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T Cell Pathways Involving CTLA4 Contribute To a Model of Acute Lung Injury

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Acute lung injury (ALI) is a frequent pulmonary complication in critically ill patients. We characterized a murine model of LPS-induced ALI, focusing on Th cells. Following LPS administration, bronchoalveolar lavage lymphocytes, neutrophils, IL-6, TNF-α, and albumin were increased. Analysis of LPS-induced T cells revealed increased Th cell-associated cytokines (IL-17A, -17F, and -22), as well as increased expression of CD69 (a cell activation marker), Foxp3, and CTLA4 in CD4+ T cells. Administration of anti-CTLA4 Ab decreased LPS-induced bronchoalveolar lavage albumin and IL-17A, while increasing CD4+Foxp3+ cell number and Foxp3 expression in CD4+Foxp3+ cells. These data suggest that pulmonary LPS administration promotes CD4+ T cells and that T cell pathways involving CTLA4 contribute to ALI. The Journal of Immunology, 2010, 184: 5835–5841.

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cute lung injury (ALI) is associated with acute respiratory distress syndrome (ARDS), a major cause of severe respiratory failure with high morbidity and mortality in critically ill patients (1, 2). The pathogenesis of ARDS remains ill defined, and the treatment of ARDS remains largely supportive. ALI models include characterization of LPS-induced pulmonary immune responses (3). LPS, a component of Gram-negative bacteria, binds to a signal-transducing integral membrane protein, TLR4, which is expressed on APCs (e.g., alveolar macrophages and lung dendritic cells). TLRs allow vertebrates to recognize a vast range of microbial products in a rapid innate immune response (4, 5). TLRs were also recently found to be expressed on T cells, suggesting a potential pathway by which LPS may directly affect T cell activity (6–8). Innate and adaptive immune responses to invading microbes (e.g., Gram-negative bacteria) are tightly interwoven and engage the total immunological capability of the host. Innate immunity is the first line of lung defense, and it includes structural barriers, alveolar macrophages, neutrophils, NK cells, and dendritic cells. Adaptive immunity, which is promoted by innate immunity, is composed of Ag-specific lymphocytes that eliminate or prevent pathogenic challenges. Lymphocytes, including T and B cells, are major cells of the adaptive immune system. T lymphocytes (CD4+ Th cells) have no cytotoxic or phagocytic activity and cannot kill infected cells or clear pathogens, but they manage the immune response by directing other cells to perform these tasks.

Materials and Methods

Mice
Female BALB/c mice (8–10 wk old) were purchased from Harlan Breeders (Indianapolis, IN). T and B cell-deficient RAG knockout (KO) mice (BALB/c background) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained according to the guidelines of the University of California, San Diego Animal Care Program.

LPS-induced ALI
Mice were anesthetized with isoflurane (Minrad, Bethlehem, PA). The tongue was gently extended, and the tip of an otoscope was introduced to reach the trachea. Once in the trachea, LPS (100 µg) in 50 µl PBS was administered through the cone of the otoscope. In the RAG KO mice experiments, LPS was administered intratracheally (i.t.) to wild type (WT)
or RAG KO mice, and the mice were harvested at day 2 after LPS administration. In the time-course experiments, LPS was administered i.t. to WT mice. PBS was administered to controls. Mice were harvested at indicated time points (day 1–6) after LPS or PBS administration.

**Treatment with anti-CTLA4**

Mice were injected i.p. with anti-CTLA4 (100 µg) in 200 µl PBS 1 d prior to i.t. administration of LPS. Hamster IgG1 (IgG, 100 µg) was administered to controls. Anti-CTLA4-treated mice were harvested at indicated time points (day 1–6) after LPS administration. IgG-treated mice were harvested at days 2 and 4.

**Bronchoalveolar lavage harvest and cell count**

Bronchoalveolar lavage (BAL) fluid was obtained by cannulating the trachea and lavaging the lungs three times with 1 ml PBS containing 0.6 mM EDTA. BAL fluid cells were pelleted, and the supernatant was stored at −80°C until use.

BAL fluid cells were counted using a hemocytometer and resuspended in RPMI 1640 (5 × 10³ cells/ml). Slides for differential cell counts were prepared with Cytospin (Thermo Scientific, Waltham, MA) and then fixed and stained with Diff-Quick (Imeb, San Marcos, CA). For each sample, an investigator blinded to the treatment groups performed two counts of 100 cells.

