Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling

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Lymphodepletion with total body irradiation (TBI) increases the efficacy of adoptively transferred tumor-specific CD8+ T cells by depleting inhibitory lymphocytes and increasing homeostatic cytokine levels. We found that TBI augmented the function of adoptively transferred CD8+ T cells in mice genetically deficient in all lymphocytes, indicating the existence of another TBI mechanism of action. Additional investigation revealed commensal gut microflora in the mesenteric lymph nodes and elevated LPS levels in the sera of irradiated mice. These findings correlated with increased dendritic cell activation and heightened levels of systemic inflammatory cytokines. Reduction of host microflora using antibiotics, neutralization of serum LPS using polymyxin B, or removal of LPS signaling components using mice genetically deficient in CD14 and TLR4 reduced the beneficial effects of TBI on tumor regression. Conversely, administration of microbial ligand–containing serum or ultrapure LPS from irradiated animals to nonirradiated antibody-lymphodepleted mice enhanced CD8+ T cell activation and improved tumor regression. Administration of ultrapure LPS to irradiated animals further enhanced the number and function of the adoptively transferred cells, leading to long-term cure of mice with large B16F10 tumors and enhanced autoimmune vitiligo. Thus, disruption of the homeostatic balance between the host and microbes can enhance cell-based tumor immunotherapy.

Introduction

The mutualistic microorganisms that colonize the gastrointestinal tract are crucial for health (1–3). Disruption of the homeostatic balance between the host and microflora is now understood to be part of the pathogenesis of HIV infection and inflammatory bowel disease (4, 5). Immune-based treatments for cancer, particularly those involving profound lymphodepletion, adoptive transfer of immune cells, or radiation have high potential to disrupt the host/microflora relationship and change it from mutualistic to pathogenic (6, 7). As cancer immunotherapy develops, it is particularly important to understand the impact of these treatments on host/microbe homeostasis and the role of microorganisms in tumor immunity.

Bacteria share conserved molecular patterns (e.g., lipopeptides, lipoteichoic acid, flagellin, peptidoglycan, LPS, and bacterial DNA) that can ligate pattern recognition receptors, such as the TLRs of the innate immune system (8–10). Engagement of TLRs promotes DC maturation and migration to lymph nodes, where these cells activate antigen-specific T cells (11, 12). LPS derived from commensal bacteria has been implicated in exacerbation of graft-versus-host disease (13–17), but its impact on T cell–based antitumor immunotherapies has not been fully elucidated.

Adoptive transfer of tumor-specific T cells is emerging as a potent cancer therapy (18). Lymphodepleting preparative regimens are a critical recent advance in this approach (19, 20). Lymphodepletion not only diminishes host inhibitory cells but also increases the availability of homeostatic cytokines, thus augmenting the activation and function of adoptively transferred cells (21–25). However, these mechanisms might not fully account for the dramatically improved tumor regression resulting from lymphodepleting preparative regimens with chemotherapy or total body irradiation (TBI).

We found that Rag2$^{-/-}\gamma_c^{-/-}$ mice, deficient in all lymphocyte subpopulations, benefited from TBI preconditioning. Here we describe how TBI caused mucosal barrier injury resulting in microbial translocation and systemic liberation of LPS. TLR4 engagement by LPS resulted in increased DC and self/tumor-specific CD8+ T cell activation, leading to greater tumor regression and enhanced autoimmune vitiligo. Thus, we show here that disruption of the homeostatic balance between the host and microbes plays a key role in the efficacy of tumor treatment by adoptively transferred T cells.

Results

TBI enhances the efficacy of adoptively transferred tumor-reactive T cells in the absence of Tregs and lymphocytes that consume homeostatic cytokines. We have previously reported that administration of a nonmyeloablative lymphodepleting preparative regimen with 5 Gy TBI prior to an adoptive cell transfer (ACT) regimen can induce significant destruction of large, established, poorly immunogenic B16F10 melanoma by removing cytokine sinks (capable of consuming homeostatic cytokines) and suppressive Tregs (22, 26). To explore whether TBI potentiates the antitumor immunity and autoimmunity of adoptively transferred cells by additional mechanisms, we

Nonstandard abbreviations used: ACT, adoptive cell transfer; PMB, polymyxin B; rFPhgp100, recombinant fowlpox virus encoding human gp100; rhIL-2, recombinant human IL-2; TBI, total body irradiation.

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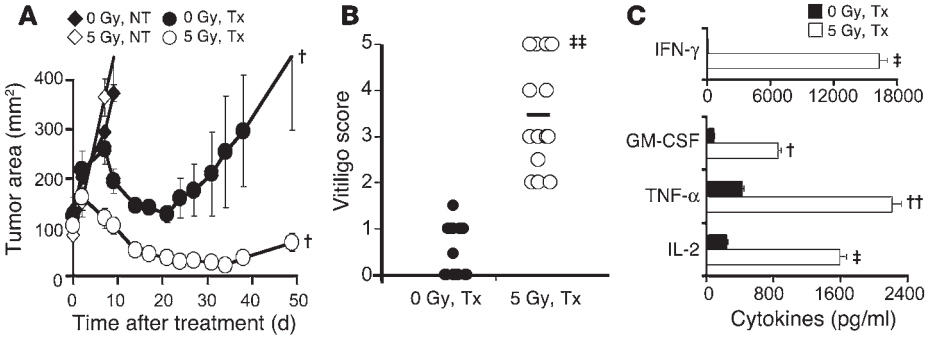

