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CD154 Blockade Abrogates Allospecific Responses and Enhances CD4+ Regulatory T Cells in Mouse Orthotopic Lung Transplant

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Abstract

Acute cellular rejection (ACR) is a common and important clinical complication following lung transplantation. While there is a clinical need for the development of novel therapies to prevent ACR, the regulation of allospecific effector T cells in this process remains incompletely understood. Using the MHC-mismatched mouse orthotopic lung transplant model, we investigated the short-term role of anti-CD154 mAb therapy alone on allograft pathology and alloimmune T cell effector responses. Untreated C57BL/6 recipients of BALB/c left lung allografts had high-grade rejection and diminished $CD4^+$:$CD8^+$ graft ratios, marked by predominantly $CD8^+$>$CD4^+$ IFN-γ^+ allospecific effector responses at day 10, compared to isograft controls. Anti-CD154 mAb

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therapy strikingly abrogated both $CD8^+$ and $CD4^+$ alloeffector responses and significantly increased lung allograft $CD4^+$:$CD8^+$ ratios. Examination of graft $CD4^+$ T cells revealed significantly increased frequencies of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the lung allografts of anti-CD154-treated mice and was associated with significant attenuation of ACR compared to untreated controls. Together, these data show that CD154/CD40 costimulation blockade alone is sufficient to abrogate allospecific effector T cell responses and significantly shifts the lung allograft toward an environment predominated by $CD4^+$ T regulatory cells in association with an attenuation of ACR.

Keywords

CD154; Mouse Orthotopic Lung Transplantation; Effector T Cell; Allograft Rejection; Regulatory T Cell

INTRODUCTION

Lung transplantation is the final therapeutic option for select patients with various end-stage lung diseases, though its long-term success is limited by the bronchiolitis obliterans syndrome (BOS) or chronic allograft rejection (1–3). Despite the use of broad immunosuppression, at least one episode of acute cellular rejection (ACR) of the lung allograft occurs in over 50% of lung transplant recipients, and is recognized as a major risk factor for the development of BOS (4). While evidence supports an important role for T cells in ACR, T cell effector mechanisms and their regulation in this process remain incompletely understood. The recent development of the orthotopic mouse lung transplantation model has been a significant advancement in the field, and enables studies in the immuno-pathogenesis of ACR, as well as mechanistic studies in lung allograft tolerance (5).

The CD154/CD40 costimulation pathway has been a focus of investigation since early studies demonstrated the potent efficacy of its blockade in establishing long-term allograft acceptance across multiple experimental transplant models (6–9). Activated $CD4^+$ T cells are a major source of CD154 (CD40L), and their interaction with CD40 on antigen presenting cells is important in the development of Th1 responses (10, 11). While several studies have shown that CD154/CD40 blockade impairs $CD4^+$ T cell-dependent rejection, the role of this pathway in $CD8^+$ T cell regulation is more variable with several reports of costimulation blockade-resistant $CD8^+$ T cell-mediated rejection (12–16). Early seminal studies by Wells et al. (17) and Li et al. (18) revealed an important role for activation-induced cell death in prolonged graft survival following costimulation blockade using anti-CD154 and cytotoxic T lymphocyte antigen 4-fusion protein (CTLA4-Ig). Subsequent work by Graca et al., showed that anti-CD154-induced tolerance could not be broken by the adoptive transfer of non-tolerant naïve cells, and provided evidence for infectious transplant tolerance through an unknown regulatory population (19). Studies by several groups later described a critical role for $CD4^+CD25^+$ regulatory T cells in the induction of tolerance to alloantigen (20, 21). Recently, costimulation blockade with anti-CD154 and CTLA4-Ig has been shown to attenuate acute cellular rejection pathology following MHC-mismatched mouse orthotopic lung transplant (22). In this study, we evaluated the effects of CD154 blockade alone on allospecific T cell responses following mouse orthotopic lung transplant. Herein, we report that CD154 blockade abrogates both $CD8^+$ and $CD4^+$ allospecific effector T cell responses and strikingly enhances intragraft $CD4^+CD25^+Foxp3^+$ regulatory T cells.

MATERIALS AND METHODS

Mice

The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols. Pathogen-free male C57BL/6 (I-a^b, H-2^b) and BALB/c (I-a^d, H-2^d) mice 25–30g were purchased from Charles River (Wilmington, MA). All mice were housed in the Johns Hopkins University animal facilities under specific pathogen-free conditions, and experiments conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee.

Orthotopic Lung Transplant

Syngeneic transplantations were performed in the C57BL/6 → C57BL/6 strain combination and allogeneic transplantations were performed in the BALB/c → C57BL/6 strain combinations. Donor mice were sedated with etomidate (1 mg, intraperitoneal), intubated, and maintained on inhaled isoflurane until sacrifice. Recipients were both initially sedated and maintained on inhaled isoflurane. Transplantation was performed using a cuffed technique as previously described (23). Mice received subcutaneous buprenorphine (0.03–0.05 mg/kg) prior to extubation and every 6 hrs thereafter as needed. Unless otherwise specificed, animals were sacrificed for analysis at 10 days posttransplant.