**BAL albumin and cytokines**

Albumin levels in BAL fluid were measured by ELISA (Alpco Diagnostics, Salem, NH). Samples of BAL fluid were aliquoted in duplicate into 96-well plates (100 µl/well) precoated with Ab to specific murine albumin and assayed according to the manufacturer’s instructions. OD was measured at 450 nm. Levels of TNF-α and IL-2, -4, -6, -13, and -17A in BAL fluid were measured by a multiplexed immunoassay (Millipore, Bedford, MA), and IL-17F, -22, -23, and -27 were measured by a multiplexed immunoassay (Biologend, San Diego, CA). Mean fluorescence intensity (MFI) was measured by Luminex 100 total system (Luminex, Austin, TX).

**Preparation of lung homogenates**

Following the BAL procedure, the lungs were perfused with 20 ml saline and isolated. The lungs were cut into small pieces with scissors in a petri dish, finely chopped, and incubated at 37°C for 60 min in 5 ml per lung of a digestion mixture consisting of collagenase from *Clostridium histolyticum*, type IV (1 mg/ml, Sigma-Aldrich) and DNase I from bovine pancreas (0.5 mg/ml, Sigma-Aldrich). Cell suspensions were obtained by passing the homogenate through a 70-µm nylon Falcon cell strainer (BD Biosciences) to remove pieces of tissue and debris. The pellets were resuspended in 5 ml 10 mM EDTA in PBS. After lysis of erythrocytes with ammonium chloride potassium bicarbonate lysing buffer, cell pellets were resuspended and washed in RPMI 1640 medium.

**Flow cytometric analysis**

Samples of lung homogenates were resuspended in staining buffer (PBS containing 2% FBS and 0.05% sodium azide) to be 1 × 10⁶ cells/ml; 100 µl prepared cells (1 × 10⁵ cells) were added to each tube. After incubating for 30 min at 4°C with anti-mouse Abs (CD3 [PECy7], CD4 [FITC], CD8 [allophycocyanin], CD25 [allophycocyanin], CD19 [PE], or CD69 [PE]) or their respective immunoglobulin isotypes (eBioscience, San Diego, CA), samples were washed twice with staining buffer, fixed by Fixation/Permeabilization solution (eBioscience), and incubated overnight. Intra-cellular staining with PE-conjugated anti-mouse CTLA4 and Foxp3 Abs was performed using a Mouse T regulatory cell staining kit (eBioscience). Samples were run on a BD FACSCalibur flow cytometry system (BD Biosciences). Data yielded were analyzed on FlowJo v8 software (Tree Star, Ashland, Oregon).

**Statistical analysis**

Data are expressed as means ± SEM. All parameters were evaluated with the two-tailed unpaired Student t test or compared by one-way ANOVA, followed by the Bonferroni test. A p value <0.05 was considered significant.

**Results**

Lymphocytes are present and contribute to the severity of ALI

We analyzed a murine model of LPS-induced ALI in WT and lymphocyte-deficient (RAG KO, T and B cell deficient) mice. Compared with WT mice, RAG KO mice exhibited a significant decrease in BAL neutrophils (Fig. 1A). The decrease in LPS-induced neutrophils in RAG KO mice suggests that lymphocytes contribute to ALI. Upon further characterization of ALI in WT mice over 6 d, we analyzed increased pulmonary vascular

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**FIGURE 1.** Analysis of pulmonary inflammatory responses in a model of LPS-induced ALI. A, LPS (100 µg) was administered i.t. to WT BALB/c mice or RAG KO mice. BAL fluid was collected at day 2 after LPS administration, and cell counts were determined by differential staining for neutrophils and macrophages. Data shown are geometric mean ± SEM (n = 3 per group). ‡p < 0.05; RAG KO versus WT. B, LPS (100 µg) or PBS was administered to WT mice (BALB/c) i.t. BAL fluid was collected at the indicated days (D1–D6). Levels of albumin in the BAL fluid were determined by ELISA. C, Absolute neutrophil count in the BAL fluid was determined by differential staining of cells. Levels of IL-6 (D) and TNF-α (E) in BAL fluid were determined by a multiplexed immunoassay. Data are geometric mean ± SEM (n = 3–6 per group). *LPS group versus respective PBS group (p < 0.05); †p < 0.05.
permeability, defined by increased BAL levels of albumin (Fig. 1B). BAL albumin production increased at day 1, peaked at day 4, and returned to baseline by day 6. BAL neutrophils in LPS-exposed mice also increased at day 1, but they exhibited more rapid kinetics: peaked at day 2 and decreasing by day 4 (Fig. 1C). LPS-induced ALI is associated with proinflammatory cytokines (e.g., IL-6 and TNF-α). LPS increases BAL IL-6, with similar kinetics to the neutrophil count (Fig. 1D). LPS also increased TNF-α, which decreased by day 2 (Fig. 1E). LPS administration increased CD69 expression on CD4+ T cells, which was greatest at day 4, in parallel with increased IL-17A, -17F, and -22 (Fig. 3D, 3E).