Figure 1

TBI enhances the function of adoptively transferred self/tumor-reactive pmel-1 T cells in mice genetically deficient in cytokine sinks and Tregs. (**A**) TBI augmented antitumor responses in mice genetically deficient in cytokine sinks and Tregs. Rag2$^{-/-}\gamma_c^{-/-}$ mice (deficient in T, B, and NK cells) bearing s.c. B16F10 tumors established for 10 days received nonmyeloablative 5 Gy TBI or were not irradiated (0 Gy). One day later, mice received an ACT treatment regimen consisting of the adoptive transfer of 10^5 cultured self/tumor reactive pmel-1 T cells, rFPhgp100 vaccination, and rhIL-2 or were left untreated (NT). Data (mean ± SEM; $n = 5$ per group) are representative of 4 independent experiments. (**B**) TBI enhanced autoimmune vitiligo in Rag2$^{-/-}\gamma_c^{-/-}$ mice. Twenty-eight days after treatment, nonirradiated and irradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice were evaluated in a blinded fashion for the development of vitiligo. Each mouse was scored for degree of hypopigmentation on a scale of 0–5. Data ($n = 14$ per group) are representative of 2 independent experiments. Horizontal bars indicate means. (**C**) TBI enhanced the function of adoptively transferred pmel-1 CD8$^+$ T cells. Five days after treatment, pmel-1–Thy1.1$^+$ cells were isolated from spleens of irradiated and nonirradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice and were cocultured with irradiated splenocytes pulsed with 1 μM hgp100$_{25–33}$. Secretion of IFN-γ, GM-CSF, TNF-α, and IL-2 in pmel-1 cells was analyzed. Unpulsed splenocytes were used as controls. Data (mean ± SEM; $n = 3$ per group) are representative of 2 independent experiments. †$P = 0.05$, ††$P < 0.05$, ‡$P < 0.01$, ‡‡$P < 0.001$ versus nonirradiated treated mice. Tx, treatment.

evaluated the ACT treatment regimen in Rag2$^{-/-}\gamma_c^{-/-}$ mice, which are genetically deficient in Tregs and cytokine sinks, and irradiated them with 5 Gy TBI. Because an ACT regimen consisting of adoptive transfer of 10^6 TCR Tg CD8$^+$ T cells (pmel-1 cells) reactive against the self/tumor antigen gp100, vaccination with a recombinant fowlpox virus encoding human gp100 (rFPhgp100), and IL-2 can eradicate large B16F10 tumors in both nonirradiated and irradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice (22), we transferred 10-fold fewer cells in order to generate a treatment window to address whether TBI affects the ACT treatment in Rag2$^{-/-}\gamma_c^{-/-}$ mice. Interestingly, we found that the effectiveness of tumor treatment was significantly improved in irradiated compared with nonirradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice ($P < 0.05$; Figure 1A). Enhanced autoimmune vitiligo was also observed in irradiated compared with nonirradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice 28 days after ACT ($P < 0.001$; Figure 1B). These data indicated that the mechanisms by which TBI enhances antitumor treatment and autoimmune responses by transferred T cells were not restricted to the elimination of cytokine sinks and Tregs.

Homeostatic expansion and activation of T cells have been proposed to explain the enhanced antitumor responses observed after ACT into irradiated hosts (27). Thus, the question arose as to whether TBI also augmented the quantity and functional quality of the transferred cells in Rag2$^{-/-}\gamma_c^{-/-}$ mice. No significant difference in the absolute number of pmel-1 T cells was found in the spleens or lymph nodes of nonirradiated and irradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice analyzed every 2–3 days for 2 weeks after ACT treatment (data not shown). These data corroborate our previous findings in WT mice showing that TBI prior to ACT treatment did not significantly increase the absolute number of the transferred cells (22). In contrast, significant differences were observed in the function of the transferred cells recovered from irradiated versus nonirradiated Rag2$^{-/-}\gamma_c^{-/-}$ mice on day 5 after ACT. The pmel-1 T cells recovered from irradiated hosts produced significantly higher amounts of IFN-γ ($P < 0.01$), GM-CSF ($P = 0.05$), TNF-α ($P = 0.01$), and IL-2 ($P < 0.01$) than did pmel-1 T cells from nonirradiated hosts after restimulation in vitro (Figure 1C). These data showed that irradiating Rag2$^{-/-}\gamma_c^{-/-}$ mice with 5 Gy TBI greatly enhanced the functional quality of adoptively transferred T cells. This improved T cell functionality may account for the pronounced tumor destruction and autoimmune vitiligo observed in Rag2$^{-/-}\gamma_c^{-/-}$ mice irradiated with 5 Gy TBI compared with nonirradiated mice (Figure 1, A and B).

Disruption of intestinal homeostasis by TBI activates the innate immune system. We next sought to better understand the mechanism underlying the enhanced function of CD8$^+$ T cells after transfer into an irradiated Rag2$^{-/-}\gamma_c^{-/-}$ host. Because preconditioning a WT host with 15 Gy TBI has previously been reported to activate the innate immune system, as indicated by an increase in the absolute number of activated host DCs (28), we hypothesized that the increased functionality of the pmel-1 T cells may be the result of innate activation induced by 5 Gy TBI. Thus, we first investigated whether 5 Gy TBI triggers the innate immune system by analyzing endogenous DCs for markers of activation in WT mice irradiated with 5 Gy TBI. Irradiation of WT mice with 5 Gy TBI significantly increased the absolute number of activated host CD11c$^+$CD86^{high} DCs in the recipient spleens ($P < 0.001$; Figure 2A) and lymph nodes ($P < 0.001$; Figure 2B) over that of nonirradiated mice. Furthermore, the level of IL-12p70, a cytokine produced by activated DCs (29), was significantly increased in the sera of irradiated WT mice compared with nonirradiated WT mice ($P < 0.05$; Figure 2C). The increase in absolute number of activated DCs and serum IL-12 levels was transient: the cells reached their maximum number at day 1 and declined rapidly afterward (data not shown). Collectively, these data showed that preconditioning WT hosts with 5 Gy TBI activated the innate immune system, as demonstrated by a significant increase in the absolute number of host CD11c$^+$CD86^{high} DCs and serum levels of IL-12.

