Central memory CD8+ T lymphocytes mediate lung allograft acceptance

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Introduction
While transplantation has become an accepted form of therapy for end-stage organ failure, formidable immunologic barriers limit long-term allograft survival. The currently accepted clinical immunosuppression protocols, consisting of life-long administration of calcineurin inhibitors, steroids, and antimetabolites, decrease immunosurveillance for both malignancies (1) and infectious diseases (2). Perioperative inhibition of lymphocyte activation through blockade of costimulatory pathways mediates acceptance of several types of allografts in murine models (3–5). Clinical data point to the efficacy of costimulatory blockade for the treatment of autoimmune diseases in humans (6, 7). Based on these data, costimulatory blockade is being actively evaluated in human solid organ transplantation (8). This would be very advantageous for lung transplantation, in which patients incur higher rates of graft loss compared with recipients of other solid organs (9) and suffer more infectious complications due to constant exposure of lung allografts to the external environment (10, 11).

Alloreactive memory T cells are generated through previous blood transfusions, pregnancy, or cross reactivity to viral or environmental antigens in a process known as heterologous immunity (12). When compared with naive T cells, memory T cells have lower activation requirements and can rapidly trigger alloimmune responses through the synthesis of multiple inflammatory cytokines and cytolytic effector molecules (12). Furthermore, this cell population is relatively resistant to immunosuppression, such as costimulatory blockade (13, 14). Multiple studies have established that early infiltration of CD8+ memory T cells into allografts, such as hearts, kidneys, and livers, facilitates accelerated rejection and presents a barrier to immunosuppression-mediated long-term graft survival (12, 15–19). Therefore, preclinical studies have focused on targeting this cell population in an effort to improve the survival of solid organ allografts, such as kidneys (17, 20, 21). However, some reports have indicated that CD8+ T cells with phenotypic and functional characteristics of memory cells can also downregulate immune responses. While it is well recognized that CD8+ memory T cells express CD122, the β subunit of the IL-2 receptor, recent work has shown that CD122-expressing CD8+ T cells can have regulatory capacity (22). CD8+CD122+PD1+ T lymphocytes, for example, suppress T cell proliferation and production of inflammatory cytokines in an IL-10–dependent fashion (22). Another study showed that memory-like CD8+ T cells expressing the ectonucleotidase CD38 suppress CD4+ T cell activation in antigen-independent fashion in vitro and attenuate autoimmune responses in vivo (23). Immune regulation by these CD8+ T cells was dependent on production of IFN-γ and direct contact with the effector CD4+ T cells. Similarly, rapid bystander production of IFN-γ by memory CD8+CD44hiCD62Lhi T cells has been shown to inhibit the development of Th2-driven allergic airway inflammation (24).

Here, we report that, in contrast to what has been described for other organ transplants, early infiltration of CD8+CD44hiCD62LhiCCR7+ central memory T cells is critical for lung allograft acceptance, due to IFN-γ–mediated induction of local NO. Our findings identify a novel mechanism of allograft acceptance that challenges the currently accepted paradigm of global T cell depletion as induction therapy for lung transplant recipients.

Results
Both CD4+ and CD8+ T lymphocytes can mediate lung allograft rejection. Lung allograft rejection is diagnosed and graded based on histological findings of cellular infiltrates (25). A wide variety of leukocytes, including B cells, macrophages, neutrophils, and natural killer cells, have been shown to contribute to rejection of solid organs (26–28), and to date it has not been established whether T lymphocytes are necessary to mediate lung allograft rejection. To
address this issue, we transplanted BALB/c lungs into allogeneic athymic nude mice and determined that, in contrast to wild-type recipients (29), these grafts remain ventilated, with little inflammation 1 week after transplantation (Figure 1A) and long term (30). We have shown previously that, unlike the case for cardiac transplants, lung allografts can be rejected in the absence of CD4+ T cells (31). To test whether CD8+ T cells are essential for the rejection of pulmonary allografts, we transplanted BALB/c lungs into CD8+ T cell–deficient B6 recipients (referred to herein as B6 Cd8–/– recipients) and noted histological changes of severe acute rejection with perivascular lymphocytic infiltrates comparable to those seen in CD8+ T cell–sufficient B6 recipients (Figure 1B). Immunostaining of these grafts demonstrated extensive infiltration with CD4+ T cells and no detectable CD8+ T cells (Figure 1C). Furthermore, reconstitution of nude mice with CD4+ T cells led to rejection of lung allografts by ISHLT A grade (32) (Figure 1D). Taken together with previously published data from our laboratory (31), we conclude that thymically derived T lymphocytes are necessary for lung allograft rejection and that either CD4+ or CD8+ T cells are sufficient to mediate this process.