Medium and Reagents

Cell culture medium RPMI 1640 (Biofluids, Rockville, MD) was supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 2mM glutamine, 1mM Sodium Pyruvate, 1% NEAA, 100 U/ml Penicillin, 100 MGC/ml Streptomycin, 50μM β-mercaptoethanol, and 25mM HEPES (Biofluids, Rockville, MD).

Cell Preparations, Stimulation, and Cytokine Detection

Spleen, draining lymph nodes (LN), and lungs were harvested from mice on day 10 after transplant. Lungs were minced and incubated at 37°C in an enzyme cocktail of RPMI containing 2.4 mg/ml of Collagenase I and 20μg/ml DNAse (Invitrogen, Carlsbad, CA), then mashed through a 70μm nylon cell strainer (BD Falcon). A 23% and 70% bilayer Percoll (Amersham Biosciences, Uppsala, Sweden) gradient was performed, and the interface collected. Isolated responder cells from allografts, LN, spleen, or lung mononuclear cells (LMNC) were cultured for 5h in medium alone, with BALB/c splenocytes (1:1), or in the presence of phorbol myristate acetate/ionomycin (PMA/ionomycin) (50ng/ml/500ng/ml) with brefeldin A (10 μg/ml; Sigma) present for the final 2h of stimulation. Following stimulation, cells were harvested for surface and intracellular staining.

Flow Cytometry

The following antibodies were purchased from BD PharMingen (San Diego, CA) or Ebioscience: Phycoerythrin (PE)-labeled anti-IL-17A; Allophycocyanin (APC)-labeled anti-IFN-γ; FITC-labeled anti-TNF-α; Peridinin-chlorophyll-protein complex (PerCP) Cy5.5-labeled anti-CD8; Alexa-700-labeled anti-CD4; Biotinylated anti-H2D^d; Pacific Blue-labeled streptavidin; APC-labeled Foxp3; PE-labeled anti-CD25; and respective isotype Abs. Surface antibody staining and intracellular cytokine staining (ICCS) was performed as previously described (36, 37). Flow cytometry analysis was performed using a FACSAria instrument and Flowjo software for analysis (Tree Star Inc, San Carlos, CA).

Histopathology and Acute Rejection Pathology Scoring

Grafts were fixed in 10% formalin, embedded in paraffin, sectioned and stained using Hematoxylin & Eosin. Hematoxylin and Eosin stained sections of grafts were scored by 3

blinded observers using a 5 point system developed to grade the severity of rejection. One point was given for the presence of each of the following characteristics: (1) Perivascular mononuclear cell infiltrate, (2) Peribronchial mononuclear cell infiltrate, (3) Interstitial inflammatory infiltrate, (4) Alveolar inflammatory infiltrate, (5) Hemorrhage/parenchymal necrosis. The acute rejection score is the sum of the points given (range 0–5).

Statistical analysis

Data were compared with two-tailed student's t-test using Graphpad software. A p value of less than 0.05 was considered statistically significant.

RESULTS

Acute rejection in MHC-mismatched murine orthotopic lung allografts is associated with a decreased CD4:CD8 ratio in infiltrating lymphocytes

To evaluate the adaptive T cell response during acute rejection of murine orthotopic lung allografts, we compared graft pathology and T cell infiltration in C57BL/6 recipients of C57BL/6 [H-2b] isografts and BALB/c [H-2d] allografts. At day 10, allogeneic lung allografts demonstrated severe lung injury on gross pathology in striking contrast to syngeneic lung isografts (Fig 1A). Allogeneic allografts had massive mononuclear cell infiltration surrounding vessels and airways with inflammation extending into the interstitium and alveolar spaces and evidence of hemorrhage and necrosis often present, in striking contrast to isografts (Fig 1B). There was a significant difference in acute rejection scores at day 10 (Fig 1C). We isolated lung mononuclear cells and found a significant four-fold increase in the mean recovery of mononuclear cells from day 10 allografts compared to isografts or the native lungs of allograft recipients (Fig 1D). We next characterized the T cell subsets in lung grafts using flow cytometry and found a significant reduction in the CD4:CD8 ratio in allografts compared to isografts (Fig 1E). Together, these data show quantitative and qualitative differences in the T cell populations between lung allografts and isografts 10 days following transplantation.