We next investigated the mechanisms underlying the activation of the innate immune system by 5 Gy TBI. In allogenic models (13), preconditioning mice with TBI damages the gastrointestinal tract, leading to translocation of microbes into the systemic circulation that can activate the innate immune system. We thus measured for the presence of microbes in the systemic circulation of WT mice irradiated with 5 Gy TBI prior to ACT treatment. We found that 5 Gy TBI compromised the morphological integrity of the gut by pathological score (Figure 2D), leading to translocation of *Enterobacter cloacae*, *E. coli*, *Lactobacillus*, and *Bifi-*

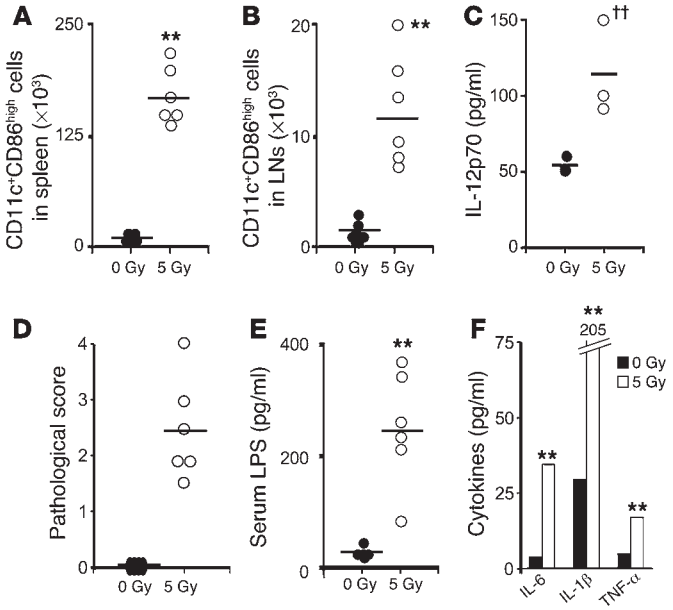

Figure 2

TBI activates the innate immune system and promotes translocation of microorganisms from the radiation-injured gastrointestinal tract. (**A** and **B**) TBI induced activation of DCs. Splenocytes and inguinal LNs were isolated from 5 Gy irradiated or nonirradiated mice 1 day after TBI. Absolute numbers of activated $CD11c^+CD86^{high}$ DCs in the spleens (**A**) and inguinal LNs (**B**) of TBI and nonirradiated C57BL/6 mice were enumerated. Data (n = 3–6 per group) are representative of 3 independent experiments. (**C**) TBI induced production of inflammatory cytokine IL-12p70. Serum was collected from nonirradiated and 5 Gy TBI mice 1 day after TBI, and IL-12p70 was measured by ELISA. Data (n = 3 per group) are representative of 2 independent experiments. (**D**) TBI damaged the colon. Colons of mice were analyzed 3 days after TBI and scored by a pathologist blinded to treatment group. Data (n = 3–6 per group) are representative of 2 independent experiments. (**E**) LPS was elevated in irradiated mice. Serum from nonirradiated and 5 Gy irradiated mice were collected and analyzed for the presence of LPS using a limulus amebocyte lysate assay 6 days after TBI. Data (n = 3–6 per group) are representative of 3 independent experiments. Horizontal bars indicate means. (**F**) Inflammatory cytokines were elevated in irradiated mice. Serum was collected from nonirradiated and 5 Gy TBI mice 1 day after TBI, and IL-6, IL-1β, and TNF-α cytokines were measured using ELISA. Data are representative of 2 independent experiments. $^{††}P < 0.05$, $^{**}P < 0.001$ versus nonirradiated mice.

dobacterium bacteria into the mesenteric lymph nodes of irradiated but not nonirradiated mice. Moreover, a significant amount of LPS, a major component of Gram-negative bacterial cell walls commonly measured to determine the degree of microbial translocation (4, 5), was detected in the sera of irradiated mice ($P < 0.001$; Figure 2E) 6 days after TBI. We found that the elevations of serum LPS and bacteria in the mesenteric lymph nodes after TBI were transient: levels reached their maximum around day 6 or 7 and declined rapidly afterward (data not shown). In addition, marked increases in levels of inflammatory cytokines associated with infection (30–32) — i.e., IL-6, IL-1β, and TNF-α — were also detected in the sera of irradiated mice compared with nonirradiated mice (Figure 2F). Collectively, these data demonstrated that irradiating mice with 5 Gy TBI damaged the gastrointestinal tract, permitting translocation of bacteria associated with the activation of the innate immune system.

Reduction of host microflora or genetic removal of LPS signaling components impairs the function of adoptively transferred $CD8^+$ T cells. We hypothesized that the translocated microbes induced by TBI were principally responsible for activating the innate immune system. To address this question, we assessed whether removing translocated microbes with a gut-decontaminating antibiotic would reduce innate immune activation that transpired after TBI. WT mice were treated with the broad-spectrum antibiotic ciprofloxacin — active against Gram-positive and Gram-negative bacteria — in their drinking water for the duration of the experiment and then analyzed for the presence of serum LPS and activated host DCs. We found that treating irradiated mice with ciprofloxacin eliminated the translocated bacteria in the mesenteric lymph nodes (data not shown) and reduced the levels of LPS in their sera ($P = 0.05$; Figure 3A). Consequently, the absolute number of activated $CD11c^+CD86^{high}$ DCs was significantly reduced in the spleens of irradiated mice treated with ciprofloxacin 1 day after TBI ($P < 0.05$; Figure 3B). In contrast, ciprofloxacin treatment did not affect the level of LPS or the absolute number of activated host $CD11c^+CD86^{high}$ DCs in nonirradiated mice (Figure 3, A and B). Together these results revealed that microbial translocation induced by 5 Gy TBI were responsible for activating the innate immune system.

To address whether circulating microbial products that translocated from the radiation-injured gut were responsible for the enhanced effectiveness of TBI, we determined whether their removal with ciprofloxacin would affect the ACT tumor treatment. Tumor-bearing WT mice were treated with ciprofloxacin in their drinking water from 2 days prior to TBI until 2 weeks after. Mice received the ACT treatment regimen 1 day after TBI. While the antibiotic alone had no effect on B16F10 tumor growth, it significantly inhibited the tumor destruction mediated by the transferred pmel-1 T cells in irradiated mice ($P < 0.001$; Figure 3C). Conversely, no difference in the effectiveness of tumor treatment was observed in nonirradiated control mice treated with or without ciprofloxacin (data not shown). These results revealed that the translocated microbes that activated the innate immune system were important for the improved tumor regression by ACT after TBI.