CD8+ T lymphocytes are critical for lung allograft acceptance. We have previously demonstrated that immunosuppression through blockade of the CD28/B7 and CD40/CD154 costimulatory pathways leads to long-term lung allograft acceptance in the BALB/c→B6 (31, 33) as well as other strain combinations (30). Regulatory CD4+ T cells have been shown to play a critical role in costimulatory blockade–mediated acceptance of heart, skin, and islet allografts as well as amelioration of autoimmune diseases (4, 5, 34–38). However, recipient bulk CD4+ T cell antibody-mediated depletion did not affect the fate of immunosuppressed lung allografts with rejection grades comparable to wild-type costimulatory blockade–treated hosts (Figure 2, A and B). While regulatory B cells have been described in some models of solid organ transplantation (39), we were still able to induce BALB/c lung allograft acceptance in B6 B cell–deficient mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI71359DS1). Surprisingly, pulmonary allografts transplanted into costimulatory blockade–treated B6 CD8+ T cell–depleted mice (Figure 2C) or B6 Cd8–/– mice (Figure 2D) were acutely rejected, with severe graft inflammation. The appearances of these grafts and rejection grades were similar to what we have reported previously for lungs transplanted into nonimmunosuppressed allogeneic recipients (40). Adoptive transfer of wild-type B6 CD8+ T cells into immunosuppressed B6 Cd8–/– recipients restored acceptance of BALB/c lungs (Figure 2E). Seven days after engraftment, we observed an increase in CD4+Foxp3+ T cells in lung allografts of immunosuppressed B6 Cd8–/– recipients compared with lungs from untransplanted controls (Figure 3A). However, the proportion of CD4+ T cells expressing Foxp3 in lung allografts was higher in the presence of CD8+ T cells. No such differences were evident in spleens of
transplanted mice (Supplemental Figure 2). Thus, CD8+ T cells play a critical role in mediating lung allograft acceptance.

Based on the finding that CD4+ T cells can trigger lung rejection in the absence of CD8+ T cells, we next decided to evaluate CD4+ T lymphocyte responses in the presence or absence of CD8+ T cells. We injected CFSE-labeled congenic B6 CD45.1+CD4+ T cells into costimulatory blockade–treated B6 wild-type or B6 Cd8–/– recipient mice at the time of BALB/c lung transplantation and observed enhanced proliferation of this cell population in B6 Cd8–/– hosts compared with that in B6 wild-type hosts (Figure 3B). We found that, after transfer into B6 Cd8–/– recipients, several other costimulatory receptors, such as CD27, ICOS, and OX40, were upregulated on CD4+ T cells compared with B6 wild-type hosts, while levels of CD28 and CD154 were comparable in these two groups (Figure 3B). We considered the possibility that costimulatory requirements of CD4+ T lymphocytes may be altered in the absence of CD8+ T cells and blockade of CD40/CD154 and CD28/B7 pathways may be insufficient to ameliorate CD4+ T cell–mediated rejection (41). To address this, we inhibited CD27/CD70, ICOS/ICOS ligand, and OX40/OX40 ligand pathways in addition to blocking CD28/B7 and CD40/CD154 pathways in B6 Cd8–/– recipients of BALB/c lungs. However, this treatment regimen did not prevent lung allograft rejection when recipients lacked CD8+ T cells (Figure 3C). These findings directly contrast with previous observations that depletion or deletion of CD8+ T cells prolongs survival of skin and heart allografts when recipients are treated with costimulatory blockade (13, 37).

Accepting lung allografts are heavily infiltrated with central memory CD8+CD44hiCD62LhiCCR7+ T cells that can downregulate alloimmune responses. Costimulatory blockade has been described to mediate graft acceptance through the generation of regulatory T lymphocytes (4, 5, 34–38). In order to evaluate whether CD8+ T lymphocytes with regulatory capacity develop in costimulatory blockade–treated lung recipients, we isolated CD8+ T cells from the lung grafts and spleens of such mice and used them as “regulators” in in vitro mixed lymphocyte reactions (MLRs) (Figure 4A). We found that CD8+ T lymphocytes isolated from accepting BALB/c→B6 lung allografts, but not spleens of these recipients, inhibited proliferation and blasting of B6 CD45.1+CD4+ (Figure 4B) and B6 CD45.1+CD8+ T lymphocytes (Figure 4C) when stimulated with BALB/c splenocytes. These findings suggested that CD8+ T cells with regulatory capacity accumulate in accepting lung allografts. While described to have regulatory function in other models (42–44), very few CD8+IL-10+ or CD8+Foxp3+ cells were detectable in accepting lung allografts (Figure 5A). Notably, however, a large portion of CD8+ T cells in accepting grafts had the capacity to produce IFN-γ and expressed phenotypic markers consistent with central memory T lymphocytes (CD44hiCD62LhiCCR7+) (45). By contrast, most CD8+ T cells in the spleens of graft-accepting recipients were naive (CD44loCD62Lhi), with lower levels of IFN-γ production (Figure 5B).