Allospecific $CD8^+IFN\text{-}\gamma^+$ effector T cell responses predominate during acute cellular rejection in MHC-mismatched murine orthotopic lung allografts

Next, we evaluated lung allograft T cells for allospecific cytokine responses. $CD8^+$ T cells spontaneously secreting the type 1 effector cytokine IFN-γ were detectable in lung allografts. In vitro re-stimulation with BALB/c splenocytes dramatically increased the percentage of $CD8^+$ T cells from lung allografts secreting IFN-γ. These findings are in striking contrast to $CD8^+$ T cells from isografts, which rarely produced IFN-γ spontaneously or after in vitro re-stimulation with BALB/c alloantigen (Fig 2A, B), but had similar percentages of IFN-γ^+ cells in response to PMA/ionomycin re-stimulation (Fig 2A). Constitutive production of IFN-γ could also be detected in $CD4^+$ T cells from lung allografts but only modestly increased with alloantigen re-stimulation (Fig 2C, D). IFN-γ production from $CD4^+$ T cells was nevertheless significantly increased in allografts compared to isografts, both constitutively and following re-stimulation (Fig 2D). Comparison of $CD8^+$ and $CD4^+$ allospecific responses (after restimulation with alloantigen) demonstrated that $CD8^+IFN\text{-}\gamma^+$ responses predominated during acute cellular rejection of lung allografts (Fig 2E). We also detected low frequencies of allospecific $CD8^+TNF\text{-}\alpha^+$ cells in lung allografts, but were unable to detect allospecific IL-2 production in $CD4^+$ or $CD8^+$ T cells (data not shown). Finally, we were unable to detect allospecific IL-17 production by $CD4^+$ T cells (Fig 3A, B) or $CD8^+$ T cells (data not shown). Lung mononuclear cell cultures from allografts and isografts stimulated with PMA/Ionomycin had similar frequencies of polyclonal $CD4^+IL\text{-}17^+$ cells, but these were significantly higher than age-matched littermate controls who did not undergo lung transplant surgery (Fig 3C). Together, these

data indicate that $CD8^+IFN\text{-}\gamma^+$ T cells are the predominant allospecific effector responses during acute cellular rejection in MHC-mismatched murine orthotopic lung allografts, though $CD4^+$ T cells also contribute allospecific effector responses.

Allospecific effector T cell responses are detectable in secondary lymphoid tissue and the native lung following murine orthotopic lung transplantation

In the same experiments, we determined whether allospecific effector T cell responses were detectable in other systemic tissues at day 10. The predominant $CD8^+IFN\text{-}\gamma^+$ allospecific response was consistently detectable in the mediastinal lymph node and spleen, as well as the native lung, shown in Fig 4A. Moreover, allospecific responses in the native lung were detectable at significantly higher frequencies (Fig 4B). In contrast, $CD4^+IFN\text{-}\gamma^+$ alloresponses were not consistently detectable in these tissues (data not shown).

Anti-CD154 costimulation blockade attenuates acute rejection pathology and abrogates $CD8^+$ and $CD4^+$ allospecific effector responses

Because the CD154/CD40 costimulation pathway has been shown to play an important role in allograft rejection, we evaluated orthotopic lung allografts following treatment with anti-CD154 Ab therapy (500 μg i.p. at days 0 and 2). At day 10, the gross appearance of lung allografts from anti-CD154 Ab-treated recipients had significantly less hemorrhagic injury (Fig 5A). Histology revealed remarkable preservation of lung architecture in the allografts of anti-CD154 Ab-treated mice compared to untreated allografts, though some mononuclear cell infiltration primarily surrounding vessels persisted (Fig 5B). There was a significant decrease in the acute rejection scores and mean mononuclear cell recovery in allografts from anti-CD154 Ab-treated mice compared to untreated allografts (Fig 5C, 5D). Next, we evaluated whether these histologic changes were associated with differences in allospecific T cell responses at day 10. The predominant lung allograft $CD8^+IFN\text{-}\gamma^+$ allospecific responses were abrogated in anti-CD154 Ab-treated recipients compared to untreated allograft recipients, as well as allograft $CD4^+IFN\text{-}\gamma^+$ allospecific T cell responses (Fig 5E, F). In contrast, anti-CD154 Ab therapy had no effect on PMA/ionomycin-induced $CD8^+IFN\text{-}\gamma^+$ responses. Together, these data indicate a critical role for the CD154/CD40 pathway in the regulation of T cell allospecific effector responses following MHC-mismatched mouse orthotopic lung transplantation.

Anti-CD154 costimulation blockade increases the CD4:CD8 ratio and the frequency of $CD4^+CD25^+Foxp3^+$ T cells in murine orthotopic lung allografts

We next evaluated the lung CD4:CD8 ratio in anti-CD154 Ab-treated allograft recipients compared to untreated lung allograft recipients. As shown in Fig 6A, we observed significantly increased CD4:CD8 ratios in lung allografts from anti-CD154-treated recipients compared to recipients receiving no treatment. We then examined whether there were differences in the frequencies of $CD4^+$ T cells with a regulatory phenotype, i.e. $CD4^+CD25^+Foxp3^+$ T cells between these groups. We found that lung allografts from anti-CD154-treated mice contained significantly increased frequencies of $CD25^+Foxp3^+$ cells as a percent of total $CD4^+$ T cells compared to control mice (Fig 6B, 6C).