Because we detected LPS in the sera of irradiated mice, we examined whether this bacterial ligand was involved in augmenting tumor destruction by ACT therapy. For this purpose, we investigated the effect of treatment with polymyxin B (PMB), a cyclic cationic polypeptide antibiotic known to specifically block the biological effect of Gram-negative LPS (33) on the ACT treatment in irradiated WT animals. A significant decrease in the effectiveness of the tumor treatment was observed in irradiated mice treated with PMB compared with irradiated mice that did not receive PMB ($P < 0.01$; Figure 4A). However, no difference in the effectiveness of tumor treatment was observed in nonirradiated control mice treated with or without PMB (data not shown). These data implied that microbial LPS played a role in the enhanced ACT treatment effectiveness of TBI.

LPS, which binds to the soluble LPS-binding protein, is thought to exert its immune-activating effects primarily through TLR4, a molecule that acts in concert with CD14 and MD2 (34–37). To determine whether TLR4 signaling was involved in the enhancement of ACT treatment by TBI, we analyzed the ACT treatment in tumor-bearing WT, $CD14^{-/-}$, and $TLR4^{-/-}$ mice irradiated with 5 Gy TBI. We found that tumor destruction was significantly

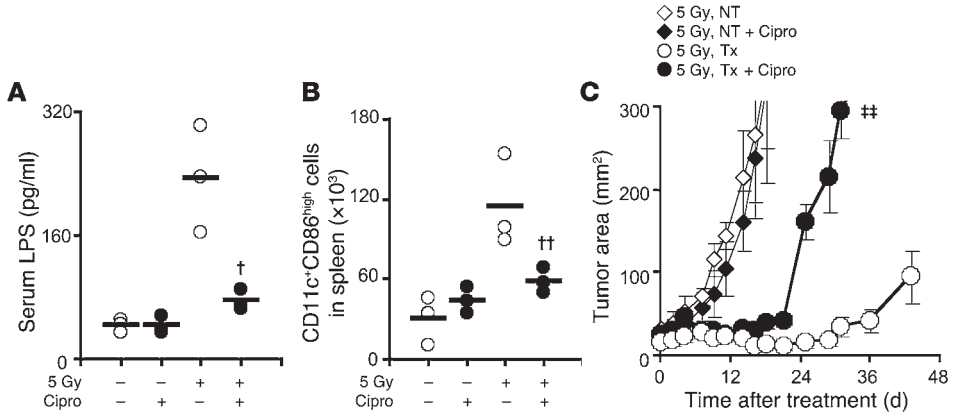

Figure 3

Ciprofloxacin treatment impairs the effectiveness of ACT therapy and reduces activation of the innate immune system in irradiated mice. (**A**) Ciprofloxacin reduced the detectable level of LPS in serum. Serum from nonirradiated and 5 Gy irradiated mice left untreated or treated with ciprofloxacin (Cipro) was collected and analyzed for the presence of microbial LPS using a limulus amebocyte lysate assay. Data (n = 3 per group) are representative of 2 independent experiments. (**B**) Ciprofloxacin treatment reduced the absolute number of host DCs. One day after TBI, splenocytes were isolated from nonirradiated and 5 Gy irradiated mice left untreated or treated with ciprofloxacin. Absolute numbers of $CD11c^+CD86^{high}$ DCs were determined in the spleens of nonirradiated and irradiated mice. Data (n = 3 per group) are representative of 2 independent experiments. Horizontal bars indicate means. (**C**) Treatment of irradiated hosts with ciprofloxacin reduced effectiveness of ACT treatment. C57BL/6 mice bearing s.c. B16F10 tumors established for 10 days received 5 Gy TBI. One day later, mice received an ACT treatment consisting of adoptive transfer of 10^6 cultured pmel-1 T cells, rFPhgp100 vaccination, and rhIL-2 or were left untreated. Administration of ciprofloxacin as indicated began 2 days prior to ACT and continued for 2 weeks after treatment. Data (mean ± SEM; n = 4–5 per group) are representative of 2 independent experiments. †P = 0.05, ††P < 0.05 versus 5 Gy TBI without ciprofloxacin; ‡‡P < 0.001 versus 5 Gy TBI plus treatment without ciprofloxacin.

reduced in irradiated $CD14^{-/-}$ mice (P < 0.05) and $TLR4^{-/-}$ mice (P < 0.01) compared with irradiated WT mice (Figure 4, B and C). In contrast, no difference in the effectiveness of tumor treatment was observed in nonirradiated control WT, $CD14^{-/-}$, and $TLR4^{-/-}$ mice (data not shown). Thus, these data indicated that translocation of microbial LPS enhanced ACT tumor treatment via TLR4 signaling in irradiated mice.

Innate immune activation, removal of Tregs, and elimination of cytokine sinks enhance the function of adoptively transferred $CD8^+$ T cells. Our data suggested that preconditioning the host with TBI improved the effectiveness of ACT by 3 mechanisms: (a) activation of the innate immune system mediated by TLR4 signaling, (b) removal of cytokine sinks, and (c) removal of Tregs. Thus, we sought to test whether applying these 3 TBI mechanisms — removing cytokine sinks and Tregs with antibodies while concomitantly activating DCs with exogenous serum obtained from irradiated mice — could mimic the effectiveness of TBI in nonirradiated recipients. To harvest microbial LPS released from the radiation-injured bowel, we collected sera from irradiated mice 6 days after TBI and administered it 1 day after ACT into nonirradiated mice depleted of cytokine sinks and Tregs using NK- and CD4-specific antibodies. Administration of serum obtained from irradiated mice into nonirradiated lymphodepleted mice resulted in enhanced tumor responses similar to those seen after 5 Gy TBI conditioning (Figure 4D). Interestingly, removal of LPS from the serum prior to its administration into nonirradiated lymphodepleted mice impaired the ACT antitumor response (P < 0.01; Figure 4D). These data highlighted the specific role of LPS released in the serum.