While the vast majority of studies suggest that memory T lymphocytes potentiate alloimmune responses and inhibit tolerance induction (46, 47), it is possible that certain subsets of these cells...
may suppress alloreactivity (48). It is noteworthy that CD8+ T lymphocytes, including memory CD8+ T cells, were present in the lung at baseline, even in the absence of acute inflammation or alloantigen stimulation (Figure 6A). This has been attributed to the lung’s constant exposure to the environment and need to mount rapid responses to pathogens (49). We next set out to investigate whether memory CD8+ T cells from lungs of resting mice also had regulatory capacity. For this purpose, we flow cytometrically sorted CD8+CD44hiCD62Lhi central memory and CD8+CD44hiCD62Llo effector memory T lymphocytes (45) from resting B6 mice and used them as regulators in in vitro MLRs, using methods similar to those described above (Figure 4A). Interestingly, even without prior in vitro stimulation, central memory CD8+ T lymphocytes could suppress proliferation of B6 CD45.1+ T cells stimulated with BALB/c splenocytes (Figure 6B), albeit to a lesser extent than those derived from transplanted grafts (Figure 4B). However, freshly isolated CD8+ effector memory T lymphocytes had no effect on B6 CD45.1+ T cell proliferation (Figure 6B).

To further evaluate whether different subsets of memory T cells could influence the alloimmune response, we generated central memory or effector memory CD8+ T cells in vitro by culturing B6 splenocytes with irradiated BALB/c stimulators in the presence of IL-15 or IL-2, respectively, as previously described (50, 51). B6 Cd8–/– mice reconstituted with central memory CD8+ T cells accepted, while those reconstituted with effector memory CD8+ T cells rejected, BALB/c lung allografts after costimulatory blockade (Figure 6, C and D). Collectively, these data demonstrate that subtypes of memory CD8+ T cells can differentially influence the alloimmune response and that central memory CD8+ T cells play a critical role in lung allograft acceptance.

Central memory CD8+ T cells suppress alloimmune responses through IFN-γ-mediated production of NO. Since central memory T cells are known to be rapid producers of proinflammatory cytokines, we next examined whether CD8+ T lymphocytes mediate lung allograft acceptance through secretion of IL-15 or IL-2, respectively, as previously described (50, 51). B6 Cd8–/– mice reconstituted with central memory CD8+ T cells accepted, while those reconstituted with effector memory CD8+ T cells rejected, BALB/c lung allografts after costimulatory blockade (Figure 6, C and D). Collectively, these data demonstrate that subtypes of memory CD8+ T cells can differentially influence the alloimmune response and that central memory CD8+ T cells play a critical role in lung allograft acceptance.

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**Figure 3**

CD4+ T lymphocyte responses in CSB-treated lung transplant recipients. (A) The proportion of lung-resident CD4+Foxp3+ T cells is not different in resting B6 and B6 Cd8–/– lungs. These numbers do increase, however, after allograft transplantation, and a higher abundance of graft-infiltrating CD4+Foxp3+ T cells is detectable in B6 compared with B6 Cd8–/– lung graft recipients (comparison between resting and transplanted lungs by ANOVA and comparison between B6 and B6 Cd8–/– groups by unpaired t test). (B) Proliferation of B6 CD4+CD45.1+ T cells was greater after injection into B6 Cd8–/– (51.3% ± 5%) than B6 wild-type (20.6% ± 4%) recipients (P = 0.0017 by unpaired t test). Proliferating CD4+CD45.1+ T cells in B6 Cd8–/– recipients upregulated CD27, ICOS, and CD40 but not CD28 or CD154 compared with wild-type mice. Numbers in contour plots represent the percentages of adoptively transferred CD4+CD45.1+ T cells that have undergone proliferation (shaded gray, isotype controls; black lines, B6 wild-type; red lines, B6 Cd8–/– recipients). (C) Inhibiting CD27/CD70, ICOS/ICOS ligand, and OX40/OX40 ligand in addition to blocking CD40/CD154 and CD28/B7 does not prevent rejection in the absence of CD8+ T cells (P = 0.00074 vs. Figure 2A by Mantel-Haenszel χ² test). All gross and histological appearances as well as rejection grades represent grafts at 7 days after transplantation (original magnification, ×200 [histology, H&E staining]). TXP denotes graft, and the arrow points to perivascular infiltrates.
CD8+ T cell–mediated suppression of CD4+ T cell proliferation was also abrogated in vitro in the presence of IFN-γ–neutralizing antibody (Figure 7B). Ifng levels were significantly elevated in grafts after transplantation of BALB/c→B6 lung transplants and adding them as “regulators” to cocultures of BALB/c splenocytes (stimulators) and CFSE-labeled B6 CD45.1+ T cells (responders). (B) After 5 days of coculture the majority of (B) B6 CD4+CD45.1+ T cells or (C) B6 CD8+CD45.1+ T cells proliferate and blast, as evidenced by size (forward scatter) (top row). Proliferation and blasting is inhibited if CD8+ T cells isolated from accepting lung allografts are added to the MLRs (second row). No inhibition is evident if CD8+ T cells are isolated from the spleens of accepting mice (third row). Numbers in histograms represent percentages of (B) CD4+CD45.1+ or (C) CD8+CD45.1+ T cells that have undergone proliferation. Proliferation and size (forward scatter) in the respective groups are summarized in the bottom panels of B and C, with pair-wise comparison between groups performed by t test. 

