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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 naive 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 MR1 (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), or heat-killed dose equivalent (heat-killed P. aeruginosa [hkPA]; 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 clone 1A8 Ly6G (Bio X Cell) neutrophil-depleting Abs was administered iv. 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 iv. 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
Two-photon microscopy

On POD 7, BALB/c → B6 CD11c-EYFP Ly6M-GFP mice received *P. aeruginosa* and $5 \times 10^5$ 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 digestes 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+CD252CD62LhiCD44loCD4+ 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 Ly6GhiGr1hiCD11b+CD1152 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 IAα (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 2.5 × 10^5 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-

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**FIGURE 1.** *P. aeruginosa*-induced neutrophilia prevents established lung allograft tolerance. BALB/c → B6 lung allografts analyzed by H&E graft histology (representative of *n* ≥ 4) (A) (original magnification ×100) or for the accumulation of indicated intragraft T lymphocytes (n ≥ 4) (B) up to 3 wk after either saline or *P. aeruginosa* airway instillation. (C) Intragraft CD4+ T cells, isolated 3 wk after either saline or *P. aeruginosa* airway instillation into BALB/c → B6 recipients, were stimulated with BALB/c splenocytes and analyzed for IL-17 or IFN-γ expression (n ≥ 3). BALB/c → B6 lung allograft BALF neutrophil numbers after either saline or *P. aeruginosa* airway instillation (n ≥ 4) (D) or CFU after *P. aeruginosa* infection (n = 3) (E). (F) BALF neutrophils quantified 1 d after hhPA or saline airway instillation into BALB/c → B6 recipients (n = 4) treated with indicated Abs or 1 d after airway instillation into BALB/c → CD11b−/− recipients (n = 3). Allograft tissue isolated 3 wk after *P. aeruginosa* infection from lung recipients treated as in (F) and analyzed by H&E histology (n ≥ 3) (G) (original magnification ×100) or for indicated intragraft T lymphocyte accumulation (n ≥ 3) (H).
Neutrophil B7 directly enhances naive CD4+ T cell activation. (A) BALF neutrophils from uninfected (black line) or *P. aeruginosa*-infected (red line) BALB/c → B6 recipients were stained with indicated Abs or isotype (shaded graph) Abs (*n* = 4). (B) IL-2 levels from naive CD4+ T cells cultured with neutrophils from *P. aeruginosa*-infected mice (B6(PA)) or stimulated by CD3 beads in the absence (no PMN) or presence of neutrophils from uninfected mice (B6), B6(PA) mice, *P. aeruginosa*-infected CD80−/−86−/− mice (CD80−/−86−/−(PA)), or B6(PA) and 15 μg/ml CTLA4lg mice. (C) CFSE-labeled naive CD4+ T cells cultured as in (B) for 72 h and analyzed for responder frequency; mitotic divisions are shown below the x ordinate (*n* = 4).
intragraft CD4 + T cells from uninfected BALB/c
plane (\(n\) is neutrophil B7 dependent. (A) Graft histology (\(n\) = 4) (original magnification \(\times 100\)) and indicated intragraft T lymphocyte accumulation (\(n\) = 4) of BALB/c \(\rightarrow\) B6 lung recipients 3 wk after receiving PA and G-CSF Abs, as well as either B6 or CD80\(^{+/+}\) neutrophils. (C) Intravital two-photon imaging within allografts of BALB/c \(\rightarrow\) B6 CD11c-EYFP LysM-GFP lung recipients that received CellTracker Red-labeled CD4^+ T cells 1 d after infection with \(P.\ aeruginosa\) and neutrophils from \(P.\ aeruginosa\)-infected mice (Fig. 3D). B6 neutrophils were significantly better at stimulating IL-2 production than were CD80\(^{+/+}\)/86\(^{+/+}\) neutrophils. However, when B6 neutrophils were separated by Transwells from DC-stimulated CD4^+ T cells, IL-2 expression decreased sharply, underscoring the requirement for neutrophils to have direct contact with intragraft T cells to promote alloimmunity.

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 intragraft 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.
Disclosures
The authors have no financial conflicts of interest.

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