The combination of all 3 mechanisms was required to induce antitumor effectiveness similar to that seen with TBI. The removal of Tregs alone, removal of cytokine sinks alone, activation of the innate immune system alone, or any 2 of the 3 mechanisms underlying the effectiveness of TBI induced nominal to weak antitumor immune responses by the adoptively transferred cells (Figure 5A). These data demonstrated that all 3 mechanisms underlying the effectiveness of TBI, removal of cytokine sinks and Tregs as well as activation of the innate immune system, are necessary to enhance the function of adoptively transferred tumor-specific $CD8^+$ T cells.

We next investigated whether administration of ultrapure LPS instead of serum microbial LPS could improve ACT treatment in nonirradiated lymphodepleted mice. We found that administration of ultrapure LPS to nonirradiated lymphodepleted mice significantly enhanced ACT treatment (P = 0.009, nonirradiated, treated, and anti-CD4 versus irradiated treated mice), similar to the results seen after administration of serum microbial LPS (Figure 5, A and B). Furthermore, results of ACT treatment in nonirradiated lymphodepleted mice receiving ultrapure LPS were comparable to those seen after 5 Gy TBI conditioning (Figure 5B). Note that the groups shown in Figure 5, A and B, are from the same experiments and can be directly compared. Administration of ultrapure LPS improved treatment in nonirradiated $Rag2^{-/-}\gamma_c^{-/-}$ mice (P = 0.05), similar to what we observed after 5 Gy TBI conditioning (Figure 5C). Collectively, these data indicated that ultrapure LPS could mimic the effectiveness of TBI-mediated microbial translocation in the absence of endogenous inhibitory lymphocytes, i.e., cytokine sinks and Tregs.

Administration of ultrapure LPS after TBI enhances antitumor immunity and autoimmunity. In an effort to use the model in a clinically relevant manner, we sought to determine whether administering LPS would further improve the antitumor response of adoptively transferred $CD8^+$ T cells in mice irradiated with 5 Gy TBI. One day after TBI, WT mice were given the ACT therapy and then treated the following day with commercial ultrapure LPS. As observed in 10 independently performed experiments, the administration of LPS significantly enhanced tumor destruction (P < 0.001; Figure 6A) and autoimmune vitiligo (P < 0.001; Figure 6B) in irradiated mice. Furthermore, long-term cure was observed in all of the irradiated mice treated with LPS.

Although administration of ultrapure LPS reproducibly mediated potent destruction of tumors in irradiated mice receiving the ACT treatment (Figure 6A), we found that LPS alone did not replace individual components of the tripartite ACT regimen con-

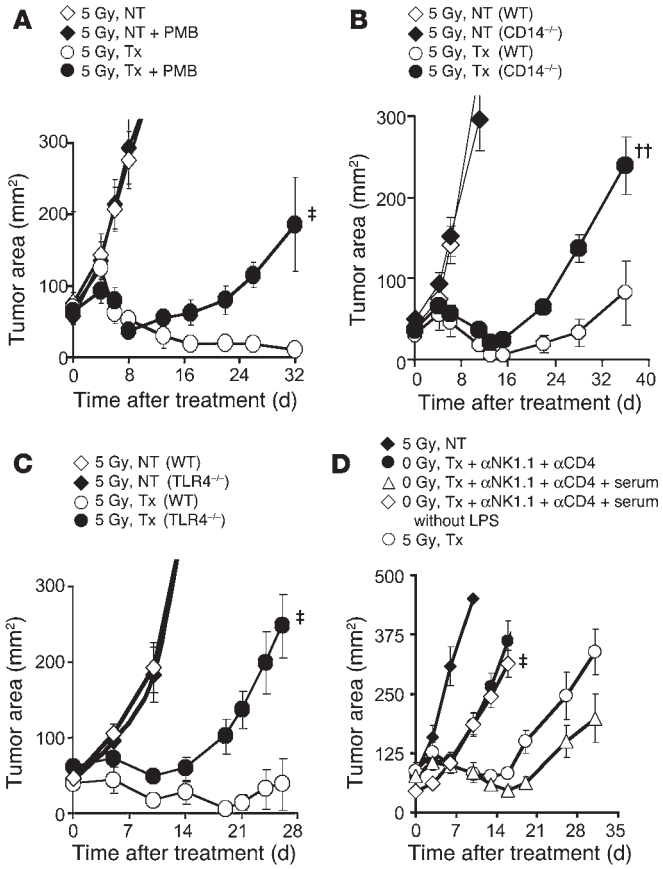

Figure 4
TLR4 signaling triggered by TBI improves the effectiveness of ACT therapy. (**A**) Administration of PMB decreased the tumor treatment effectiveness of TBI. Mice bearing s.c. B16F10 tumors established for 10 days received 5 Gy TBI. One day later, mice received an ACT treatment consisting of adoptive transfer of 10^6 cultured pmel-1 T cells, rFPhgp100 vaccination, and rhIL-2 or were left untreated. For the duration of the experiment, mice were treated or not with PMB in their water. Data (mean ± SEM; $n = 5$ per group) are representative of 2 independent experiments. (**B** and **C**) The effectiveness of treatment was decreased in irradiated mice genetically deficient in CD14 (**B**) and TLR4 (**C**). WT, CD14$^{-/-}$, and TLR4$^{-/-}$ tumor-bearing mice were irradiated and then received the ACT treatment described above or were left untreated. Data (mean ± SEM; $n = 5$ per group) are representative of 2 independent experiments. (**D**) Serum from irradiated mice improved treatment when transferred to nonirradiated antibody-lymphodepleted mice. Tumor-bearing mice received 5 Gy TBI or were left unirradiated. Alternatively, mice were depleted of lymphocytes with CD4 and NK antibodies and received 5×10^5 cultured pmel-1 T cells, rFPhgp100 vaccination, and IL-2 1 day later. Mice received serum with translocated LPS or serum removed of LPS with Detoxi-gel beads 1 day after ACT. Data (mean ± SEM; $n = 4$–5 per group) are representative of 2 independent experiments. ††$P < 0.05$, ‡$P < 0.01$ versus irradiated treated WT mice (**A**–**C**) or recipients of serum with LPS (**D**).

sisting of adoptive transfer of pmel-1 T cells reactive against the self/tumor antigen gp100, vaccination, and IL-2 (data not shown). Thus, administration of ultrapure LPS to irradiated hosts significantly enhanced but did not replace individual components of the tripartite ACT therapy.

