interestingly, several molecules responsible for negative costimulation of T cells (16), including HVEM, CD152, ICOS, and PD-1, were up-regulated in adapted T cells. The most striking difference was observed for the expression of the PD-1 gene. Although PD-1 mRNA could not be detected in naive T cells, it was estimated that adapted T cells contained at least 1010 times more copies of PD-1 mRNA. We also noticed the up-regulation of IL-10 and IL-21 expression. Both cytokines have been described for their inhibitory activity on Th1 cells and IFN-γ production (10, 17).

T cell phenotype was also analyzed by flow cytometry (Fig. 2). Again, among cell surface proteins expressed by adapted T cells, PD-1 was the one whose expression distinguished the most naive and adapted CD4+ T cells. PD-1 was indeed constitutively expressed by adapted CD4+ T cells and in vitro anti-CD3/CD28 stimulation increased even further the intensity of PD-1 expression. On the contrary, cell surface PD-1 was not seen on naive T cells or T cells immunized 1 day after transfer into immunodeficient female recipients and subsequent TCR-mediated activation did not induce its expression (Fig. 2 and supplemental data in Fig. S3). Thus, considering that the interaction between PD-1 and its ligand PD-L1 is involved in the inhibition of T cell responses, we hypothesized the participation of PD-1/PD-L1 in the process of T cell adaptation to persistent antigenic stimulation. Moreover, the possibility of a bidirectional inhibitory interaction between PD-L1 and CD80 expressed on T cells was recently described by Sharpe and coworkers (18) for the control of T cell responses. Therefore, we also analyzed the expression of CD80 and PD-L1 on naive and adapted T cells (Fig. 2). Adapted T cells constitutively expressed high levels of CD80 and PD-L1. Naive T cells, however, expressed lower levels of PD-L1 and CD80. Their activation by CD3/CD28-mediated stimulation, though increasing PD-L1 expression, did not modify significantly the expression of CD80. Thus, if one considers the function of PD-1/PD-L1 and CD80/PD-L1 interactions in the inhibition of T cell responses, PD-L1 could represent an important mediator for the maintenance of immune unresponsiveness that characterized Ag-adapted T cells.

We also analyzed by flow cytometry the expression of other molecules known to play a role in the costimulation of T cells. In vitro CD3/CD28-mediated activation also up-regulated ICOS, CD152, and CD40L expression at the surface of both naive and adapted T cells (Fig. 2). As also depicted in Fig. 2, whereas, once activated, naive T cells up-regulated cell surface expression of activation markers CD25 and CD69; adapted T cells had impaired expression of these molecules either before or after activation, confirming previous observations made by Tanchot et al. (3). Naive and adapted T cells could also be differentiated by their expression of the L-selectin CD62L. Naive CD4+ T cells expressed CD62L at their surface and, following activation, some of the T cells downregulated their expression of CD62L (Fig. 2). In contrast, none of the adapted T cells expressed CD62L and CD3/CD28-mediated activation did not modify this pattern of expression (Fig. 2). Adapted T cells, as opposite to naive T cells, however, were CD44 positive. We did not observe Foxp3 expression in any T cell subsets analyzed in this study. Thus, Ag-adapted T cells exhibited a phenotype very similar to that expressed by memory effector CD4+ T cells.

Blocking negative costimulation stimulates adapted CD4+ T cells for the Ag-specific secretion of IFN-γ and promotes the development of severe GVHD

Engagement of negative costimulatory molecules at the surface of T cells regulates activation and effector function. Adapted T cells did not respond to anti-male stimulators even at high responder:stimulator ratios (Fig. 3), demonstrating that anti-male CD4+ T cells had adapted their activation threshold to male stimulator cells. However, blocking PD-L1/PD-1 interaction strongly stimulated the specific production of IFN-γ by adapted anti-male T cells (Fig. 3). This response was Ag specific because blockade in cocultures of adapted T cells and female stimulators did not trigger IFN-γ secretion (data not shown). However, IL-2 secretion in response to male Ag stimulation was not restored by PD-L1/PD-1 blockade (data not shown), suggesting that the effect observed after blocking PD-L1/PD-1 interaction was not the result of reversed anergy but rather the lifting of specific inhibitory signals on IFN-γ synthesis. Blockade of CTLA-4 with specific Abs weakly stimulated the production of IFN-γ by adapted T cells (Fig. 3). Again, the production of IL-2 was not induced by anti-CTLA-4 treatment. Combining anti-CTLA-4 and anti-PD-1 Abs had an additive effect on abrogating the inhibition on IFN-γ secretion but did not restore IL-2 secretion (Fig. 3 and data not shown).