Thus, LPS exposure increased CD4+ T cell number and activity. To define the T cell population modified by LPS exposure, we analyzed lung tissue for two markers expressed by T cells: CTLA4 and Foxp3. LPS exposure increased MFI of CTLA4 expression (Fig. 4A, 4B). LPS exposure also increased Foxp3 expression, which was greatest at day 6 (Fig. 4C, 4D).

Administration of anti-CTLA4 and analysis of CD4+ Foxp3+ cell activation

LPS exposure increased CTLA4 expression in CD4+ T cells. To examine the potential role of CTLA4 in ALI, we analyzed the impact of anti-CTLA4. BAL albumin, increased at day 4 in LPS-exposed mice, was significantly decreased by anti-CTLA4 administration, suggesting that vascular permeability is reduced (Fig. 5A). Anti-CTLA4 significantly reduced LPS-induced BAL IL-17A (Fig. 6A) without impacting neutrophil count, lymphocyte number (Fig. 5B, 5C), IL-6, or TNF-α (data not shown). Interestingly, the reduction in Th17 cells at day 4 by anti-CTLA4 was counterbalanced by a significant increase in CD69 and Foxp3 expression in CD4+ T cells at day 2 (Fig. 6B–D). We then focused on CD4+Foxp3+ cells at day 2 following LPS administration. Absolute cell count and the ratio of CD4+Foxp3+ cells in the CD4+ cell population were increased by anti-CTLA4 (Fig. 7A). In contrast, the number of CD4+Foxp3− cells was not significantly increased. Also, the ratio of CD4+Foxp3− cells in the CD4+ cell population was decreased by anti-CTLA4 (Fig. 7B). Anti-CTLA4 also increased LPS-induced Foxp3 in CD4+Foxp3+ cells (Fig. 7C, 7D) and LPS-induced CD69 in CD4+CD25+ cells (Fig. 7E, 7F).

Discussion

ALI is classically characterized as an innate response, predominantly involving neutrophils. In a murine model of ALI, we focused on the analysis of T cell-dependent pathways. Consistent with lung injury models, LPS administration induces BAL neutrophils in WT mice. Lymphocyte-deficient mice were unable to increase neutrophils in response to LPS, suggesting that T cells may contribute to ALI. LPS also increased a number of T cell-associated parameters, including lymphocyte number and activity and IL-17A production. Notably, LPS exposure, an Ag-independent pathway, also increased two molecules found primarily on T cells: CTLA4 and Foxp3. Testing the functional role of CTLA4, anti-CTLA4 administration to LPS-exposed mice resulted in decreased IL-17A and albumin production. These findings suggest that CTLA4 and T cells may contribute to pulmonary inflammatory pathways in ALI.

We analyzed LPS-induced T cells over a 6-d time course, providing information on the early and late influx of cells and the secretion of cytokines. As expected, LPS administration increased BAL neutrophils and inflammatory cytokines (i.e., IL-6 and TNF-α) within 2 d (early phase). LPS administration increased lymphocytes within 4–6 d (late phase). Other investigators showed that LPS-induced TNF-α secretion in macrophages is decreased when CD4+ T cells are depleted (17) and that γδ T cells increase BAL neutrophils by producing IL-17A (18), suggesting that lymphocytes may facilitate neutrophil migration to the lungs. In our study, the kinetic analysis of neutrophils and lymphocytes supports the
concept that lymphocytes may play roles in addition to facilitating neutrophil migration, perhaps in dampening inflammation in ALI. In a prior study, adrenaline attenuated ALI by diminishing the recruitment of CD4+ cells to lung (19). The acute phase of ALI is characterized mainly by the influx of protein-rich edema fluid into the air space as a consequence of increased permeability of the alveolar–capillary barrier (20). Following LPS exposure, albumin levels peaked later than neutrophils, indicating that neutrophil