We next sought to determine the effect of ultrapure LPS on the quantity and function of adoptively transferred cells in irradiated mice given the ACT treatment. We found that administration of ultrapure LPS significantly increased the number of tumor-reactive pmel-1 T cells in irradiated animals receiving ACT treatment ($P < 0.05$), as determined by enumeration of the absolute number of congenically marked transferred pmel-1 lymphocytes in the spleen assayed every 2–3 days for 2 weeks (Figure 6C). Additionally, pmel-1 T cells isolated from ultrapure LPS–treated mice on day 5 after ACT secreted markedly higher levels of IFN-γ ex vivo in the presence of specific antigen than did pmel-1 T cells isolated from untreated mice (Figure 6D). These data indicated that LPS further increased the number and enhanced the function of the transferred cells in the irradiated mice, leading to long-term cure of mice with large B16F10 tumors and enhanced autoimmune vitiligo.

Discussion

Lymphodepletion with a nonmyeloablative preparative regimen by 5 Gy TBI prior to the adoptive transfer of self/tumor-reactive CD8$^+$ T cells can significantly improve the effectiveness of tumor treatment in mice (22). The translation of these findings into clinical trials yielded an objective response rate of approximately 50% in patients with advanced metastatic disease (19, 20). The mechanisms underlying the effectiveness of lymphodepletion prior to ACT have only begun to be elucidated. TBI depletes elements of the cellular immune system that are capable of acting as sinks for homeostatic cytokines (e.g., CD8 and NK cells) (38–41). Depletion of cytokine sinks resulted in the increased availability of homeostatic cytokines IL-7 and IL-15 for the adoptively transferred cells, which led to their enhanced functional quality (22, 23). Tregs have been reported to maintain tolerance to self antigens and can block the functionality of effector T cells (42–44). Their removal is a mechanism that contributes to the effectiveness of TBI (23, 26). Myeloid-derived suppressor cells have previously been reported to promote tumor growth and induce lymphocyte dysfunction. Thus, their removal might also contribute to the effectiveness of TBI (45, 46).

We found that removal of host Tregs and cytokine sinks were not the only mechanisms underlying the improved antitumor treatment effectiveness of TBI. Indeed, the function of adoptively transferred cells was enhanced in Rag2$^{-/-}\gamma_c^{-/-}$ mice, genetically deficient in all lymphocytes, that were irradiated with TBI. These data indicated the presence of another TBI mechanism of action. We identified that disruption of host/microbe homeostasis by TBI activated the innate immune system and that the transient bacterial infection was a key mechanism in driving CD8$^+$ T cell reactivity to self tissue and tumor. Microbial LPS liberated from the radiation-injured gut was responsible for activating the innate immune system via engagement of TLR4. Removal of translocated LPS or of critical components of the TLR4 signaling pathway impaired the effectiveness of TBI. Conversely, administration of LPS to nonirradiated mice that were lymphodepleted

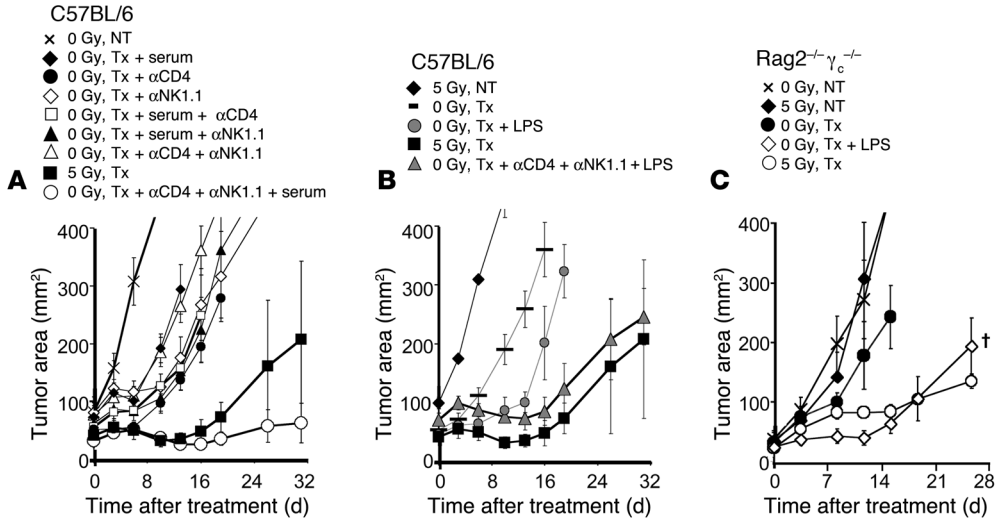

Figure 5

Depletion of cytokine sinks, removal of Tregs, and activation of the innate immune system recapitulate the effectiveness of TBI. (**A**) Activation of the innate immune system with serum LPS, depletion of Tregs with anti-CD4 antibody, and removal of cytokine sinks with anti-NK antibody are required to improve the efficacy of adoptively transferred in nonirradiated mice. Tumor-bearing C57BL/6 mice received 5 Gy TBI or were left nonirradiated. Alternatively, mice were depleted of lymphocytes with CD4 and NK antibodies; 1 day later mice received 5×10^5 cultured pmel-1 T cells, rFPhgp100 vaccination, and IL-2. Serum for irradiated mice containing LPS was harvested and transferred into nonirradiated recipients. (**B**) Ultrapure LPS enhanced treatment in lymphodepleted nonirradiated mice. C57BL/6 mice were irradiated as a control. Mice received serum with translocated LPS or ultrapure LPS alone, CD4 alone, or NK-depleting antibody alone 1 day after ACT. Data (mean ± SEM; $n = 4$–5 per group) are representative of 2 independent experiments. (**C**) Ultrapure LPS recapitulated the effectiveness of TBI in Rag2$^{-/-}\gamma_c^{-/-}$ mice genetically deficient in all lymphocytes. Data (mean ± SEM; $n = 5$ per group) are representative of 2 independent experiments. †$P = 0.05$ versus irradiated treated mice. The difference between the nonirradiated, treated, LPS-administered group and the irradiated treated group was not significant ($P < 0.2$).

with antibodies enhanced the antitumor efficacy of the adoptively transferred cells. Our results demonstrated that TLR4-mediated activation of the innate immune system by TBI plays a crucial role in enhancing the effectiveness of the adoptively transferred self/tumor-reactive CD8$^+$ T cells.