We then analyzed the effect that PD-L1/PD-1 or CTLA-4/B7 blockade could have on T cell-mediated GVHD. Immunocompromised male recipients were reconstituted with anti-male CD4+ T cells. Eighteen days later, once T cell adaptation was established, neutralizing anti-mouse PD-L1 or anti-mouse CTLA-4 or isotype control Abs were injected. After 10 days of treatment, animals were sacrificed and organs taken for analysis. As illustrated in
Fig. 3, blocking PD-L1 stimulated a dramatic increase in the number of splenic T cells. T cell number, however, was slightly increased after anti-CTLA-4 treatment. Histology revealed an aggressive form of GVHD with severe tissue damages in anti-PD-L1-treated animals (Fig. 3). Extensive periportal inflammation along with granuloma formation and large areas of coagulative necrosis were observed in the liver (Fig. 3). High mitotic index, indicative of active hepatic regeneration, characterized anti-PD-L1-treated animals (Fig. 3). Anti-PD-L1 treatment also induced an important inflammation of the kidney with tubular necrosis and glomerulonephritis (Fig. 3). Focal cellular infiltration of the skin and destruction of hair follicles with granuloma formation were apparent in anti-PD-L1-treated mice (Fig. 3). Cellular edema and inflammatory ulcers could also be seen in the colon (Fig. 3). Histology of organs isolated from anti-CTLA-4-treated animals did not reveal signs of inflammation. Thus, negative costimulation blockade, more particularly one directed toward PD-L1/PD-1, converted a quiescent T cell inflammation of most organs into an aggressive immunopathology.
Neutralizing anti-PD-L1, anti-CTLA-4, anti-PD-1, or control isotype Abs were added to the assay as indicated. Data are plotted as medians ± interquartile range (n = 4 mice/group). B. Number of transgenic CD4+ T cells in the spleen of male mice reconstituted 28 days earlier. Animals received blocking anti-PD-L1 (n = 7 mice; ▲), or isotype control (n = 7 mice; ○) or no (n = 8 mice; ●) Abs, or anti-CTLA-4 (n = 8 mice; ●), or isotype control (n = 5 mice; □) Abs for 10 days from day 18 after T cell injection (t = 0). *, p < 0.001. C. Histology of liver (a, e, i, and j), skin (b, f, and k), kidney (c and g), and intestine (d and h) of male mice reconstituted 28 days earlier with anti-male transgenic CD4+ T cells. The liver of anti-PD-L1-treated animals showed extensive coagulative necrosis (e; original magnification, ×100) as well as portal granuloma characterized by lymphocytes, epitheloid cells, giant cells (i; original magnification, ×400), and pericentral regenerative changes with mitosis (green arrow, j; original magnification, ×600). In the skin, anti-PD-L1 treatment was characterized by focal perivascular and perifascicular lymphocytic infiltration (f; original magnification, ×200) and perianal granulomatous reaction (k; original magnification, ×400).

**FIGURE 3.** Blockage of negative costimulation stimulates IFN-γ secretion, in vivo proliferation by Ag-adapted CD4+ T cells, and the development of severe T cell-mediated wasting disease. A. IFN-γ secretion by purified Ag-adapted CD4+ T cells stimulated with T cell-depleted I-Ak male spleen cells. Neutralizing anti-PD-L1, anti-CTLA-4, anti-PD-1, or control isotype Abs were added to the assay as indicated. Data are plotted as medians ± interquartile range (n = 4 mice/group). B. Number of transgenic CD4+ T cells in the spleen of male mice reconstituted 28 days earlier. Animals received blocking anti-PD-L1 (n = 7 mice; ▲), or isotype control (n = 7 mice; ○) or no (n = 8 mice; ●) Abs, or anti-CTLA-4 (n = 8 mice; ●), or isotype control (n = 5 mice; □) Abs for 10 days from day 18 after T cell injection (t = 0). *, p < 0.001. C. Histology of liver (a, e, i, and j), skin (b, f, and k), kidney (c and g), and intestine (d and h) of male mice reconstituted 28 days earlier with anti-male transgenic CD4+ T cells. The liver of anti-PD-L1-treated animals showed extensive coagulative necrosis (e; original magnification, ×100) as well as portal granuloma characterized by lymphocytes, epitheloid cells, giant cells (i; original magnification, ×400), and pericentral regenerative changes with mitosis (green arrow, j; original magnification, ×600). In the skin, anti-PD-L1 treatment was characterized by focal perivascular and perifascicular lymphocytic infiltration (f; original magnification, ×200) and perianal granulomatous reaction (k; original magnification, ×400).

