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Recipient T Cells Mediate Reperfusion Injury after Lung Transplantation in the Rat

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Leukocytes have been implicated in ischemia-reperfusion (IR) injury of the lung, but the individual role of T cells has not been explored. Recent evidence in mice suggests that T cells may play a role in IR injury. Using a syngeneic (Lewis to Lewis) rat lung transplant model, we observed that recipient CD4+ T cells infiltrated lung grafts within 1 h of reperfusion and up-regulated the expression of CD25 over the ensuing 12 h. Nude rats (rnu/rnu) and heterozygous rats (rnu/+ ) were used to determine the role of T cells in IR injury. No significant difference in lung function was observed between nude and heterozygous recipient rats after 2 h of reperfusion. However, after 12 h of reperfusion, recipient nude rats had significantly higher oxygenation and lower peak airway pressure than recipient heterozygous rats. This was associated with significantly lower levels of IFN-γ in transplanted lung tissue of recipient nude rats. Reconstitution of recipient nude rats with T cells from heterozygous rats restored IR injury after 12 h of reperfusion. The effect of T cells was independent of neutrophil recruitment and activation in the transplanted lung. These results demonstrate that recipient T cells are activated and mediate IR injury during lung transplantation in rats. The Journal of Immunology, 2003, 171: 4995–5002.

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2 Abbreviations used in this paper: IR, ischemia-reperfusion; PEEP, positive end-expiratory pressure; PawP, peak airway pressure; PaO2, arterial pressure of oxygen; MP-2, macrophage inflammatory protein-2.

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Cytokine measurements
At the end of the reperfusion period, lung tissue samples were immediately snap frozen in liquid nitrogen and stored at −70 °C. Tissues were subsequently homogenized and incubated at 4 °C in lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DT T, 0.5 mM PMSF, and 0.06% octylphenolpolyethoxylate (Nonidet P-40) (26). Homogenates were then sonicated and centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants and plasma samples were assayed in duplicate using specific ELISA kits for rat IFN-γ and rat macrophage inflammatory protein-2 (MIP-2), according to the manufacturer’s instruction. Specific Cytoscreen Immunoassay Kits (BioSource International, Camarillo, CA) were used for all cytokines. The OD of each well was read at 450 nm with an NM-600 microplate reader (Dynatech Laboratories, Chantilly, VA). The final concentration was calculated by converting the OD readings against a standard curve. The protein content was determined by the Bradford method (27). Data are presented as units of cytokine per mg protein.

T cell adoptive transfer
Spleens were collected from immune competent heterozygous rnu/+ rats. Freshly isolated spleens were placed into RPMI (HEPES) medium containing 10% FCS and cells were separated by filtration through a 200-μm mesh screen. Splenic cells were collected by centrifugation and RBC were removed by lysis in NH4Cl at room temperature for 5 min in the dark. Mononuclear cells were harvested by centrifugation and washed three times with 10% FCS-containing medium. T cell enrichment was performed using nylon wool column chromatography. Briefly, sterile nylon wool-packed columns were equilibrated in RPMI with 10% FCS, and cells were incubated on the column for 1 h at 37 °C in 5% CO2. Nonadherent cells (predominantly T cells) were then eluted in 30 ml of RPMI and collected by centrifugation. The percentage of CD4+ and CD8+ T cells was assessed in nylon wool T cell-enriched fraction by flow cytometry. Enriched T cells were then washed three times with PBS to remove serum from the preparation. Enriched T cells (~25 × 106) were injected i.v. via the tail vein of each rnu/rnu rat. The rats were then kept in sterile condition for 6 days before being used as donor or recipient animals. The 6-day time point was based on preliminary experiments showing that longer time points of up to 3 wk did not enhance the efficiency of T cells reconstitution.

FACS
The left lung was crushed in 5 ml of HBSS, and cell aggregates were separated by filtration through a 200-μm pore size nylon screen. Cells were collected by centrifugation for 5 min at 1500 rpm. They were resuspended in NH4Cl for 5 min at room temperature in the dark to remove RBC. Cells were then washed three times in PBS-BSA. For each analysis, the total number of lymphocytes in the left lung was counted, and 107 cells were stained with 1 μg of FITC anti-rat CD3 Ab, 1 μg of PE anti-rat CD8 Ab, and 1 μg of quantum red anti-rat CD4 Ab (BD Pharmingen) for 20 min at 4 °C in the dark. T cell activation was determined by staining a separate aliquot of 105 cells with 1 μg of PE anti-CD25 Ab and 1 μg of PE anti-CD25 Abs (BD Pharmingen). Neutrophil infiltration in lung tissue was determined by CD11b staining (PharMingen). After a washing in PBS-BSA, cells were resuspended in 300 μl of PBS-BSA and analyzed by flow cytometry (Beckman Coulter, Fullerton, CA).