Our finding that microbial translocation augments the function of adoptively transferred CD8$^+$ T cells brings to mind findings reported by William B. Coley over a century ago (47). Hypothesizing that tumor regression in some patients was the result of bacterial infection, Coley designed a mixture of bacteria consisting of killed cultures of *Streptococci* and *Bacillus prodigiosus* known as Coley's toxins. He reported occasional success using these toxins in patients with cancer. Our findings may be consistent with the notion that activation of the innate immune system can trigger tumor regression with bacterially derived products.

Bacteria that translocate from the gut contain of a variety of TLR ligands including lipoproteins, lipoteichoic acid, peptidoglycan, flagellin, bacterial DNA, and LPS (10, 12, 48). It was possible that any or all of these ligands might have been responsible for improving tumor regression with TBI; however, neutralizing LPS with PMB or removing LPS signaling pathways through use of TLR4$^{-/-}$ or CD14$^{-/-}$ mice significantly diminished the advantage conferred by TBI in this model. Although our results do not rule out the effects of other TLR ligands, they indicate that LPS ligation of TLR4 substantially accounts for the TBI-mediated enhancement of ACT treatment efficacy.

Administration of TLR ligands to lymphoreplete mice can inhibit tumor establishment and growth by adoptively transferred cells in tumor prevention models (49–52). However, LPS alone was insufficient to trigger the regression of established tumors in our experiments. The superior antitumor treatment effectiveness promoted by TBI after ACT could only be accomplished in nonirradiated mice by the combination of (a) Treg removal by a CD4-deleting antibody, (b) cytokine sink elimination by a NK-depleting antibody, and (c) innate immune system activation by microbial LPS obtained from the sera of irradiated mice. These data are important because they suggest that alternative regimens to chemotherapy or TBI may be used to safely treat patients with advanced disease and promote tumor regression comparable to that seen with nonmyeloablative preparative regimens.

Finally, it is interesting to consider the impact of TBI on models of lymphodepletion. Many investigators use TBI in order to induce a lymphopenic state to study lymphocyte maturation and homeostasis (53, 54). TBI is often used interchangeably with antibody depletion experiments or gene-knockout mice to study immune cell reconstitution, but different methods of lymphodepletion cannot be assumed to be immunologically equivalent (55). The induction of homeostatic expansion of the T cell compartment after TBI preconditioning cannot be viewed as merely expanding to fill empty space (56). Rather, TBI induces a complex set of events, which includes the liberation of endogenous LPS capable of triggering TLR4 signals that enhance the efficacy of adoptively transferred T cells.

Methods

Mice and tumor lines. All mice were bred and housed at NIH facilities. Female pmel-1 TCR Tg mice were crossed with C57BL/6-Thy1.1 Tg mice to derive pmel-Thy1.1 double Tg mice (C57BL/6–pmel-1–Thy1.1 mice; The Jack-