**Adapted CD4+ T cells promote lethal LPS-induced shock**

It is well established that T cell activation requires a non-Ag-specific second signal to be delivered by the APCs (19, 20). We reasoned that failure for Ag-adapted T cells to mediate severe disease in immunocompromised animals might also reflect the fact that chronic Ag stimulation occurred without second signal. To investigate this possibility, we reconstituted male or control female mice with 1 × 10^6 anti-male CD4+ T cells. Twenty-eight days later, TLR4 ligand (purified bacterial LPS), was injected and, after 3 h, animals were bled and sera tested for their content of cytokines known to be secreted in response to TLR4 stimulation. As seen in Fig. 4, LPS injection induced the production of IL-12, IFN-γ, and TNF-α. Cytokine production appeared, however, specific to the presence of T cells since it was poorly detected in the serum of nonreconstituted animals. Moreover, the low production of these cytokines in reconstituted female recipients indicated that T cell exposure to persistent Ag potentiated cytokine production following LPS injection (Fig. 4). On the contrary, IL-6 production after LPS stimulation was detected in both reconstituted and nonreconstituted hosts (Fig. 4). Taken together, these results supported the conclusion that the presence of Ag-adapted CD4+ T cells did not prevent APCs to respond to TLR stimulation and produce proinflammatory cytokines, but directly conditioned the intensity of the response.

Apart from a transient increase in perportal inflammation (data not shown), there was no sign of enhanced illness after LPS injection in lymphopenic male mice reconstituted with H-Y-specific CD4+ T cells. This suggested that T cells present in these mice were not being activated to trigger lethal shock. Thus, providing second signal through LPS injection along with blocking PD-L1 activity could increase the symptoms of illness. As shown in Fig. 4, the majority of mice with adapted T cells that received anti-PD-L1 Abs died shortly after LPS injection. This observation again supported a major role played by PD-L1-mediated inhibition in controlling in vivo the activity of Ag-adapted CD4+ T cells. The role played by T cell-negative costimulation in the regulation of LPS-stimulated response was further investigated in vitro. Spleen cells isolated from animals with Ag-adapted T cells produced low amounts of IFN-γ following LPS stimulation. However, combining LPS treatment with blockade of PD-1/PD-L1 interaction stimulated a strong production of IFN-γ (Fig. 4), confirming our in vivo data that negative costimulation controlled the activity of adapted T cells.

**Negative costimulation blockade stimulates skin graft rejection by adapted T cells**

mHA expression can be expected to be shared by transformed malignant cells and normal healthy tissues. Therefore, immunoregulation induced by chronic Ag exposure of T cells specific to recipient’s mHA expressed at the surface of tissue parenchyma cells might impair their capacity to reject mHA-positive tumor cells and to mediate the graft-versus-leukemia effect, often observed in bone marrow-transplanted patients. To investigate this possibility, we tested the capacity of Ag-adapted H-Y-specific T cells to reject male skin grafts. As depicted in Fig. 5, immunodeficient female recipients reconstituted with anti-male CD4+ T cells rejected acutely male skin grafts. On the contrary, none of the male recipients reconstituted with anti-male CD4+ T cells were capable of mounting a rejection response and male skin grafts survived indefinitely on these recipients without signs of rejection (Fig. 5)
Given our observations that PD-L1 blockade induced severe T cell-mediated chronic GVHD as well as lethal LPS shock in mice carrying adapted T cells, we decided to analyze its effect on the survival of male skin grafted on this type of recipients. Fig. 5 shows that administrating anti-PD-L1 Abs stimulated the rejection of ~60% of male skins grafted onto male mice with adapted anti-male CD4 T cells (see also supporting information in Fig. S4). Thus, PD-L1 controlled also the capacity of adapted T cells to mediate the rejection of grafted skin.