DISCUSSION

The regulation of allospecific T cell effector responses following solid organ transplant remains incompletely understood. Our studies show that $CD8^+$ T cells are the predominant allospecific responders in MHC-mismatched (BALB/c → B6) mouse orthotopic lung allograft rejection, with these cells producing predominantly IFN-γ in response to alloantigen. Our results are consistent with previous studies in the mouse orthotopic lung transplant model showing a high percentage of $CD8^+$ T cells have the capacity for IFN-γ

following re-stimulation using the calcium ionophore, PMA/ionomycin (5, 24). It should be pointed out however, that PMA/ionomycin-induced responses are not specific to allograft recipients, given that we found $CD8^+$ T cells from isografts also demonstrate a high capacity for IFN-γ production under these re-stimulation conditions. In contrast, we show that constitutive IFN-γ responses and increased IFN-γ responses in response to re-stimulation with BALB/c alloantigen were very specific to allograft recipients. It is not surprising, however, that $CD8^+$ T cells (and $CD4^+$ T cells) produced constitutive IFN-γ in allografts, as these allograft-derived cultures already contain some alloantigen (25). Our studies also demonstrate a decrease in the CD4:CD8 ratio in day 10 lung allografts compared to isografts due to increased numbers of $CD8^+$ T cells. Our results are consistent with the report by Gelman et al. that showed $CD8^+$ T cells outnumbering $CD4^+$ T cells in mouse lung allografts despite differences in the timing (day 7) and mouse strains used (B6 → CBA) (24). Our current results are also similar to our previous report in the murine heterotopic tracheal transplant model of predominant $CD8^+$IFN-γ^+ allospecific effectors at the peak of inflammation (25). Together, our results suggest a predominant role for $CD8^+$ allospecific effector T cells in the acute rejection response following MHC-mismatched mouse lung transplant.

We also found $CD4^+$ allospecific IFN-γ responses in the lung allograft indicating these cells are capable of participating in the alloimmune response, albeit at lower frequencies compared to $CD8^+$ T cells. While a report by Gelman et al. reported $CD4^+$ T cells were not necessary for acute cellular lung rejection mediated by $CD8^+$ T cells using CD4-depletion and CD4-deficient mice in this model (24), allospecific $CD4^+$ responses were not evaluated in that study. As activated $CD4^+$ T cells are a major source of CD154 costimulation in providing 'help', it is possible that $CD8^+$ T cell-mediated rejection in the absence of $CD4^+$ T cells may be facilitated by other sources of CD154, such as activated platelets (26). Alternatively, $CD4^+$ T cell deficiency may actually promote acute cellular rejection in the absence of regulatory T cells. Thus, the role(s) of $CD4^+$ T cells in allogeneic mouse othotopic lung transplant need to be more fully elucidated.

Because IL-17 production has been implicated in chronic lung allograft rejection in humans (27), we asked whether it was produced in response to alloantigen. While we were unable to detect allospecific IL-17 at day 10 (or day 21), approximately 17–35% of $CD4^+$ T cells in isografts and allografts had the capacity to secrete IL-17 in response to PMA/Ionomycin stimulation. We found that the lung transplant surgery alone was sufficient to induce these polyclonal IL-17 responses, which were increased compared to lower responses found in control mice that had not undergone surgery. While the explanation for this finding remains unclear at this time, it is possible that surgery-related injury could unmask autoantigens capable of driving IL-17 induction in rat lung isografts (28). Together, these findings indicate that $CD4^+$ T cells do not produce high levels of IL-17 in response to alloantigen in the acute rejection response under these conditions of MHC-mismatched mouse orthotopic lung transplant. However, it is conceivable that other antigens or exogenous stimuli may elicit IL-17 secretion, and therefore our studies do not necessarily exclude a potential role for this cytokine in chronic lung allograft rejection.

Several studies in the past few years have demonstrated the capacity for tertiary lymphoid organs (TLO), such as the lung, to establish aggregates of antigen-specific T and B cells during inflammation independent of secondary lymphoid organs (SLO) (29–31). Recently, work by Gelman et al. showed the capacity for acute cellular rejection in the mouse orthotopic lung transplant model to occur in the absence of secondary lymphoid organs (32). Our studies detected allospecific $CD8^+$IFN-γ^+ T cells in the mediastinal lymph node and spleen consistent with systemic priming in SLO following orthotopic lung transplant. Further, we found increased allospecific $CD8^+$IFN-γ^+ cells in the native lung indicating that

effector memory cells can stochastically traffic to non-inflamed effector sites. This is reminiscent of our prior findings in the heterotopic tracheal transplant model where allospecific $CD8^+$ effectors were detectable in the recipient lungs posttransplant (25). Thus, while T cell priming may occur in the lung allograft itself, our findings indicate that systemic T cell allosensitization following mouse orthotopic lung transplantation also occurs.