Figure 4
Graft-infiltrating CD8+ T cells play a critical role in downregulating alloimmune responses. (A) In vitro MLRs were established by isolating CD8+ T cells from CSB-treated BALB/c→B6 lung transplants and adding them as “regulators” to cocultures of BALB/c splenocytes (stimulators) and CFSE-labeled B6 CD45.1+ T cells (responders). (B) After 5 days of coculture the majority of (B) B6 CD4+CD45.1+ T cells or (C) B6 CD8+CD45.1+ T cells proliferate and blast, as evidenced by size (forward scatter) (top row). Proliferation and blasting is inhibited if CD8+ T cells isolated from accepting lung allografts are added to the MLRs (second row). No inhibition is evident if CD8+ T cells are isolated from the spleens of accepting mice (third row). Numbers in histograms represent percentages of (B) CD4+CD45.1+ or (C) CD8+CD45.1+ T cells that have undergone proliferation. Proliferation and size (forward scatter) in the respective groups are summarized in the bottom panels of B and C, with pair-wise comparison between groups performed by t test.

7A and Supplemental Figure 3). CD8+ T cell–mediated suppression of CD4+ T cell proliferation was also abrogated in vitro in the presence of IFN-γ–neutralizing antibody (Figure 7B). Ifng levels were significantly elevated in grafts after transplantation of BALB/c lungs into immunosuppressed B6 wild-type recipients compared with B6 Cdx8−/− recipients (Figure 7C). Finally, injection of Ifng−/− CD8+ T cells into Cdx8−/− mice failed to rescue BALB/c lung allografts from rejection, despite costimulatory blockade (Figure 7D). Taken together, these data demonstrate that IFN-γ production by CD8+ T cells plays a critical role in lung allograft acceptance.

In in vitro MLRs described above (Figure 4A), we noted that the majority of CD4+CD45.1+ responder T lymphocytes were not
viable as measured by 7-AAD uptake when CD8+ T cells obtained from accepting lung allografts were added to the cultures (Figure 8A). Moreover, sensitivity of antigen-presenting cells to IFN-γ was critical for CD8+ T cell–mediated suppression, as proliferation of IFN-γ receptor–deficient CD4+ T cells was inhibited by allo-graft-derived CD8+ T cells, but no inhibition was evident if IFN-γ receptor–deficient antigen-presenting cells were used (Figure 8B). Also, CD8+ T cell–mediated suppression was not observed when T cells were activated with anti-CD3 and anti-CD28 antibodies in an antigen-presenting cell–free system (Figure 8C). Taken together, these data indicate that CD8+ T cells require antigen-presenting cells to downregulate T lymphocyte responses and the process depends on death of alloreactive cells.

Since metabolism of essential amino acids is a common mechanism of immunoregulation by antigen-presenting cells (52), we next added various pharmacologic inhibitors to in vitro MLRs and noted that only L-NNA (Nω-nitro-L-arginine), an inhibitor of endothelial, neuronal, and inducible NO synthase (eNOS, nNOS, and iNOS, respectively), and L-nil [N6-(1-iminoethyl)-L-lysine, dihydrochloride], a selective iNOS inhibitor, were able to attenuate and iNOS, respectively), and L-nil [N6-(1-iminoethyl)-L-lysine, dihydrochloride], a selective iNOS inhibitor, were able to attenuate the capacity to produce IFN-γ and expressed a central memory phenotype (CD44hiCD62LloCCR7+). Fewer cells in spleens of lung graft recipients had the capacity to produce IFN-γ, and only few cells had a central memory T cell phenotype. Numbers in density plots in A and B represent percentages of CD8+ T cells expressing indicated markers. Phenotype of CD8+ T cells is representative of at least 4 separate experiments.

Figure 5
IFN-γ–producing central memory CD8+CD44hiCD62LloCCR7+ T cells infiltrate accepting lung allografts. (A) Flow cytometry of CD8+ T lymphocytes in lung allografts of acceptors demonstrated few Foxp3+ or IL-10–producing cells. A large proportion of lung-resident CD8+ T cells had the capacity to produce IFN-γ and expressed a central memory phenotype (CD44hiCD62LloCCR7+). (B) Fewer cells in spleens of lung graft recipients had the capacity to produce IFN-γ, and only few cells had a central memory T cell phenotype. Numbers in density plots in A and B represent percentages of CD8+ T cells expressing indicated markers. Phenotype of CD8+ T cells is representative of at least 4 separate experiments.
spleens of lung graft recipients (Supplemental Figure 5). Similar to central memory CD8+ T lymphocytes, graft infiltration of in vitro–generated anti-BALB/c CD8+ effector memory T cells was impaired after PTX treatment (Figure 9A). However, the absolute number of anti-donor effector memory T cells accumulating in the lung was significantly lower than that of anti-donor central memory T cells (Figure 9A). Collectively, these data suggest that chemokine receptor signaling as well as alloantigen recognition play a role in graft infiltration by CD8+ central memory T lymphocytes.