**FIGURE 3.** LPS activates CD4+ T cells. LPS (100 μg) or PBS was administered to mice (BALB/c) i.t. BAL fluid and lungs were harvested at the indicated days (D1–D6). Lungs were digested as described in Materials and Methods. Levels of IL-17A (A), IL-17F (B), and IL-22 (C) in BAL fluid were determined by a multiplexed immunoassay. Expression levels of CD69 in the CD3+CD4+ cell population in lung tissue were determined by flow cytometry; representative bar graphs of CD69 expression (shaded areas represent isotype control) (D) and MFI for CD69 (E). Data are geometric mean ± SEM (n = 3–6 per group). *LPS group versus respective PBS group (p < 0.05); † p < 0.05.

**FIGURE 4.** LPS induces Th cell-dependent suppressive pathways. LPS (100 μg) or PBS was administered to mice (BALB/c) i.t. Lungs were harvested at the indicated days (D1–D6). Lungs were digested as described in Materials and Methods. Expression levels of CTLA4 (A) and Foxp3 (C) in the CD3+CD4+ cell population in lung tissue were determined by flow cytometry; representative graphs of CTLA4 and Foxp3 expression (shaded areas represent isotype control). MFI for CTLA4 (B) and Foxp3 (D). Data are geometric mean ± SEM (n = 3–6 per group). *LPS group versus respective PBS group (p < 0.05); †LPS group versus LPS D6 (p < 0.05); #p < 0.05.
infiltration may not directly correspond with lung vascular permeability. The time difference between the LPS-induced neutrophil influx and the albumin increase may be due to several possibilities. First, proteolytic enzymes released from neutrophils, such as elastase and free-oxygen radicals, require time to exert their effects. Second, the peak of tissue damage may be earlier than the peak of albumin levels. The kinetics of ALI indicate that lung inflammation peaks at day 2 (acute phase), with the resolution process starting at day 4. Third, the severity of ALI may not be determined solely by neutrophils; different cell types may contribute to the lung damage. Expression of CD69, a cell-activation marker, on T cells was increased by LPS throughout the time points analyzed. Together, these data suggest that T cells may impact acute and late phases of inflammation in ALI.

Th17 cell-related cytokines (i.e., IL-17A, -17F, and -22) were increased following LPS exposure. Interestingly, LPS-induced Th17 cell-related cytokines increased in a pattern similar to increased lymphocyte number and CD69 expression. Other Th cell-related cytokines (i.e., IL-2, -4, and -13) were not increased (data not shown), which implies that, among the subsets of Th cells, Th17 cells may play a role in ALI. IL-17 is important for host defense against infection, as well as neutrophil recruitment (21). Indeed, IL-17R–deficient mice succumb to infection (22). In contrast, elevated and prolonged expression of IL-17 is found in autoimmune disease (23). In murine models of LPS-induced airway inflammation, previous reports showed that neutralization of IL-17 significantly reduced neutrophil infiltration (24–26). Recently, other investigators demonstrated that IL-17R antagonist KO mice show reduced neutrophilic inflammation of ALI in the setting of influenza infection-induced ALI (18). Together, these studies in ALI suggest that IL-17 is increased at an early phase, induces inflammatory cytokines, and affects neutrophil kinetics. In our model, the time course for LPS-induced IL-17 was delayed compared with the increase in neutrophil infiltration. IL-17 is primarily considered a proinflammatory cytokine; however, in an allergic model, IL-17 suppresses eosinophil migration (27). In a bleomycin-induced lung injury model, IL-17 produced by γδ T cells controls resolution of inflammation (28). In a Helicobacter pylori-induced gastritis model, IL-17 exerts anti-inflammatory effects (29). Thus, IL-17 may play pathogenic and protective roles in immune responses. Of note, the kinetics of LPS-induced IL-17 family members and Foxp3 differ, with IL-17 secreted earlier than Foxp3, which may impact their biological activities.