**Discussion**

Adaptation to Ag is a process by which T cells become desensitized when Ag stimulation persists following an initial immune response in vivo. In fact, it has been shown that adapted T cells are characterized by their ability to calibrate their responsiveness to a persistent Ag (2–4). In this study, we show for the first time that negative costimulation was critical for CD4 T cells to maintain their adaptation to systemic mHA and that negative costimulation blockade by Ab treatment converted adapted T cells into aggressive effector cells. Importantly, our results also demonstrated that negative costimulation did not prevent the differentiation of Th1 effector cells, but rather appeared to limit the effector function of adapted CD4 T cells. This was more particularly seen for the production of IFN-γ. Interestingly, IFN-γ is known to stimulate the expression of negative costimulatory molecules, such as PD-L1 or B7 molecules, by various non-T cells (21). Our study suggests that expression of these molecules could create a negative loop to control the activity of Th1 cells and the production of IFN-γ. Indeed, breaking this regulatory loop transformed mild chronic GVHD-like immunopathology into an acute wasting disease. The type of tissue lesions that developed after negative costimulation blockade, such as granuloma formation in the liver and glomerulonephritis, supported the idea that they were indeed primarily the work of IFN-γ activity (22–24).

We also observed that, although they were not able to respond to Ag presentation by T cell-deficient spleen cells, T cells adapted to persistent Ag were responsive to strong TCR-mediated signals and produced IFN-γ upon anti-CD3 stimulation. Moreover, by their presence in vivo, adapted T cells conditioned the intensity of the response to LPS injection. These observations supported the idea that chronically stimulated CD4 T cells were not anergic and could promote immune responses if activation signals were
sufficient to overcome the negative costimulatory pathways that mediated their adaptation to the persistence of cognate Ag. Moreover, our study also showed that following an Ag-driven initial expansion, CD4+ T cells under chronic antigenic stimulation persisted for a long period of time. Thus, chronic TCR stimulation combined with negative costimulation might represent the mechanism allowing the long-term survival of Ag-specific CD4+ T cells.

What is the mechanism responsible for the effect of LPS/costimulation pathway on adapted T cells? There is the possibility that adapted T cell-dependent LPS-induced toxic shock observed in our study could result from the direct stimulation of adapted T cells by LPS. Several articles have reported TLR expression by T cells (reviewed in Ref. 25). However, expression patterns of different TLRs often vary between studies and the role played by TLR molecules in T cell function remains to be clarified. Another possibility is that LPS-activated APCs, which express TLR4, could up-regulate at their surface positive costimulatory ligands (26), such as B7 or CD40 molecules, as well as secreted cytokines (IL-12), known to counteract negative regulatory signals and, in this way, would lower the activation threshold of adapted T cells. As a consequence of this, T cells would then be able to respond to persistent antigenic stimulation and produce IFN-γ. However, it is also possible that LPS-induced IFN-γ production might come from the direct stimulation of other cells than the adapted T cells themselves. Because IFN-γ production in response to LPS stimulation was conditioned by the presence in vivo of adapted T cells, one would then have to postulate that the activity of these non-T IFN-γ-producing cells be tightly controlled by adapted T cells. Experiments designed at studying these possibilities are currently being conducted. Finally, the observed capacity of adapted T cells to condition innate immune response to LPS suggest that stimulation of TLR signaling by opportunistic infection could trigger deleterious inflammatory reaction in bone marrow-transplanted patients (27). This hypothesis is also supported by the observation that bone marrow-transplanted mice with stable mixed blood chimerism exhibit a high sensitivity to LPS challenge (28, 29). It remains to be determined whether this situation results from the activity of T cells adapted to recipient’s mHA.