Statistical analysis
All data are expressed as mean ± SD. A one-way ANOVA was used to determine statistical significance. For differences in PAwP, the Friedman repeated measures ANOVA was performed, p < 0.05 was considered statistically significant. The Graphpad software package (Graphpad Software, San Diego, CA) was used for all statistical analyses.

Results
Kinetics of T cell infiltration in the lung during ischemia and after reperfusion
The large majority of studies analyzing the infiltration of leukocytes in IR of the lung have focused their attention on neutrophils. T cells have generally not been sought or rigorously identified with definite techniques, and very early times after reperfusion have not been examined. Using Lewis rats as donors and recipients, we quantified the number of lymphocytes and CD3+ T cells and determined the proportion of CD3+, CD4+, and CD8+ T cells in the left lung before the retrieval procedure (normal lung), at the end of

ruu), and heterozygous rats (rnu+/+) (Harlan Breeders, Indianapolis, IN). Five animals were used in each group for all experiments. All animals received care in compliance with the Principles of Laboratory Animal Care (21) and the Guide to the Care and Use of Experimental Animals (22). The experimental protocol was approved by the Animal Care Committee of the Toronto General Hospital Research Institute.

Lung transplantation model
The rat single lung transplant model used in this study has been described in greater detail by us elsewhere (23). Briefly, donor rats were anesthetized by an i.p. injection of 1 ml of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and intubated through a tracheostomy with a 14-gauge i.v. catheter. The tracheostomy tube was then connected to a volume-controlled ventilator (Harvard Rodent Ventilator Model 683; Harvard Apparatus, South Natick, MA), and the animals were ventilated at a rate of 80 breaths/min, a tidal volume (VT) of 10 ml/kg, an inspired fraction of oxygen of 1.0, and a positive end-expiratory pressure (PEEP) of 2 cm H2O. Lungs were flushed in situ with 20 ml of low-potassium-dextran-glucose preservation solution (Perfadex; Vitroflö, Göteborg, Sweden). The left lung was placed into 40 ml of low-potassium-dextran-glucose solution at 4 °C for 12 h.

Recipient animals were anesthetized in a halothane chamber and intubated with a 14-gauge i.v. catheter. All recipient animals were ventilated with 100% O2 mixed with 1.5% halothane at a rate of 80 breaths/min, a PEEP of 2 cm H2O, and a VT of 10 ml/kg. The left pulmonary artery, left main bronchus, and pulmonary vein were anastomosed with the corresponding recipient structures using the cuff technique. If the transplanted lung was reperfused for 12 h, animals were then awakened after closure of the left thoracotomy.

At the end of the 12-h reperfusion period, recipient animals were anesthetized in a halothane chamber and intubated with a 14-gauge i.v. catheter. A 22-gauge catheter was inserted into the carotid artery for continuous measurement of the systemic blood pressure. For functional assessment of the transplanted left lung, the right hilum was dissected and the pulmonary artery, vein, and bronchi were occluded. The transplanted lung was then ventilated with 100% O2 mixed with 1.5% halothane at a rate of 80 breaths/min, a PEEP of 2 cm H2O, and a VT of 4.5 ml/kg for 5 min before any functional measurement was performed.

If the transplanted lung was reperfused for 2 h, we used a modification of our model to measure the airway pressure from the transplanted lung throughout the reperfusion period. This model has been described in details elsewhere (24). Briefly, the left main bronchus of the transplanted lung was connected to a second ventilator and ventilated with 100% O2 mixed with 1.5% halothane at a rate of 80 breaths/min, a PEEP of 2 cm H2O, and a VT of 4.5 ml/kg.

Assessment of lung graft function
The peak airway pressures (PAwP) and arterial pressure of oxygen (PaO2) of the transplanted lung have been shown to be reliable markers of lung function in a rat left single lung transplant model (23, 25). PAwPs from the transplanted lung were measured with a three-way tap inserted between the endotracheal tube of the transplanted lung and the ventilator and connected to a pressure transducer. PAwPs were measured continuously during the 2-h reperfusion period or at the end of the 12 h reperfusion during 5 min to achieve a steady state. At the end of the reperfusion period after the last PAwP measurement was performed, a blood gas sample was drawn from the left pulmonary vein of the transplanted lung or from the carotid artery if the right hilum was clamped.