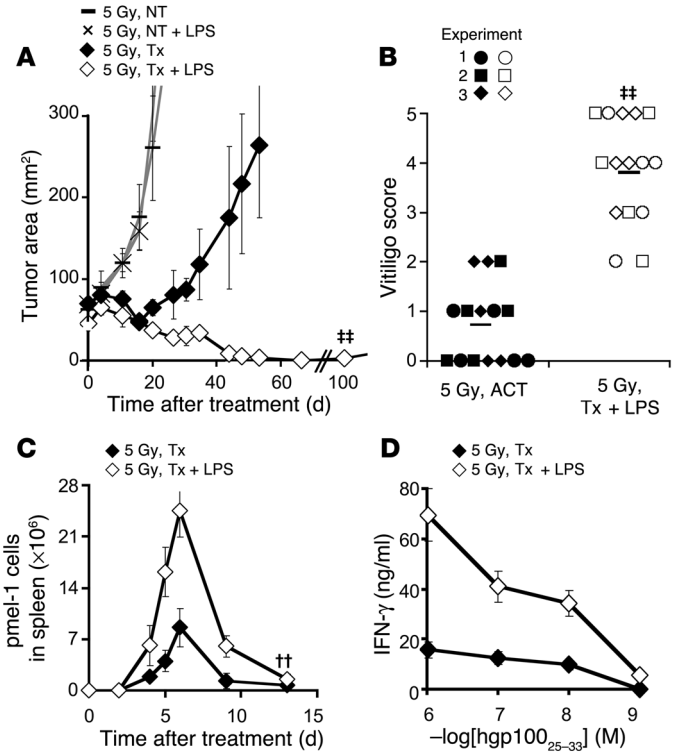

Figure 6
Administration of ultrapure LPS after TBI significantly enhances antitumor immunity and autoimmunity. (**A**) Ultrapure LPS augmented antitumor responses in irradiated mice. Mice bearing s.c. B16F10 tumors established for 10 days received 5 Gy TBI. One day later, mice received an ACT treatment consisting of adoptive transfer of 10^6 cultured pmel-1 T cells, rFPhgp100 vaccination, and rhIL-2 or were left untreated. The next day, mice received ultrapure LPS or were left untreated. Data (mean ± SEM; n = 5–10 per group) are representative of 10 independent experiments. (**B**) LPS enhanced autoimmune vitiligo in irradiated mice. One month after ACT treatment, irradiated mice treated with or without ultra-pure LPS were evaluated for the development of vitiligo. Mice were scored for the degree of hypopigmentation on a scale of 0–5. Data (n = 4–5 per group) are from 3 independent experiments. Horizontal bars indicate means. (**C**) Ultrapure LPS increased the absolute number of transferred pmel-1 T cells in the irradiated host. Absolute numbers of transferred pmel-1 T cells ($CD8^+Thy1.1^+$) in irradiated host. Data (mean ± SEM; n = 3–5 per group) are representative of 2 independent experiments. (**D**) Ultrapure LPS enhanced the function of adoptively transferred cells in irradiated mice. Five days after ACT treatment, pmel-1–$Thy1.1^+$ splenocytes were cocultured with irradiated splenocytes pulsed with the indicated doses of $hpg100_{25–33}$. Unpulsed splenocytes were used as controls. Data (mean ± SEM; n = 3 per group) are representative of 2 independent experiments. ††$P < 0.05$, ‡‡$P < 0.001$ versus irradiated treated mice.

son Laboratory). C57BL/6, Rag-$2^{-/-}\gamma_c^{-/-}$ (Taconic), $CD14^{-/-}$ (Taconic), and $TLR4^{-/-}$ (kindly provided by A. Akpinarli, NIH, Bethesda, Maryland, USA) mice were used as recipients in ACT experiments. Experiments were conducted with the approval of the NCI Animal Use and Care Committee. B16F10 (H-2b), a spontaneous, transplantable $gp100^+$ murine melanoma, was maintained in culture media.

In vitro activation of pmel-1 T cells. Isolation of pmel-1 splenocytes was performed as described previously (57), and isolated cells were cultured in the presence of 1 M $hgp100_{25–33}$ and culture media containing 30 IU/ml of recombinant human IL-2 (rhIL-2; Chiron Corp.). Cells were transferred 6 days after the start of the culture.

ACT, vaccination, cytokine administration, and TLR agonist LPS. Six 10-week-old mice were injected s.c. with 2–5 × 10^5 B16F10 melanoma cells and treated 10 days later with i.v. adoptive transfer of pmel-1 T cells in vitro–activated splenocytes. Mice received 5 Gy TBI on the day of ACT. NK and CD4 cells were depleted by i.p. administration of anti-NK1.1 and anti-CD4 antibodies (0.1 mg/mouse; BD Biosciences) 1 day before ACT. Isotypes were used as controls. Mice were vaccinated with 2 × 10^7 PFU of rFPhgp100 (Therion Biologics). Administration of rhIL-2 was performed by i.p. injection twice daily at 3.6–36 g/dose for a total of 5 doses. Ultrapure LPS (1 g i.v.; Invivogen) was administered 1 day after ACT. Tumors were measured with calipers, and the perpendicular diameters were recorded. Experiments were performed in a blinded, randomized fashion.

Vitiligo scoring. Vitiligo on treated mice was scored on a scale of 0–5 as follows: 0, no vitiligo (wild-type); 1, depigmentation detected; 2, >10% vitiligo; 3, >25% vitiligo; 4, >50% vitiligo; 5, >75% vitiligo. Mice were evaluated and scored by 2 independent investigators blinded to group at approximately 1 month after ACT.

Detection and purification of serum LPS. A limulus amebocyte lysate (LAL) assay (QCL-1000; Cambrex) was used to analyze serum LPS on days 1–8 after ACT. To harvest microbial LPS, 40–60 mice were irradiated; on day 6 after TBI, their serum was pooled and transferred into 5–10 nonirradiated mice. Detoxi-gel beads were used to remove endotoxin from the serum of TBI mice (Pierce Inc.).

Antibiotic treatment. Two days before TBI, ciprofloxacin (50 mg/kg/d for 1–2 weeks; Bayer) or PMB (1 mg/kg/d for 3–4 weeks; Invivogen) was added to the drinking water of irradiated mice.

Enumeration of adoptively transferred cells and host $CD11c^+CD86^{high}$ DCs and ex vivo cytokine release assay. At the indicated times, adoptively transferred pmel-1–Thy1.1 cells were enumerated (22). The number of transferred pmel-1–Thy 1.1 cells was calculated by multiplying the percentage of Thy1.1/$CD8^+$ T cells in the spleen by the absolute spleen cell count. Enumeration of host $CD11c^+$ $CD86^{high}$ DCs was similarly performed. Six days after ACT, pmel-1–Thy1.1 cells were used for cytokine release assay. The pmel-1–Thy1.1 cells were isolated from splenocytes (26) and were cocultured at a 1:2 ratio with 10^5 irradiated splenocytes pulsed with titrated doses of $hgp100_{25–33}$ peptide or unpulsed as negative controls. Supernatants were collected after 18 hours and assayed by mouse IFN-γ, IL-2, TNF-α, and GM-CSF with Mouse IFN gamma Colorimetric, Mouse IL-2 Chemiluminescent, Mouse GM-CSF Colorimetric, Mouse TNF alpha Colorimetric and Mouse IL-12 (p70) Colorimetric ELISA kits (all from Pierce). Ex vivo IL-12p70 production was determined by ELISA in the serum of mice after TBI.

Mucosal barrier score. Colons were removed from mice, placed in 10% formalin for 48 hours, and embedded in methylacrylate. Sections (4–5 mm) were taken along the papillary-optical axis and scored by a pathologist blinded to group as follows: 0, normal architecture; 1, some signs of edema; 2, mild cell infiltration and reduction of crypts and goblets; 3, severe cell infiltration and profound reduction of crypts and goblets; 4, severe cell infiltration and visually undetectable crypt and goblets.

Statistics. Tumor growth rate measurements were examined using the ANOVA between-groups test. Statistical analyses comparing frequencies of vitiligo, DCs, cytokines, and LPS between 0 Gy TBI and 5 Gy TBI groups were performed using 1-way ANOVA with Bonferroni's correction for multiple comparisons. A P value of 0.05 or lower was considered significant

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