**CCR7 expression on CD8+ T cells is critical for lung allograft acceptance.** As the expression of the Gαi-coupled chemokine receptor C-C chemokine receptor type 7 (CCR7) is a hallmark of central memory T cells, and a large portion of CD8+CD44hiCD62Llo T cells in accepting lung allografts express CCR7 (Figure 5A), we next explored whether this specific chemokine receptor plays a role in mediating lung allograft acceptance. We first transplanted BALB/c lungs into immunosuppressed Cd8–/– recipients and observed that these grafts were acutely rejected (Supplemental Figure 6). As several cell populations in addition to T cells can express CCR7, we next focused on T lymphocytes by adoptively transferring B6 Ccr7–/– CD8+ effector memory T lymphocytes despite costimulatory blockade (P = 0.00105 compared to Figure 2E by Mantel-Haenszel χ² test). BALB/c lungs are rejected by B6 Cd8–/– recipient mice reconstituted with in vitro–generated anti-BALB/c CD8+ effector memory T lymphocytes despite costimulatory blockade (P = 0.00105 compared to Figure 2E by Mantel-Haenszel χ² test). TXP denotes graft, and the arrow points to perivascular infiltrates. All gross and histological appearances as well as rejection grades represent grafts at 7 days after transplantation (original magnification, ×200 [histology, H&E staining]).
described method to image murine lungs by 2-photon microscopy in vivo (58). We transplanted BALB/c lungs into immunosuppressed B6 CD11c-EYFP hosts, which express enhanced yellow fluorescent protein under a CD11c promoter, and injected fluorescently labeled B6 CD8+ wild-type and Ccr7–/– T cells 3 days after engraftment. When we imaged these lung grafts 24 hours later, we observed that wild-type CD8+ T lymphocytes made stable and long-lasting contacts with graft-infiltrating recipient CD11c+ cells. By contrast, in the absence of CCR7 expression, CD8+ T cells interacted with CD11c+ dendritic cells only briefly, with significantly shorter retention times (P < 0.001) (Figure 10 and Supplemental Video 1). Collectively, these findings suggest that, in addition to directing trafficking of T lymphocytes into the lung, chemokine receptor signaling regulates contact between graft-infiltrating CD8+ T cells and alloantigen-expressing cells, which is associated with decreased local production of IFN-γ and graft rejection.

Discussion
The overwhelming success of costimulation blockade (CSB) in extending graft survival in small animal models of organ transplantation has laid the foundation for translating this therapy to the clinics (59). However, kidney transplantation experiments in non-human primates demonstrated that alloreactive memory T cells, generated through heterologous immunity, may represent a barrier to long-term graft survival in animals raised outside the confines of specific pathogen-free conditions (60, 61). This has been suspected to be especially problematic in recipients with a high frequency of CD8+ memory T cells, due to rapid graft infiltration by this cell population (13, 15). Based on these observations, strategies have been developed to either globally deplete T lymphocytes during the perioperative period (62) or specifically target memory T cells (21). We have previously reported that treatment of lung allograft recipients with CTLA4-Ig alone does not prevent acute rejection regardless of presence of CD4+ T cells (31). Additional treatment with anti-CD154 prevents rejection after transplantation of lungs into wild-type or even CD4+ T cell–depleted allogeneic hosts, possibly due to transient expression of this costimulatory molecule on CD8+ T lymphocytes or other cells (13, 63).

The unique features of the lung, such as the rapidity and local initiation of the immune response, have allowed us to unravel a previously unrecognized and critical role for CD8+CD44hiCD62Lhi CCR7+ T cells in the induction of graft acceptance. We and others have shown that lungs provide a suitable environment for the activation of adaptive immunity in the absence of secondary lymphoid organs (64–66). Our recent studies have demonstrated that innate and adaptive immune cells rapidly infiltrate lung grafts and that their interactions within the graft determine the fate of this organ (58, 67). Of particular relevance to the current findings, we have shown recently that immune responses contributing to lung allograft acceptance are established locally in the graft shortly after transplantation (30), while other tissue and organ grafts require the presence of secondary lymphoid organs for the initia-
Our findings, with regard to trafficking requirements of CD8+ T cells to pulmonary allografts, further extend the notion that lungs differ immunologically from other transplanted organs. It has been demonstrated recently that antigen recognition regulates trafficking of effector CD8+ T cells into murine heart grafts (71). Consistent with these data, we now demonstrate that in vitro–generated central memory CD8+ T lymphocytes infiltrate lung allografts to a significantly larger extent compared with anti-third party central memory CD8+ T cells. However, in direct contrast to