Immunohistochemistry
Frozen sections (7 μm) where prepared on sialized treated glass slides. Sections where air dried and fixed in acetone for 10 min at room temperature. Immunohistochemical procedure on frozen sections for mouse anti-rat CD3 or CD11b (BD PharMingen, San Diego, CA), at a dilution of 1/50, was performed on the NEXES autoimmunoventr (Ventana Medical Systems, Tucson, AZ). CD3 is a marker for T cells and CD11b is a marker of activated neutrophils and macrophages that is absent on lymphocytes. To avoid cross-reactivity between similar species, detection was conducted using a modification of the M.O.M ABC system (Vector Laboratories, Burlington, CA). CD3 in combination with the Ventana DAB detection system, using biotinylated goat anti-mouse polyclonal IgGs at a 1/50 dilution (BD Pharmingen). Negative control slides were performed with the primary Ab omitted and replaced with nonimmunized control goat serum. The counterstain of preference was hematoxylin for nuclear detail. After the entire lung section was viewed, immunoreactive cells were counted in 10 random high-powered (×400) fields, and total numbers of cells were quantified in a blinded manner.
the cold ischemic time, and after reperfusion of the transplanted left lung. The reperfusion period was divided into five time points: 10, 30, and 60 min, followed by 3 and 12 h of reperfusion. Donor lungs contained ~250,000 lymphocytes that remained unchanged during the cold ischemic time. The number of lymphocytes in the transplanted lung tissue began to rise within 10 min of reperfusion and increased up to 2 million after 1 h of reperfusion (Fig. 1). During the ensuing 12 h of reperfusion, the number of lymphocytes progressively decreased. Using immunostaining, we observed that the number of CD3+ T cells significantly increased after 1 h of reperfusion and that they were principally located in the lung parenchyma (Fig. 1). T cells infiltrating lung tissue were predominantly CD4+ T cells, which increased from 22% in donor lungs to 41% after 1 h of reperfusion (Fig. 1). In the recipient blood, the number of lymphocytes and the proportion of CD3+, CD4+, and CD8+ T cells did not significantly change during the first 12 h of reperfusion (data not shown). These findings suggest that lymphocytes and predominantly CD4+ T cells infiltrate the transplanted lung rapidly after reperfusion and may thus play an active role in mediating IR injury.

**Early reperfusion injury is dependent of donor but not recipient T cells**

Because T cell infiltration peaked in the transplanted lung after 1 h of reperfusion, we wanted to determine the role of donor and recipient T cells in the early phase of reperfusion injury. We therefore compared four groups during a 2-h reperfusion period. The first group included heterozygous rats as donor and recipient (H-H), the second group included nude rats as donor and heterozygous rats as recipient (N-H), the third group included heterozygous rats as donor and nude rats as recipient (H-N), and the fourth group included nude rats as donor and recipient (N-N). After completion of the 2-h reperfusion period, we observed that transplantation with lungs from donor nude rats was associated with more severe reperfusion injury. Indeed, the groups N-H and N-N had lower PaO2 and higher PAwP after 2 h of reperfusion than the groups H-H and H-N (Fig. 2). No significant difference in lung function, however, was observed according to whether the recipient was nude rats (group H-N) or heterozygous rats (group H-H). Hence, these data indicate that although recipient T cells infiltrate the transplanted lung rapidly after reperfusion, they do not contribute to reperfusion injury after 2 h of reperfusion. Donor parameters appeared to have more influence on the degree of injury during this early phase of reperfusion.

**Late reperfusion injury is dependent of recipient T cells**

Previous studies have suggested that IR injury can be differentiated into two phases: the early phase of reperfusion injury, which corresponds to the first few hours of reperfusion during which function of the transplanted lung is mainly dependent of donor characteristics; and the late phase of reperfusion, which occurs thereafter and is principally dependent of recipient factors (7, 8).