The early stages of T cell activation are regulated by interaction of costimulatory molecules CD80 and CD86 with their receptor CD28 on the T cell surface (30). Once activated, these T cells transiently increase CTLA4 on their cell surface, interacting with
the same CD80 and CD86 costimulatory molecules but now inhibiting cell-cycle progression and IL-2 production (31). Thus, CTLA4 signaling provides negative feedback to activated T cells, thereby dampening an immune response. In our model, LPS increased CTLA4 expression from days 1–6, indicating that Th cells were activated and susceptible to downregulation from the early to the late phase, concomitant with increased CD69 expression. Mattern et al. (32) demonstrated that monocyte-dependent activation of human T cells by LPS requires costimulatory signals via CD28 and/or CTLA4 but is not MHC restricted. TLRs are expressed on T cells, and TLR ligands act directly upon T cells (6–8). Together, our findings in an ALI model suggest that T cells may be regulated, in part, by Ag-independent pathways.

In vivo analysis of anti-CTLA4 administration resulted in decreased albumin and IL-17A levels, suggesting a beneficial effect in ALI of manipulating a T cell pathway. Although BAL cell counts were not altered by aCTLA4 administration, the severity of ALI may not always be determined solely by neutrophils. Multiple cells may contribute to the lung damage, and CD4 T cells may impact other cells (e.g., other lymphocyte subsets, macrophages, or epithelial cells). A specific subset of lymphocytes may act as anti-inflammatory cells, not proinflammatory cells: Tregs. D’Alessio et al. (16) recently demonstrated that Tregs play critical roles during the resolution phase in an ALI model. They showed that Tregs resolve lung inflammation by inducing TGF-β and neutrophil apoptosis. The contribution of other T cell subsets, the impact of T cells on the early phase in ALI, and the potential role of CTLA4 were not examined. In our study, we focused on additional T cell subsets and the early phase of ALI. We also analyzed anti-CTLA4, which may activate T effector cells, as well as Tregs. Because Tregs constitutively express CTLA4 on their cell surface (33), anti-CTLA4 may impair the suppressive function of Tregs. Prior studies in cancer patients showed that anti-CTLA4 increases activated effector CD4 T cells and Tregs (34). Inhibiting CTLA4 signaling with anti-CTLA4 enhances Treg proliferation and overall Treg frequency (35). Notably, we demonstrated that anti-CTLA4 increased LPS-induced Foxp3 in CD4+ cells, suggesting that the decrease in albumin levels by anti-CTLA4 may relate to Treg activation. Increased Treg activation is supported by an increased absolute CD4+Foxp3 cell number but not CD4+Foxp3 (effector) cells in the anti-CTLA4-treated group. Further, LPS-induced Foxp3 expression in CD4+Foxp3+ cells and LPS-induced CD69 expression in CD4+CD25+ cells were increased by anti-CTLA4. Our study suggests that anti-CTLA4 may activate Tregs more than T effector cells, perhaps suppressing inflammation or accelerating resolution. If so, the severity of ALI may depend on the T effector cell/Treg balance. Also, anti-CTLA4 suppression of IL-17A synthesis is associated with decreased albumin. Tregs, which are activated by anti-CTLA4, may suppress Th17 cell activation. Thus, the Th17/Treg balance may be important in ALI pathogenesis and warrants further analysis.

**FIGURE 7.** Anti-CTLA4 increases LPS-induced Treg number and activation. Mice were injected i.p. with anti-CTLA4 (100 μg) or hamster IgG (100 μg) 1 d prior to i.t. administration of LPS (100 μg). Lungs were collected at day 2 after the LPS exposure and digested as described in Materials and Methods. Absolute cell counts and ratio of CD4+Foxp3+ (Treg; A) and CD4+Foxp3– (T effector; B) cells in the CD4+ cell population were determined by flow cytometry. Expression of Foxp3 (C) in the CD4+Foxp3 cell population and CD69 (E) in the CD4+CD25+ cell population in lung tissue was determined by flow cytometry; representative graphs of CD69 and Foxp3 expression (shaded areas represent isotype control). MFI for Foxp3 (D) and CD69 (F). Data are geometric mean ± SEM (n = 3–6 per group). †p < 0.05.
In summary, we focused on the analysis of T cells in a model of ALI. Notably, LPS induces increased number and activation of T cells, as well as other T cell-associated markers (i.e., IL-17A, Foxp3, and CTLA4). Testing the functional role of CTLA4, anti-CTLA4 decreased LPS-induced albumin and IL-17A, while increasing the number of CD4+Foxp3+ cells and Foxp3 expression in CD4+Foxp3+ cells. These data are consistent with a role for CTLA4+ T cells in LPS-induced inflammation. The specific pathways by which T cells are activated in ALI remain to be determined. The potential therapeutic impact of manipulating T cell pathways involving CTLA4 in ALI merits further investigation.

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