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Figure 8
CD8+ T cells suppress through IFN-γ–mediated production of NO. (A) After 5 days, the majority of CD4+CD45.1+ T cell “responders” are not viable if CD8+ T cells from accepting allografts are added. CD4+CD45.1+ T cell viability (by 7-AAD uptake) and representative plots of CFSE vs. 7-AAD are shown (groups compared by unpaired t test). (B) CD4+ T cell proliferation (CFSE) and viability (7-AAD) in an MLR containing Ifngr1−/− CD4+ T cell responders or Ifngr1−/− antigen-presenting cells (n ≥ 3). Numbers in density plots in A and B represent percentages of CD4+CD45.1+ T cells within the respective quadrants, assessing their proliferation (CFSE) vs. viability (7-AAD). (C) CD4+ T cell proliferation after stimulation with platebound anti-CD3 and soluble anti-CD28 in the absence or presence of accepting allograft-derived CD8+ T cells (P = 0.55 between the 2 groups by unpaired t test). (D) CD4+ T cell proliferation with inhibitors of amino acid metabolism, arginine, or Inos−/− antigen-presenting cells (multiple group comparison performed by ANOVA). (E) NO levels in resting lungs, allografts, and right native lungs (n ≥ 3) (unpaired t test). (F) BALB/c lungs transplanted into CSB-treated Inos−/− B6 recipients (P = 0.00059 vs. Figure 2A by Mantel-Haenszel χ2 test). TXP denotes graft, and the arrow points to perivascular infiltrates. All gross and histological appearances as well as rejection grades represent grafts at 7 days after transplantation (original magnification, ×200 [histology, H&E staining]).
heart allografts, we now demonstrate that \( G_\alpha_1 \) receptor signaling is also critical for donor-primed CD8+ effector and central memory T cell infiltration into lung grafts. Our findings extend recent reports that chemokine receptor expression on T cells regulates their homing to virally infected lungs (72). We thus show that both alloantigen- and \( G_\alpha_1 \)-dependent chemokine signaling play a role in memory T lymphocyte migration into lungs.

Since their description almost 2 decades ago (73), the majority of studies investigating mechanisms of immune regulation have focused on CD4+Foxp3+ regulatory T cells (69). Despite experimental evidence dating back to the 1970s that CD8+ T cells can suppress immune responses, only recently has this cell population experienced a resurgence in the literature. This is in large part due to the phenotypic heterogeneity of CD8+ T cells with suppressive function. To this end, CD8+ T cells with both naive and memory phenotypes have been described to have regulatory capacity. Expansion of naive human CD8+CCR7+ T cells with low-dose anti-CD3 and IL-15 induces their expression of Foxp3, CD25, and CD103 and their ability to suppress activation of CD4+ T cells (74). In mice, CD8+ Foxp3+ T cells can regulate skin allosensitization responses in a contact-dependent fashion (43), and a similar population of cells that relies on direct interaction with CD4+ T cell responders has been described in humans (74). In rats, a regulatory CD8+Foxp3+CTLA4+CD45RCD38+ population has been described; however, controversy exists as to whether these cells suppress via production of cytokines or cell-to-cell contact (42, 75). Reports that CD8+ T cells can suppress through TGF-\( \beta \) also exist (48, 76). In contrast to these reports, we now describe an IFN-\( \gamma \)-dependent mechanism of CD8+CCR7+ T cell–mediated immunosuppression in the murine lung.

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CCR7 expression is a hallmark of central memory T cells and regulates their homing to lymph nodes. Investigations into the role of CCR7 in transplant rejection have yielded conflicting results, which may be in part due to this molecule regulating migration and function of multiple cell populations. Hearts and skin experienced a moderate prolongation in survival after transplantation into Ccr7–/– recipients, which is associated with reduced alloantigen-presenting cells, such as plasmacytoid dendritic cells, into draining lymph nodes (79) or CD4+Foxp3+ T cells into grafts (80).
Here, we report that CCR7-expressing CD8+ T cells are critical for lung allograft acceptance. Mechanistically, we show by intravital 2-photon microscopy that, in the absence of CCR7, CD8+ T cells are unable to form durable interactions with antigen-presenting cells within the graft, which is associated with lower expression of IFN-γ. These findings extend previous reports showing that dendritic cells express CCL21 and that surface-bound CCR7 ligands induce tethering of T lymphocytes to antigen-presenting cells during the formation of stable synapses (81, 82). It has also been shown that dendritic cells bind more CCR7 ligands on their surface than other cell populations (57). Previous reports have pointed to a role of CCR7 ligands in T cell differentiation. Stimulation of dendritic cells with CCR7 ligands induces their production of IL-12 and IL-23, which can drive Th1 and Th17 differentiation, respectively (83). Consistent with our findings, others have also observed that CCR7 expression on T cells is critical to mount an IFN-γ-dependent immune response that is required to clear pathogens (84).

Traditionally, Th1 responses have been considered to be instrumental in promoting cell-mediated rejection. We have described an accumulation of IFN-γ-producing CD4+ and CD8+ T cells in nonimmunosuppressed lung allografts that undergo acute rejection (31). Perhaps more importantly, excessive activation of Th1 responses due to ischemia/reperfusion injury abrogates immunosuppression-mediated lung graft acceptance (67). However, the absence of IFN-γ can also have deleterious effects on graft survival. Cardiac allografts undergo necrosis in the absence of recipient IFN-γ despite immunosuppression, which has been attributed to inefficient deletion of activated T lymphocytes (85). Activation of alternative pathways, such as the Th17 differentiation pathway, may also mediate aggressive proinflammatory responses in the absence of IFN-γ (86, 87).