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### FIGURE 1

A. Kinetics of lymphocyte infiltration in the transplanted lung. The total number of lymphocytes in the left lung was determined at the time of lung retrieval (normal lung), at the end of cold ischemic time (CIT) and after 10 min (10'), 30 min (30'), 1 h (1H), 3 h (3H), and 12 h (12H) of reperfusion. Five animals were used at each time point. Lymphocytes started to infiltrate the transplanted lung after 10 min of reperfusion; the number of lymphocytes peaked after 1 h of reperfusion. B. The number of T cells infiltrating the transplanted lung was specifically determined by CD3 staining. CD3+ T cells significantly increased in the transplanted lung after 1 h of reperfusion when compared with normal lung and decreased after 12 h of reperfusion. C. Immunohistochemistry for CD3 shows that CD3+ T cells were already located in the lung parenchyma after 1 h of reperfusion (arrows). D. The proportion of CD3+CD4+CD8+ T cells was determined by FACS analysis of the transplanted lung before and after reperfusion. After 1 h of reperfusion, the proportion of CD4+ T cells significantly increased in the transplanted lung and then progressively decreased over time. *, p < 0.05; **, p < 0.01; ***, and p < 0.001 when compared with normal lung. HPF, High power field.
We therefore analyzed lung function in two groups after 12 h of reperfusion. Heterozygous rats were used as donors in both groups, and recipients were divided into nude rats in one group and heterozygous rats in the other. After 12 h of reperfusion, the group that included nude rats as recipient presented with significantly better lung function characterized by lower PAwP and higher PaO2 than the group that included heterozygous rats as recipient (Fig. 3). Nude rats are genetically mutant animals lacking T cells. However, they may develop compensatory mechanism to palliate the T cell deficiency. Hence, to confirm that T cells had a role in the late phase of reperfusion injury, we reconstituted nude rats with T cells. T cell population was enriched from the spleen of heterozygous rats using nylon wool chromatography (Fig. 3).

T cell-reconstituted nude rats were then used as recipients. After 12 h of reperfusion, when T cell reconstituted nude rats were used as recipients, they demonstrated a return of the reperfusion injury as determined by PaO2 and PAwP of the transplanted lung (Fig. 3). These data indicate that despite a rapid infiltration of lymphocytes in lung tissue after reperfusion, recipient T cells mediate reperfusion injury in the late phase of reperfusion only.

**Recipient CD4+ T cells expressed CD25 after 12 h of reperfusion**

We have demonstrated that recipient T cells infiltrate lung graft and mediate reperfusion injury after 12 h of reperfusion. Hence, to confirm that reperfusion injury may activate T cells despite the absence of alloantigen stimuli, we determined the level of CD25 T cells in lung graft tissue before and after 2 and 12 h of reperfusion using Lewis rats as donors and recipients. We demonstrated that ~4% of CD4+ T cells expressed CD25 in the normal lung and that no significant change was observed after 2 h of reperfusion. However, after 12 h of reperfusion, up to 23% of CD4+ T cells expressed the marker CD25 in the transplanted lung tissue (Fig. 4). In the blood, in contrast, the proportion of CD4+CD25+ T cells remained unchanged during the 12-h reperfusion period (Fig. 4).

Using nude rats and heterozygous rats alternatively as donors and recipients, we determined that the activated CD4+ T cells originated from the recipient rats. Indeed, transplantation of lungs from heterozygous rats to nude recipient was associated with only 3% of CD4+CD25+ T cells after 12 h of reperfusion. In contrast, transplantation of lungs from nude rats into heterozygous recipients was associated with a large increase in CD4+CD25+ T cells in the graft after 12 h of reperfusion (Fig. 4).

Recipient T cells release IFN-γ in lung tissue after 12 h of reperfusion

The cytokine IFN-γ is characteristically released by activated T cells. We therefore determined the level of IFN-γ in the transplanted lung tissue from recipient nude rats and from recipient...
heterozygous rats after 12 h of reperfusion. We observed that better lung function in recipient nude rats was associated with significantly lower levels of IFN-γ in lung tissue than in recipient heterozygous rats (Fig. 5). The level of IFN-γ, however, was not significantly different from those of recipient heterozygous rats if recipient nude rats were reconstituted with a population of enriched T cells (Fig. 5). These data indicate that reperfusion injury may be mediated by the release of IFN-γ from activated T cells after 12 h of reperfusion.

**Discussion**

This study demonstrates that recipient T cells infiltrate the transplanted lung after a period of cold ischemia and mediate lung injury during the late phase of reperfusion. These findings are consistent with previous studies in T cell-deficient mice, suggesting that CD4⁺ T cells were involved in the mechanism of IR injury of the liver, kidney, and intestine (17, 19, 28). Our results for the first time provide direct evidence that recipient (but not donor) CD4⁺ T cells are activated during reperfusion injury despite the absence of alloantigen stimuli and the short period of reperfusion.

