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Cutting Edge: *Pseudomonas aeruginosa* Abolishes Established Lung Transplant Tolerance by Stimulating B7 Expression on Neutrophils

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The mechanisms that link bacterial infection to solid organ rejection remain unclear. In this study, we show that following the establishment of lung allograft acceptance in mice, *Pseudomonas aeruginosa* airway infection induces a G-CSF–dependent neutrophilia that stimulates acute rejection. Graft-infiltrating neutrophils sharply upregulate the B7 molecules CD80 and CD86, but they do not express CD40 or MHC class II in response to *P. aeruginosa* infection. Neutrophil B7 promotes naïve CD4+ T cell activation and intragraft IL-2, IFN-γ, and IL-17 T lymphocyte accumulation. Intravital two-photon microscopy reveals direct interactions between neutrophils and CD4+ T cells within pulmonary allografts. Importantly, lung rejection in *P. aeruginosa*-infected recipients is triggered by CD80/86 on neutrophils and can be prevented by B7 blockade without affecting clearance of this pathogen. These data show that neutrophils enhance T cell activation through B7 trans-costimulation and suggest that inhibiting neutrophil-mediated alloimmunity can be accomplished without compromising bacterial immune surveillance. *The Journal of Immunology*, 2012, 189: 4221–4225.

In human lung recipients, posttransplant airway colonization with *Pseudomonas aeruginosa* is associated with graft rejection (1). Airway neutrophilia that often accompanies such infections has also been linked to both chronic and acute lung allograft rejection (2). G-CSF is a critical mediator of neutrophil mobilization in *P. aeruginosa*-infected lungs (3). Accordingly, we reported that G-CSF–driven granulopoiesis leads to pulmonary tissue injury and prevents immunosuppression-mediated acceptance of mouse lung allografts (4, 5). Neutrophils have been proposed to regulate adaptive immune responses through a variety of mechanisms. Interestingly, neutrophils can express MHC class II, as well as costimulatory molecules, and several studies reported their capacity to act as APCs (6, 7). In addition to delivery of costimulatory signals by the cell that presents the Ag, adaptive-immune responses can be further enhanced by costimulatory signals expressed on bystander cells, a process referred to as trans-costimulation. Bystander APCs are thought to be the major mediators of B7 trans-costimulation and were shown to play a critical role in promoting solid organ rejection (8). In this article, we provide evidence that, in response to *P. aeruginosa* infection, G-CSF–mobilized neutrophils upregulate and provide B7 trans-co-stimulatory signals to T cells and prevent established lung allograft tolerance.

**Materials and Methods**

**Mice**

C57BL/6j (B6), BALB/c (BALB/c), and B6 CD11b−/− mice are from The Jackson Laboratory. B6 CD11c-EYFP mice were crossed with B6 LysM-GFP mice to generate double-reporter mice (B6 CD11c-EYFP LysM-GFP). All experiments were approved by the Washington University Animal Studies Committee.

Lung transplantation, infection, Abs, and neutrophil adoptive transfer

Lung transplantation was conducted as previously described (9), and all graft recipients were treated with CD154:CD40 blockade via CD154 Ab clone MR3 (250 μg, postoperative day [POD] 0 and CD28/B7 blockade via CTLA4-Ig (200 μg, POD 2; both from Bio X Cell), which we showed maintains acceptance for ≥100 d (10). A total of 2.5 × 10^6 CFU *P. aeruginosa* strain P01, live (*P. aeruginosa*; 65˚C for 1 h) was resuspended in 50 μl normal saline for airway administration. A total of 200 μg G-CSF Abs (PeproTech) or 250 μg Ab clone 1A8 Ly6G (Bio X Cell) neutrophil-depleting Abs was administered i.v. 4 h prior to *P. aeruginosa* inoculation. Neutrophils were purified by negative selection, as previously described (4). A total of 10^7 neutrophils was injected i.v. into *P. aeruginosa*-infected G-CSF Ab-treated lung recipients once a day for up to 3 d.

**Rejection assessment**

H&E sections of allograft tissue from uninfected and infected recipients were screened in a double-blind fashion for the presentation of dense perivascular infiltrates and scored by the criteria set forth by the International Society for Organ Transplantation. Bronchoalveolar lavage fluid (BALF) was collected 7 d posttransplant via a catheter introduced via the trachea. Total BALF cellularity was enumerated by hemocytometry, and mononuclear cell populations were enumerated by multicolor flow cytometry. The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; hPA, heat-killed *Pseudomonas aeruginosa*; POD, postoperative day.
Two-photon microscopy
On POD 7, BALB/c → B6 CD11c-EYFP LysM-GFP mice received P. aeruginosa and 5 × 10^6 CellTracker Red (Invitrogen)-labeled B6 CD4^+ T cells. On POD 8, time-lapse imaging was performed with a custom-built two-photon microscope running ImageWarp acquisition software (A&B Software) (5). For time-lapse imaging of neutrophil–CD4^+ T cell interactions in the lung tissue, we averaged 15 video-rate frames (0.5 s/slice).