Previous studies have shown an important role for the CD154/CD40 costimulation pathway in allograft acceptance in experimental transplant models, including our studies in the heterotopic airway transplant model (6–9, 33). Our results demonstrate a critical role for the CD154/CD40 pathway in the regulation of both allospecific $CD8^+$ and $CD4^+$ type 1 T cell responses and attenuation of acute rejection following mouse orthotopic lung transplantation. While some studies have found $CD8^+$ T cells to be resistant to costimulation blockade (12–16), our results are consistent with studies demonstrating abrogation of allospecific $CD8^+$ T cell responses with CD154/CD40 pathway disruption (34–37). Prior work in skin and bone marrow transplant models suggest allospecific $CD8^+$ T cells undergo clonal deletion (37, 38). Interestingly, a recent study using anti-CD154 MR1 Ab in conjunction with CTLA4-Ig showed that attenuation of acute rejection pathology in mouse orthotopic lung transplant was abrogated in Bcl-2 transgenic recipients resistant to T cell apoptosis, consistent with a role for deletion of allospecific T cells in costimulation blockade mediated tolerance (39). Previously, we reported activated airway allograft $CD8^+$ T cells with uncoupled effector cytokine production and proliferation following heterotopic tracheal allotransplant in the setting of CD154 deficiency (33). While these are different experimental systems, our current findings are similar in that we find 1) significantly improved allograft acceptance and 2) significantly reduced allospecific $CD8^+$ effector responses and diminished mononuclear infiltration with disruption of CD154/CD40 signaling. However, in contrast to our previous work in the airway transplant model, our current studies observed a $CD4^+$ T cell predominance in lung allografts following disruption of CD154/CD40 signaling. The potential uncoupling of T cell expansion and effector function as a mechanism for allograft acceptance was not assessed in this current study, and it remains to be determined in future studies. This is of particular interest given our current findings of the persistence of graft mononuclear infiltrates and lung $CD8^+$ T cells in anti-CD154-treated allografts, similar to earlier reports (9, 36).

Our results also show anti-CD154 therapy significantly increases the frequencies of $CD4^+CD25^+Foxp3^+$ T cells in the lung allograft. This finding is consistent with prior *in vitro* and *in vivo* studies implicating an important role for regulatory T cells (Tregs) in the establishment of tolerance to alloantigens (20, 21, 40, 41). While it remains unclear the precise role or mechanism(s) by which Tregs contribute to allograft acceptance under conditions of CD154 blockade, growing evidence supports contributory roles for both deletion and regulation of allospecific T cell responses in transplant tolerance (42–44).

In conclusion, our studies find that anti-CD154 Ab therapy alone is sufficient to attenuate acute cellular rejection in an MHC-mismatched mouse orthotopic lung transplant model. Improved lung allograft acceptance in anti-CD154 Ab-treated recipients was associated with abrogated $CD8^+$ and $CD4^+$ allospecific effector responses and increased frequencies of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the lung allografts. Further studies in the mouse orthotopic lung transplant model are required to determine whether these cellular effects following CD154 costimulation blockade represent distinct mechanisms contributing to lung allograft acceptance or are interdependent. Finally, these data indicate that novel approaches to block the CD154/CD40 costimulation pathway may be beneficial in the clinical transplant setting if this can be achieved safely (45).

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LIST OF ABBREVIATIONS

BOS	Bronchiolitis obliterans syndrome
ACR	Acute cellular rejection
CTLA4-Ig	Cytotoxic T lymphocyte antigen 4-fusion protein immunoglobulin
LN	Lymph node
LMNC	Lung mononuclear cells
PMA/ionomycin	Phorbol myrisate acetate /ionomycin
PE	Phycoerythrin
APC	Allophycocyanin
IFN-γ	Interferon-gamma
FITC	Fluorescein isothiocyanate
TNF-α	Tumor necrosis factor-alpha
IL-17	Interleukin 17
PerCPCy 5.5	Peridinin -chlorophyll-protein complex cyanine 5.5 conjugate
ICCS	Intracellular cytokine staining
Foxp3	Forkhead box P3
Tregs	Regulatory T cells
SLO	Secondary lymphoid organs
TLO	Tertiary lymphoid organs