We also observed that T cell adaptation was mainly the result of PD-L1-mediated negative costimulation. PD-L1/PD-1 interaction has been shown to regulate various T cell-mediated autoimmune diseases, including experimental autoimmune encephalomyelitis, autoimmune cardiomyopathy, lupus-like disease, and type I diabetes in nonobese diabetic mice (30–33). In transplantation, absence of PD-L1 expression on cardiac tissue was shown to stimulate acute rejection of MHC class II Ag-disparate hearts (34). Blockade of PD-L1 also accelerated GVHD disease lethality in irradiated bone marrow recipients (35). PD-1/PD-L1 appeared also to be responsible for the induction and the maintenance of T cell unresponsiveness in an in vivo model of functional energy in TCR-transgenic T cells induced by high doses of antigenic peptide (36). The other prototype of CD28 family inhibitory receptors is CTLA-4 (CD152) (37). Although to a lesser extent, CTLA-4 expression was also constitutively up-regulated by Ag-adapted T cells and, therefore, the molecule could also participate in the phenomenon of T cell adaptation. The role played by CTLA-4 in the phenomenon of T cell functional unresponsiveness in vivo is well documented (38). It appears to be directly linked to the distribution of the tolerogenic Ag and whether Ag expression is tissue localized or systemic (39). On the one hand, CTLA-4 was critical for the induction of tolerance to tissue Ag and, in these conditions, self-Ag was found to be alone sufficient to initiate pathologic autoimmune in lymphocyte-depleted recipients reconstituted with self-Ag-specific CTLA-4-deficient CD4+ T cells (40). On the other hand, the same CTLA-4-deficient T cells transferred into systemic Ag-expressing mice became just as hyporesponsive as wild-type cells and adapted their response to the persistent presence of their Ag (41, 42). Thus, CTLA-4 does not appear to be as important for T cell adaptation to systemic Ags as it is for tolerance to tissue-restricted Ag. This is not the case for PD-L1-mediated inhibitory signals since our data clearly demonstrated PD-L1 inhibitory pathway as being particularly critical for the maintenance of T cell hyporesponsiveness to systemic antigenic stimulation. This difference could reflect the fact that induction of CTLA-4-dependent inhibition is restricted to tissue-draining lymphoid organs where tissue self-Ag is transported and presented to Ag-specific T cells by professional APC that express CTLA-4 ligands, whereas PD-L1-mediated pathway, due to PD-L1 wide expression on different cell types in many different organs, including lymphoid as well as nonlymphoid ones, maintains unresponsiveness at the effector phase of the immune response (43). This possibility is also supported by the observations that CTLA-4 targets directly the production of IL-2 needed for the initial expansion of Ag-specific T cells (44), whereas PD-L1 has been shown to be a potent inhibitor of IL-2 as well as IFN-γ, a cytokine preferentially synthesized by effector CD4+ T cells (45).

The question one often asks in view of our results is whether T cell adaptation still occurs in nonlymphopenic conditions. We inspired ourselves by the model system originally developed by Schwartz and colleagues (7) for Ag adaptation where Ag-specific naive CD4+ T cells were transferred into Ag-expressing hosts crossed onto the T cell-deficient background. Our results confirmed that these T cells, in the absence of other competing T cells, persisted for extended periods of time in the adapted state. Interestingly, in the initial experiment by Schwartz and colleagues (7) after transfer into a T cell-replete Ag-expressing host, naive CD4+ T cells were still able to adapt to the persistent Ag but their expansion and persistence were limited in both amplitude and time. It was concluded that the containment of systemic pathology required host T cell-mediated extrinsic regulatory mechanisms to synergize with the cell intrinsic adaptation process. Our study demonstrates that the molecular nature of T cell Ag adaptation does not entirely rely on factors intrinsic to the T cells, but also includes the contribution of extrinsic regulatory processes involving negative costimulation, particularly that involving the molecule PD-L1.

Our study also shows that T cells exposed to systemic Ag exhibit the phenotype of effector memory CD44+CD25−CD62L−CD4+ T cells. Effector memory CD4+ T cells transferred into irradiated recipients have been shown to mediate the rejection of retrovirally induced B cell lymphoma without inducing GVHD (46). Our data support the idea that PD-L1-mediated negative regulation could represent the mechanism by which mHA-specific effector memory CD4+ T cells are prevented to induce severe GVHD. However, we also show that the capacity of Ag-adapted CD4+ T cells to promote rejection of mHA-expressing allografts is also controlled by a PD-L1-dependent mechanism. Taken together, these results suggest that T cells specific to mHA expressed by both tumor cells and healthy tissues of bone marrow-transplanted recipients might not be able to mediate antileukemia effect. This is particularly relevant if one considers the major importance of PD-L1 tumor cell expression in the phenomenon of immune evasion (47).

In summary, the major findings of this study are that 1) negative costimulation is the molecular mechanism used by mHA-specific CD4+ T cells to adapt their activity in response to persistent antigenic stimulation and 2) the effector function of CD4+ T cells
that have adapted to persistent antigenic stimulation can be activated by stimuli strong enough to overcome regulatory signals delivered to the T cells by negative costimulation. These data contain important implications for our understanding of how chronically stimulated T cells are involved in the pathophysiology of GVHD in bone marrow-transplanted patients.

Disclosures

The authors have no financial conflict of interest.

References