T cell analysis
Lung tissue digests were performed, and T cell intracellular expression of IFN-γ, IL-17A, and IL-2 was measured, as previously described (4, 12). IL-2 culture production was measured by ELISA (eBioscience). Intragraft CD4^+ T cells were isolated with anti-CD4 beads (Miltenyi Biotec) and cultured with BALB/c bone marrow-derived dendritic cells (DCs) for 36 h. Splenic naive CD4^+ T cells were isolated by flow cytometric sort on a CD90.2^+CD25^−CD62L^hiCD44^loCD4^+ gate. Alloantigen-specific CD4^+ T cell responses were generated with irradiated BALB/c T cell-depleted splenocytes for 36 h, and IL-17 and IFN-γ were determined by FACS-cytokine secretion assay (Miltenyi Biotec).

Neutrophil assessment
Neutrophils were identified as Ly6G^hiGr1^hiCD11b^+CD115^−^ cells by FACS and quantified by multiplying the percentage abundance by the total cell count in the bronchoalveolar lavage fluid (BALF), as previously described (4). Neutrophils were stained with Abs (BD Pharmingen) to CD80 (16-10A1), CD86 (AF6-120.1), CD40 (3/23), and Ia^b^ (GL1).

Statistical analysis
Data were analyzed using GraphPad Prism, version 5.0, and the results are presented as mean ± SEM. An unpaired two-tailed Student t test was used to evaluate pairs of means for significance. The p values < 0.05 were considered significant.

Results and Discussion
P. aeruginosa infection prevents established lung tolerance
Based on clinical reports that P. aeruginosa colonization shortens human pulmonary allograft survival (1), we asked whether this infection abrogates established tolerance in a model of immunosuppression-mediated BALB/c → B6 lung acceptance (4, 10). On POD 7, the lung recipients received saline or 2.5 × 10^6 CFU of P. aeruginosa intratracheally, and allograft histology, T lymphocyte intragraft accumulation, and neutrophilia were analyzed for up to 3 wk postinfection (Fig. 1A, 1B). Compared with saline-treated control recipients, there was progressive histological evidence of lymphocytic vascular rejection and the accumulation of graft
infiltrating IL-17+CD4+, IFN-γ+CD4+, and IFN-γ+CD8+ T cells in *P. aeruginosa*-infected recipients. Additionally, lung rejection was associated with more alloantigen-specific Th1 and Th17 cells compared with saline-treated lung recipients (Fig. 1C). Finally, allograft rejection could be induced by *P. aeruginosa* infection in recipients that had accepted their lungs for >100 d, suggesting the abrogation of regulatory mechanisms that also promote long-term acceptance (Supplemental Fig. 1A).

*P. aeruginosa* infection also stimulated high levels of lung neutrophilia (Fig. 1D), which resolved only partially despite the rapid clearance of this pathogen from lung airways (Fig. 1E). To assess the role of neutrophilia in lung survival, we used several approaches to prevent neutrophil graft accumulation in *hkPA*-treated lung recipients (Fig. 1F); live strain administration is lethal to neutrophil-depleted lung recipients because of their inability to clear this infection (13). Importantly, *hkPA* induced patterns of allograft rejection, as well as as intragraft Th1 and Th17 cell accumulation, comparable to the live strain (Supplemental Fig. 1B). Inhibiting neutrophil accumulation with either G-CSF Ab blockade or Ly6G Ab-mediated neutrophil depletion led to similar reductions in intragraft Th1 and Th17 cells, as well as protection against lung allograft rejection (Fig. 1G, 1H). Moreover, allograft survival was also maintained in CD11b−/− recipients, a critical mediator of neutrophil trafficking into the lung (14). Therefore, these data collectively show that *P. aeruginosa* initiates a pulmonary neutrophilia that prevents established allograft tolerance.