As memory T lymphocytes in peripheral organs provide a first line of defense against infection, mucosal barrier organs, such as the lung, are especially rich in this cell population (88, 89). In fact, independent of antigen or inflammation, memory T cells are retained in lungs, in which they are rapid producers of proinflammatory cytokines (89, 90). As uncontrolled inflammatory responses in the lung can result in potentially life-threatening pulmonary dysfunction, mechanisms have evolved to limit the extent of inflammation to prevent tissue damage (91). For example, iNOS limits pulmonary inflammation in several models of lung injury (92, 93). Thus, it is possible that our costimulatory blockade protocol relies on a naturally occurring IFN-γ- and NO-dependent “feedback mechanism” normally operational in the lung. In contrast to central memory CD8+ T cells, effector memory CD8+ T cells do not promote...
lungs as a source of NO for the lung microenvironment (41). IL-4 and IL-13 from mast cells, fibroblasts, and other cells in the airway epithelial layer or from alveolar 24-hour explants, as previously described (51). We used three different assays to detect NO production: the Griess reaction, NO measurement in vivo, and NO measurement in vitro. The Griess reaction, which is based on the formation of a dye by the reaction of diazotized sulphanilamide and nitrite, is a colorimetric assay that is sensitive to NO but not to nitrate. In the NO measurement in vivo assay, we used a NO-sensitive sensor that was placed in the lung tissue and monitored the NO concentration over time. This assay is based on the detection of NO by a NO-sensitive sensor, which is inserted into the lung tissue and monitored for changes in NO concentration over time. This assay is sensitive to both NO and nitrate, but it is specific for NO because the sensor is able to differentiate between the two compounds. In the NO measurement in vitro assay, we used a NO-sensitive sensor that was placed in the lung tissue and monitored the NO concentration over time. This assay is based on the detection of NO by a NO-sensitive sensor, which is inserted into the lung tissue and monitored for changes in NO concentration over time. This assay is sensitive to both NO and nitrate, but it is specific for NO because the sensor is able to differentiate between the two compounds. In the NO measurement in vitro assay, we used a NO-sensitive sensor that was placed in the lung tissue and monitored the NO concentration over time. This assay is based on the detection of NO by a NO-sensitive sensor, which is inserted into the lung tissue and monitored for changes in NO concentration over time. This assay is sensitive to both NO and nitrate, but it is specific for NO because the sensor is able to differentiate between the two compounds.

Methods

Animals. Wild-type, Ifng−/−, Ifng1−/−, Ccr7−/−, Gdb−/−, Inos−/−, Tnfa−/−, CD11c-EYFP, CD45.1−/−, and B cell-deficient (Ighm−/−) mice, all on a B6 (H-2Kb) background, as well as BALB/c (H-2Kb), CBA/Ca (H2Kk), and nude mice were purchased from The Jackson Laboratory. Animals were housed in a barrier facility in air-filtered cages. Left orthotopic vascularized lung transplants were performed as previously described (40) with CSB in select experiments consisting of MR1 (250 μg i.p., day 0) and CTLA4-Ig (200 μg i.p., day 2). As indicated for select experiments, CD8−/− T cells were depleted in vivo by YTS 169.1 (250 μg i.p., days –3, –1). IFN-γ was neutralized using hamster anti-mouse anti–IFN-γ antibody (clone H22) (500 g day−2, 250 μg i.p. day−1), and CD4+ T cells were depleted using GK1.5 (100 μg i.p., days –3, –1). For select experiments, OX40/OX40 ligand (clone OX-86), CD27/CD70 (clone FR-70), and ICOS/ICOS ligand (clone 17C9) pathways were inhibited as previously described (all antibodies from BioXcell) (41). For some experiments, nude mice were reconstituted with 105 CD4+ T cells isolated from the spleens and peripheral lymph nodes of B6 wild-type mice and, for others, CFSE-labeled CD4+CD45.1− T cells were adoptively transferred into B6 mice. Reconstitution of B6 Gdb−/− mice was performed with a minimum of 5 × 105 CD8−/− T cells isolated either by flow cytometric sorting or magnetic bead isolation (Miltenyi Biotech).

Memory cell generation and injection. Both central and effector memory CD8−/− T cells were generated in vitro based on previously described methods (50, 51). Briefly, central memory cells were generated by coculturing B6 CD45.1− splenocytes with irradiated BALB/c (donor) or CBA/Ca (third party) splenocyte stimulators. Sixty hours after initiation of the cocultures dead cells were removed by Ficoll-Paque density centrifugation and CD8−/− T cells were positively selected with magnetic beads. CD8−/− T cells were then expanded in 20 ng/ml IL-15 (R&D Systems) and injected intravenously approximately 2 weeks later. Effector memory cells were generated by coculturing B6 CD45.1− splenocytes with irradiated BALB/c stimulators in the presence of 1,000 U/ml IL-2 (NIH NCI-Clinical Repository). For homing studies, 5 × 106 effector memory cells and 1 × 106 central memory cells were injected per mouse 2 to 3 days after transplantation. For reconstitution experiments, B6 Gdb−/− mice were injected with 5 × 106 effector or central memory cells 48 to 72 hours prior to BALB/c lung allograft transplantation. For some experiments, memory cells were treated with 200 ng/ml PTX for 30 minutes prior to injection. For homing studies, lung grafts were flushed with 2.5 ml sterile saline prior to analysis.