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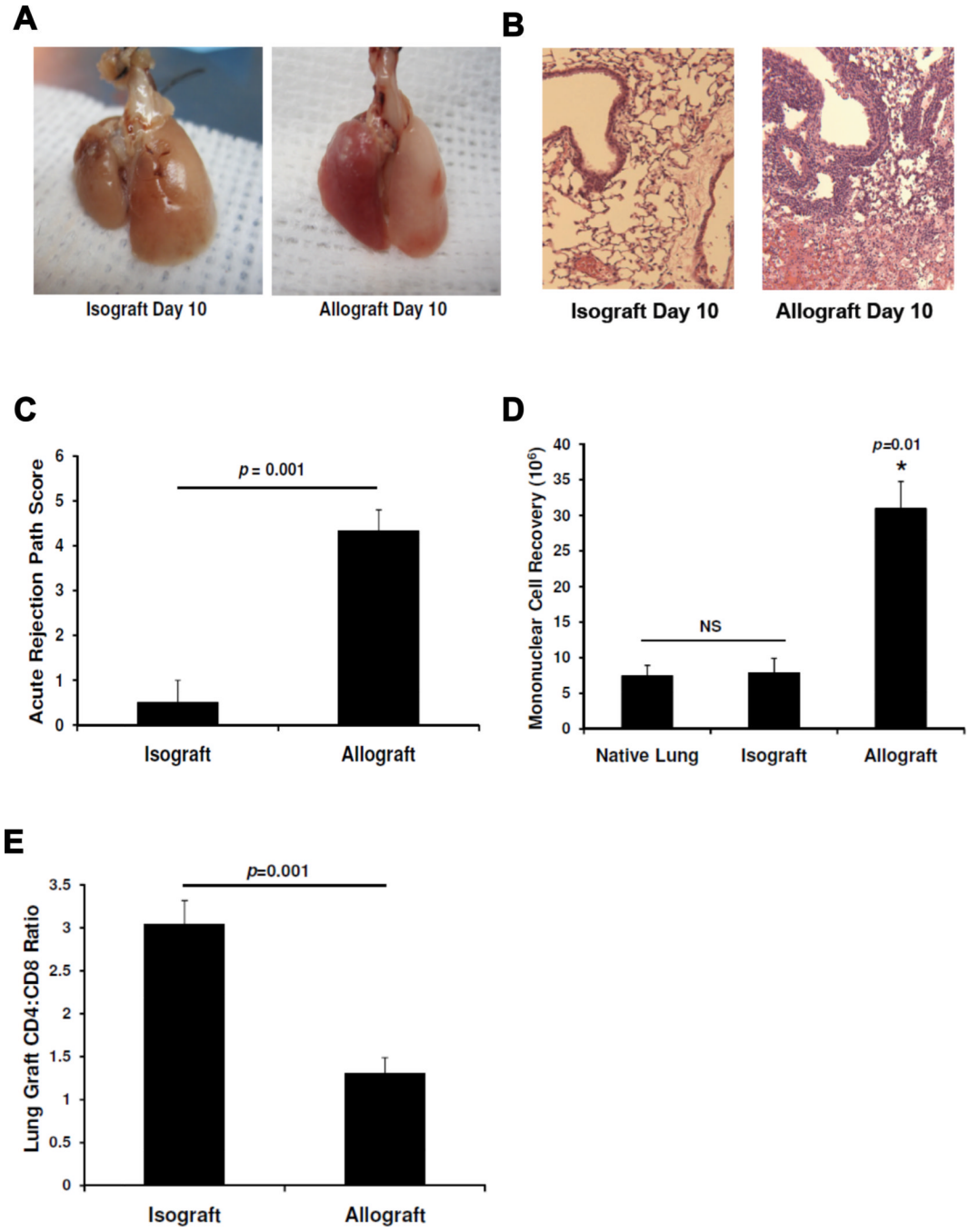

Figure 1. Acute cellular rejection following MHC-mismatched orthotopic lung transplant is associated with a decreased graft CD4:CD8 ratio

(A) Gross pathology of BALB/c → B6 allograft and B6 →B6 isograft (graft left in photos) and (B) lung histology with H&E staining (10×) at day 10. (C) Acute rejection pathology score means at day 10 in isografts versus allografts (n=4 mice per group). (D) Comparison of mean mononuclear cell recovery at day 10 in isografts versus allografts (n=3–8 mice per group). (E) Mean lung graft CD4:CD8 ratio determined by flow cytometry in isografts versus allografts (n=3–8 mice per group).