**Neutrophil B7 trans-costimulation promotes CD4+ T cell activation**

Reports of neutrophils acquiring the characteristics of APCs in both humans (7) and mice (6) led us to investigate whether these cells could directly promote naive CD4+ T cell activation. Although neutrophils did not express MHC class II or CD40 in *P. aeruginosa*-infected lung recipients, we detected high levels of CD80 and CD86 on these cells (Fig. 2A), suggesting CD4+ T cell activation potential through B7 trans-costimulation. To investigate this possibility, we cocultured neutrophils from *P. aeruginosa*-infected mice with naive CFSE-labeled CD4+ T cells in the presence or absence of latex beads coupled to CD3 Abs and analyzed IL-2 expression and proliferation (Fig. 2B, 2C). In the absence of CD3 beads, neutrophils from *P. aeruginosa*-infected mice were unable to stimulate CD4+ T cell IL-2 expression or proliferation, consistent with a lack of MHC class II expression on these cells. However, in the presence of CD3 beads, CD4+ T cell IL-2 expression and proliferation were significantly augmented by neutrophils from *P. aeruginosa*-infected mice compared with neutrophils from either uninfected or *P. aeruginosa*-infected CD80−/−/CD86−/− mice. Consistent with these observations, B7 blockade mediated by CTLA4Ig markedly reduced the ability of neutrophils from *P. aeruginosa*-infected mice to enhance CD4+ T cell IL-2 expression and proliferation to levels that were comparable to cocultures with neutrophils from *P. aeruginosa*-infected CD80−/−/CD86−/− mice. Collectively, these data show that neutrophil B7 trans-costimulation enhances naive CD4+ T cell activation.

**Neutrophil B7 prevents established lung allograft tolerance**

We next asked whether neutrophil B7 stimulated CD4+ T cell activation in vivo. To this end, we sought to normalize for APC costimulatory molecule expression by adoptively transferring neutrophils isolated from either B6 or B6 CD80−/−/CD86−/− mice into *P. aeruginosa*-infected BALB/c → B6 lung recipients that were also treated with G-CSF Abs to ablate endogenous granulocyte mobilization (Fig. 3A). Strikingly, 36 h postinfection, the percentages of intragraft IL-2+ and IFN-γ+CD4+ T cells increased to levels nearly equivalent to those in control Ig-treated *P. aeruginosa*-infected lung recipients. Also, neutrophil B7 significantly augmented intragraft IL-17+CD4+ and IFN-γ+CD8+ T cell accumulation. We then analyzed the effect of neutrophil B7 on allograft tolerance by continuing adoptive transfers of B6 or CD80−/−/CD86−/− neutrophils for two additional days into G-CSF Ab-treated *P. aeruginosa*-infected lung recipients (Fig. 3B). CD80−/−/CD86−/− neutrophils had little impact on allograft inflammation 3 wk postinfection, whereas B6 neutrophil recipients

![Figure 2](http://www.jimmunol.org/).
In murine transplantation models (16) and human kidney recipients (17), B7-CD28 blockade strategies have been used to promote allograft acceptance, but their use to maintain established tolerance in infected recipients has not been reported. Therefore, we asked whether either CD154 Ab or CTLA4Ig administration at the time of *P. aeruginosa* infection could promote established lung tolerance in B6 lung allograft recipients (Fig. 3E). CD154 Ab treatment did not prevent acute rejection consistent with the absence of CD40 expression on neutrophils in *P. aeruginosa*-infected lung recipients. In contrast, CTLA4Ig-treated recipients maintained lung survival and had patterns of intra-allograft Th1 and Th17 cell abundance that were comparable with uninfected lung recipients (Fig. 3F versus Fig. 1B). Also, CTLA4Ig treatment did not affect airway neutrophil infiltration or *P. aeruginosa* infection clearance, indicating that targeting B7 function does not impair pulmonary bacterial immune surveillance (Fig. 3G, 3H).

In summary, we showed that neutrophil B7 expression induced by *P. aeruginosa* infection plays a critical role in preventing established lung tolerance through promoting T cell trans-costimulation. In light of previous work that showed the importance of TLRs in regulating organ tolerance (18, 19), our data provide additional insight into innate immune responses to infected allografts. Because pulmonary allografts are especially vulnerable to infection given the organ’s direct exposure to the external environment, a better understanding of how neutrophils regulate alloimmunity will be important for the development of more effective immunotherapeutic strategies for lung recipients.