Histology. Transplanted mouse lungs were fixed in formaldehyde, sectioned, and stained with H&E. A lung pathologist (J.H. Ritter) blinded to the experimental conditions graded graft rejection using standard criteria (International Society for Heart and Lung Transplantation [ISHLT] A Grade) developed by the Lung Rejection Study Group (32).

Flow cytometry. All antibodies for flow cytometry were primarily fluorochrome conjugated and purchased from eBioscience. Intracellular staining was performed as previously described (31).

In vitro MLRs. In vitro MLRs were performed in round bottom 96-well plates using 3 × 105 T cell–depleted BALB/c splenocyte stimulators, with 105 CFSE-labeled B6 CD45.1−CD4+ or CD8−/− T cells responders and, as indicated above, 105 CD8−/− T cells isolated from lungs or spleens of immunosuppressed B6 recipients of BALB/c allografts. For some experiments, central and effector memory T cells were sorted from lungs of resting mice. T cell responses were evaluated flow cytometrically on day 5. All compounds inhibiting the metabolism of essential amino acids were obtained from Sigma-Aldrich and added to the cocultures as previously described for the duration of the experiment (41).

Quantitative gene expression analysis. For quantitative gene expression analysis, mRNA from whole lung grafts was isolated in accordance with the manufacturer’s instructions. Quantitative real-time RT-PCR was conducted on an ABI 7900 using TaqMan Gene Expression Assay system (Applied Biosystems) in accordance with the manufacturer’s recommendations. Amplification of target sequences was conducted as follows: 50°C for 20 minutes and 95°C for 10 minutes, followed by 38 to 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primers and MGB probes were purchased as kits from Applied Biosystems and can be identified in the following manner: IFN-γ (Mm01168134_m1) and β-2 microglobulin (Mm00437762_m1). Transcript levels of IFN-γ are expressed relative to transcript levels of β-2 microglobulin.

NO measurement in vivo. In vivo experiments were carried out using a 2 mm NO Sensor (World Precision Instruments) connected to a Free Radical Analyzer TBR-1025 (World Precision Instruments). The specifications include a 2 pA/nM sensitivity with a 1 nM minimum detection limit. Prior to the experiments, the sensor was polarized for at least 24 hours before use according to the manufacturer’s recommendations. After sedating the mouse, a 2 mm long and 1 mm deep incision was made in the lung tissue to provide an area for the sensor to rest in. Approximately 0.5 ml saline was applied to the incision in order to provide an interface between the mouse lung and sensor and also to monitor the integrity of the sensor’s NO-selective membrane. The data from each lung were recorded using a LabTrax data acquisition unit and LabScribe software for 5 minutes after reaching a stable signal. The data were then analyzed against a baseline signal from normal saline and converted from current to NO concentration in ppm using NO donor DEA-Nonoate (Cayman Chemical) dissolved in PBS buffer as a standard.

Immunostaining. Lungs were cryopreserved and then cut into 6-μm-thick sections. Sections were fixed in pure acetone for 10 minutes at −20°C and blocked with 10% normal donkey serum. Unlabeled anti-CD4 (H129.19) and anti-CD8 (53-6.7) (Pharmingen) were visualized using donkey anti-rat IgG conjugated with indocarbocyanine (Cy3) (Roche). Slides were imaged using an Olympus BX51 microscope. No detectable staining was observed with isotype-matched or species-specific control antibodies.

Intratracheal 2-photon microscopy. BALB/c lungs were transplanted into immunosuppressed B6 CD11c-EYFP recipients and, on postoperative day 3, received an injection of 105 CMTMR-labeled Ccr7−/− and 105 CD8−/− T cells isolated from wild-type B6 mice expressing cyan fluorescent protein under an actin promoter. Twenty-four hours after injection of T cells, time-lapse
imaging was performed with a custom-built 2-photon microscope running ImageWarp version 2.1 acquisition software (A&K Software). For time-lapse imaging of T-cell–CD11c⁺ dendritic cell interactions in lung tissue, we averaged 15 video-rate frames (0.5 seconds per slice) during the acquisition to match the ventilator rate and to minimize movement artifacts. Each plane represents an image of 220 × 240 μm in the x and y dimensions. Twenty-one sequential planes were acquired in the z dimension (2.5 μm each) to form a z stack. Each individual T cell was tracked from its first appearance in the imaging window and followed up to the time point at which it dislocated more than 20 μm from its starting position. T cells that did not travel were tracked for the duration of the imaging period.

Statistics. Continuous variables, such as in vitro and in vivo T cell proliferation, gene expression levels, retention times of T cells, number of memory T cells penetrating lung grafts, and NO levels, were compared among various conditions. Two-tailed Student’s t test was used for 2 comparisons and ANOVA was used for multiple comparisons, as indicated in the appropriate figure legends. For ordinal variables, such as lung allograft rejection scores, the Mantel-Haenszel χ² test was used. Data in figures are presented as mean ± SEM. A P value of more than 0.05 is assumed to be not statistically significant.

Study approval. All animal procedures were approved by the Animal Studies Committee at Washington University School of Medicine, St. Louis, Missouri, USA.


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