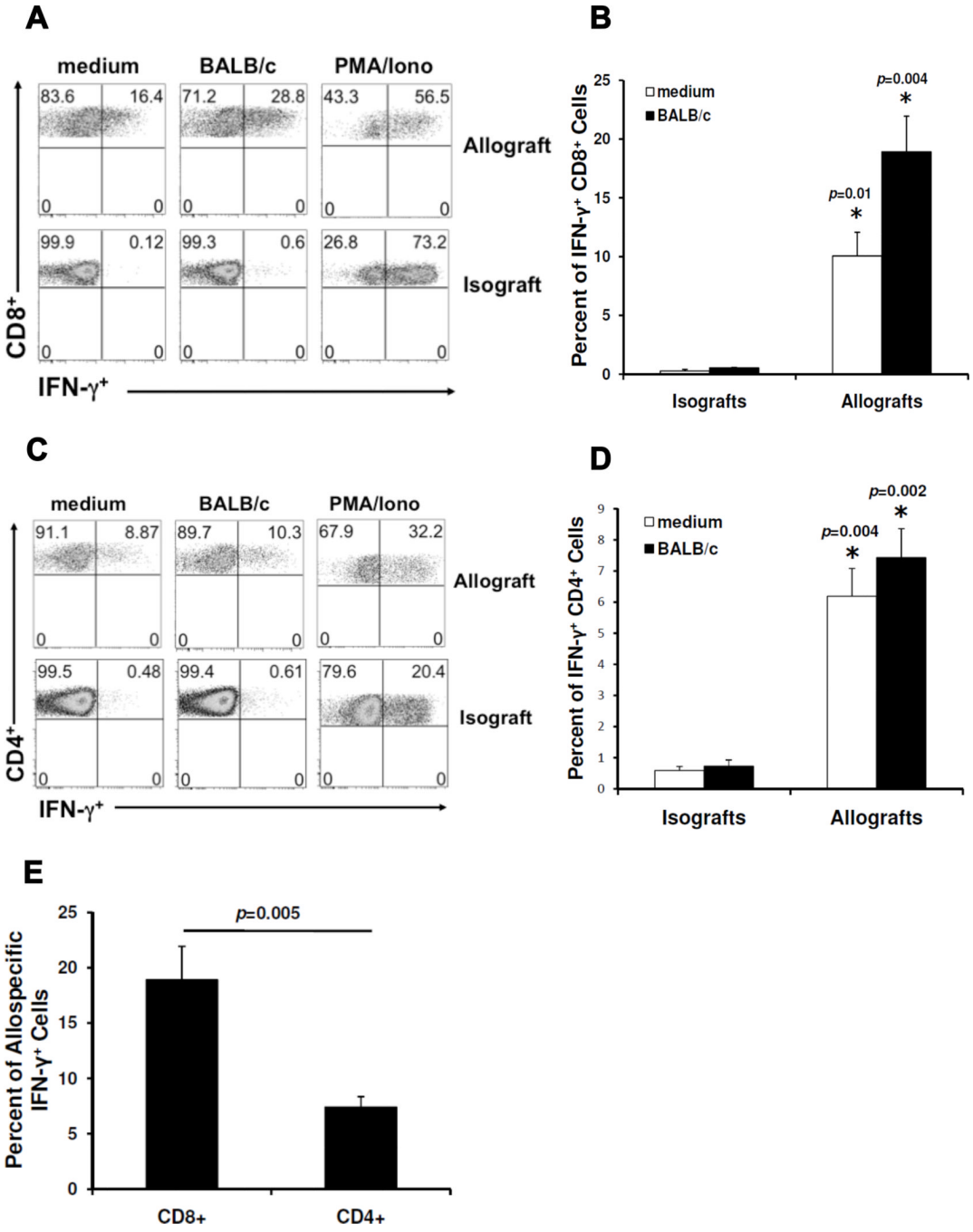

Figure 2. Allospecific CD8$^+$IFN-γ$^+$ effector responses predominate over CD4$^+$ responses in MHC-mismatched orthotopic lung allografts

(A) Representative day 10 flow plots of lung allograft or isograft mononuclear cells cultured for 5h in medium alone, in the presence of BALB/c splenocytes (1:1 ratio), or with PMA/ionomycin; gating on *H-2^d* negative, CD8$^+$ T cells in mixed cultures. (B) Cumulative day 10 mean CD8$^+$ allospecific IFN-γ responses (expressed as percent of CD8$^+$ cells positive for IFN-γ) in allografts versus isografts (n=3–6 mice per group). (C) Representative day 10 flow plots of lung allograft or isograft mononuclear cells with the same culture conditions and analysis as in (*A*); gating on *H-2^d* negative, CD4$^+$ T cells in mixed cultures. (D) Cumulative day 10 mean CD4$^+$ allospecific IFN-γ responses (expressed as percent of CD4$^+$ cells

positive for IFN-γ) in allografts versus isografts (n=3–6 mice per group). (E) Comparison of day 10 mean $CD8^+$ and $CD4^+$ allospecific IFN-γ responses in allografts after 5h culture of lung mononuclear cells with BALB/c splenocytes (n=6 mice).