(DC + B6 PMN), or cocultured with DCs separated from B6 neutrophils by Transwells (DC II B6 PMN) (n = 3). On POD 7, lung recipients received *P. aeruginosa* along with 200 μg of control Ig, CD154, or CTLA4Ig and were analyzed 3 wk later for H&E graft histology (n = 3) (E) (original magnification ×100) and intragraft T lymphocyte accumulation (n = 4) of BALB/c → B6 lung recipients 3 wk after receiving PA and G-CSF Abs, as well as either B6 or CD80<sup>−/−</sup>86<sup>−/−</sup> neutrophils. How-

![Graph](Image)

**FIGURE 3.** Lung allograft rejection in *P. aeruginosa*-infected recipients is neutrophil B7 dependent. (A) Percentage abundance of indicated intragraft CD4<sup>+</sup> and CD8<sup>+</sup> T cells 1 d after saline or *P. aeruginosa* inoculation into BALB/c → B6 lung recipients that either received control Ig or G-CSF Abs with B6 or CD80<sup>−/−</sup>86<sup>−/−</sup> neutrophils (n = 3). (B) H&E graft histology (n = 4) (original magnification ×100) and indicated intragraft T lymphocyte accumulation (n = 4) of BALB/c → B6 lung recipients 3 wk after receiving PA and G-CSF Abs, as well as either B6 or CD80<sup>−/−</sup>86<sup>−/−</sup> neutrophils. (C) Intra-allograft two-photon imaging within allografts of BALB/c → B6 CD11c-EYFP LysM-GFP lung recipients that received CellTracker Red-labeled CD4<sup>+</sup> T cells 1 d after *P. aeruginosa* infection. A zoomed view of a typical interaction between a CD4<sup>+</sup> T cell (red; yellow arrowheads), a DC (green; hollow arrowheads), and a neutrophil (blue; white arrowheads) in the y-x (left panel) and z-x (right panel) plane (n = 2). The white bar denotes a 10 μm scale. (D) IL-2 produced by intragraft CD4<sup>+</sup> T cells from uninfected BALB/c → B6 lungs cultured alone (No DC), cultured with allogeneic BALB/c bone marrow-derived DCs (DC), cultured with DCs in combination with PMN from *P. aeruginosa*-infected CD80<sup>−/−</sup>86<sup>−/−</sup> mice (DC + CD80<sup>−/−</sup>86<sup>−/−</sup> PMN), cultured with DCs in combination with B6 neutrophils from *P. aeruginosa*-infected B6 mice rejected their lungs in a manner similar to control Ig-treated *P. aeruginosa*-infected recipients. Because B7-dependent responses suggested direct association of graft-resident T cells with neutrophils, we used intravital two-photon microscopy to analyze cell–cell interactions within the allograft tissue (Fig. 3C, Supplemental Video 1). One day postinfection, we detected graft-infiltrating neutrophils making contact with CD4<sup>+</sup> T cells that were simultaneously associated with pulmonary CD11c<sup>+</sup> DCs, lending support for the B7 trans-costimulation three-cell contact model in vivo (15). To further assess whether these interactions can enhance graft-resident T lymphocyte activation, we purified CD4<sup>+</sup> T cells from BALB/c allografts and analyzed IL-2 expression following coculture with BALB/c bone marrow-derived DCs and neutrophils from *P. aeruginosa*-infected mice (Fig. 3D). B6 neutrophils were significantly better at stimulating IL-2 production than were CD80<sup>−/−</sup>86<sup>−/−</sup> neutrophils. However, when B6 neutrophils were separated by Transwells from DC-stimulated CD4<sup>+</sup> T cells, IL-2 expression decreased sharply, underscoring the requirement for neutrophils to have direct contact with intragraft T cells to promote alloimmunity.
Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure S1a and b:

(a) Histology and intragraft percent abundance of indicated CD4⁺ and CD8⁺ T cell populations from either uninfected or PA-infected Balb/c → B6 lung recipients with established tolerance for greater than 100 days. Histology and T cell analysis was conducted 3 weeks after infection and are representative of N=4. (b) Three weeks after saline or hkPA airway instillation into Balb/c → B6 lung recipients with established tolerance allograft tissue was analyzed for (left) H & E staining (N=4) or (right) indicated intragraft T lymphocyte accumulation (N=4).

Movie 1:

Intravital 2-photon imaging of a Balb/c lung allograft engrafted into a B6 CD11c-EYFP LysM-GFP lung recipient that received CMTPX-labeled CD4⁺ T cells one day after PA-infection. Blood vessels (red) were labeled by intravenous injection of non-targeted 655-nm quantum dots prior to imaging. A movie of a CD4⁺ T cell (red) while associated with a DC (green) interacting with several neutrophils (blue) in the z-x plane over a 45 minute period (representative of 2 independent experiments).