Classically, T cells are thought to require several days after transplantation to be exposed to foreign Ags by APCs and to be
activated (29). Although this holds true for naive T cells, other types of T cells may be involved in IR injury. Effector T cells and possibly some memory T cells can maintain the expression of specific cytokine receptor on their cell surface and proliferate in vivo in the absence of Ag stimulation (30). This Ag-independent proliferation of T cells is known as bystander activation. Recent studies have also shown that polarized memory Th1 cells can be reactivated and up-regulate the expression of CD4+CD25+ T cells in normal blood is similar to that in the lung and is ~4% (data not shown). E. When heterozygous rats were used as donor and nude rats as recipient, the levels of CD4+CD25+ T cells remained low in lung tissue after 12 h of reperfusion; the proportion of CD4+CD25+ T cells in normal lung tissue contains ~4% of CD4+CD25+ T cells. F. In contrast, when nude rats were used as donor and heterozygous rats as recipient, lung tissue contained ~16% of CD4+CD25+ T cells after 12 h of reperfusion, demonstrating that recipient CD4+ T cells are activated after reperfusion. QR, Quantum red.

Our results have important clinical implications in the prevention and treatment of IR injury. Humanized mAbs against IL-2Rα (CD25) are currently used in clinical transplantation as induction therapy to prevent rejection (36). However, their role in IR injury has not been analyzed systematically. In one randomized study.
involving kidney transplantation, the authors have observed that the group of patients treated with anti-IL-2Rα Ab before reperfusion resumed urine output earlier and had less renal dysfunction at 1 month than the control group treated with placebo (37). Future experimental studies should help to determine whether the administration of anti-CD25 Abs to the recipient before reperfusion protects the graft from IR injury.

This study presents some limitations due to the use of athymic nude rats. Hence, some of the changes that we observed may have been due to uncontrolled abnormalities in nude rats. T cell depletion in wild-type rats would have been optimal to confirm our results, but CD3- or CD4- T cell depletion in rats was not feasible despite our attempts with various doses of Abs. The reconstitution of IR injury by T cell transfer to recipient nude rats and the activation of recipient CD4+ T cells during the same time frame, however, strongly supports our results on the role of T cells in IR injury.

An interesting finding from our study was the enhanced IR injury when nude rats were used as donors. This suggests that T cells may have a suppressive and protective role during cold ischemia or that lungs from nude rats contain other immunological characteristics that render them more susceptible to cold ischemia (38, 39). Nude rats have a more developed innate

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**FIGURE 5.** Using the same groups as in Fig. 3 (heterozygous rats as donor and recipient in the first group, heterozygous rats as donor and nude rats as recipient in the second group, and heterozygous rats as donor and nude rats reconstituted with T cells as recipients in the third group), we analyzed the level of IFN-γ in transplanted lung tissue after 12 h of reperfusion. The level of IFN-γ was significantly lower in nude rats than in heterozygous recipients and in nude recipients reconstituted with T cells. *, p < 0.05 when compared with the groups with heterozygous recipient and reconstituted recipient (recipient nude + T cells).

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**FIGURE 6.** A. FACS analysis of the transplanted lung shows that T cells infiltrate the transplanted lung before neutrophils. T cells are already present in the transplanted lung after 1 h of reperfusion (circle A), whereas neutrophils started to be recruited only after 3 h of reperfusion (circle B). B, Levels of MIP-2 in transplanted lung tissue was not significantly different between recipient nude and heterozygous rats after 2 and 12 h of reperfusion (five animals per group). C, The number of CD11b cells in the transplanted lung was not significantly different between recipient nude and heterozygous rats after 12 h of reperfusion (five animals per group).
immune system and contain more NK cells and γδ T cells in lung tissue than immunocompetent rats (39). Future studies should determine whether NK cells and γδ T cells are involved in IR injury.

In conclusion, this study demonstrates that recipient CD4+ T cells rapidly infiltrate lung graft tissue and up-regulate CD25, a marker of T cells activation, after 12 h of reperfusion. Recipient T cells did not appear to be involved in the early phase of reperfusion injury. However, after 12 h of reperfusion, recipient T cells mediated reperfusion injury and were associated with significant release of IFN-γ in lung tissue. These findings may have important implication for the prevention and treatment of IR injury. Future studies should determine more precisely the type of T cells and their mechanism of activation.

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