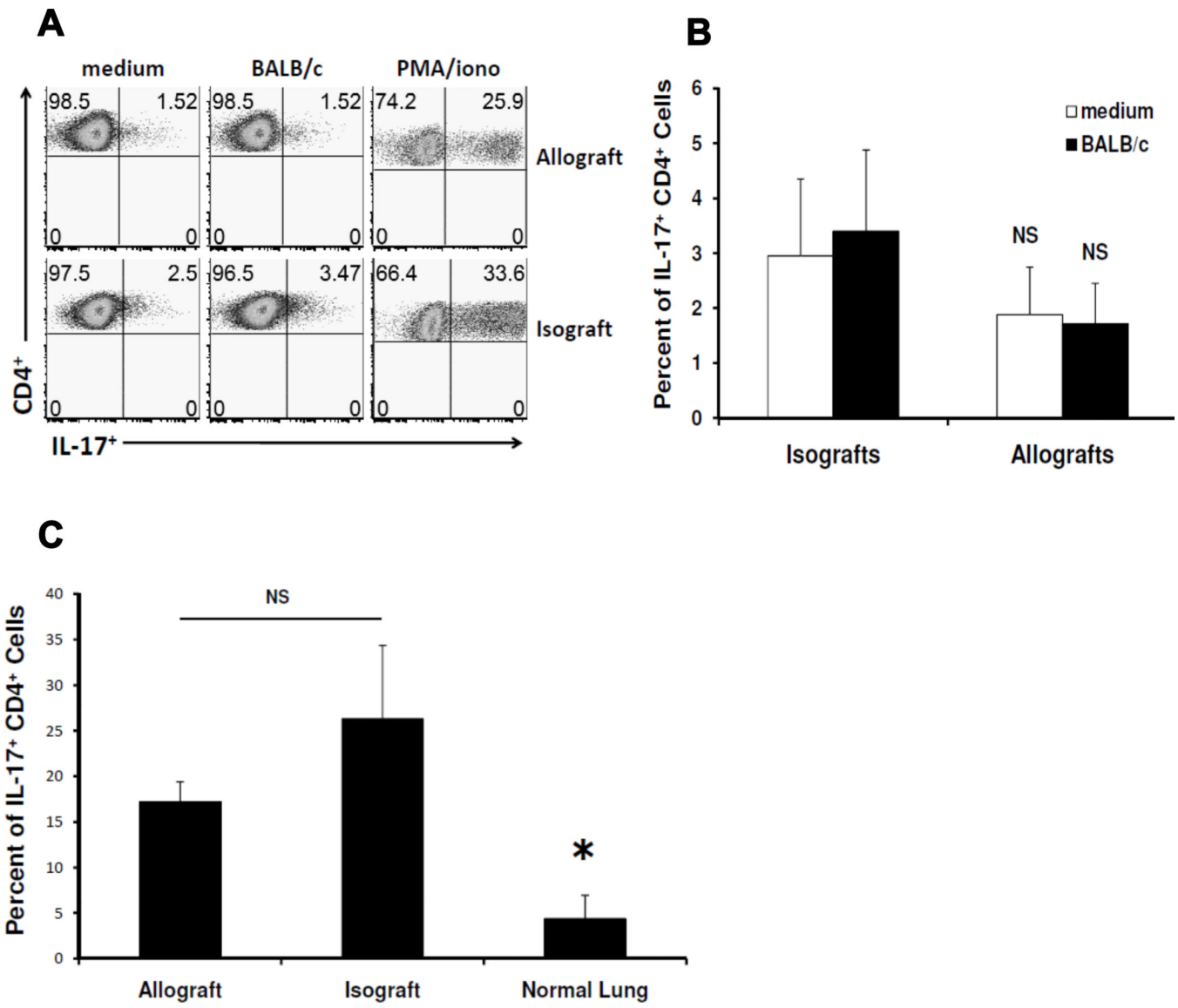

Figure 3. Lung transplant surgery is sufficient for the induction of polyclonal CD4$^+$IL-17$^+$ T cells

(A) Representative flow plots of CD4$^+$ IL-17 responses at day 10 in lung graft mononuclear cells cultured for 5h in medium alone, in the presence of BALB/c splenocytes (1:1 ratio), or with PMA/ionomycin; gating performed on *H-2^d* negative, CD4$^+$ T cells in mixed cultures. (B) Cumulative day 10 mean CD4$^+$IL-17$^+$ responses (expressed as percent of CD4$^+$ cells positive for IL-17) from isografts versus allografts after culture of lung mononuclear cells in the presence or absence of BALB/c splenocytes (n=3–5 mice per group). (C) Cumulative day 10 mean polyclonal CD4$^+$IL-17$^+$ responses (expressed as percent of CD4$^+$ cells positive for IL-17) from isografts, allografts, and left lungs of littermates receiving no transplant after culture of lung mononuclear cells with PMA/ionomycin.

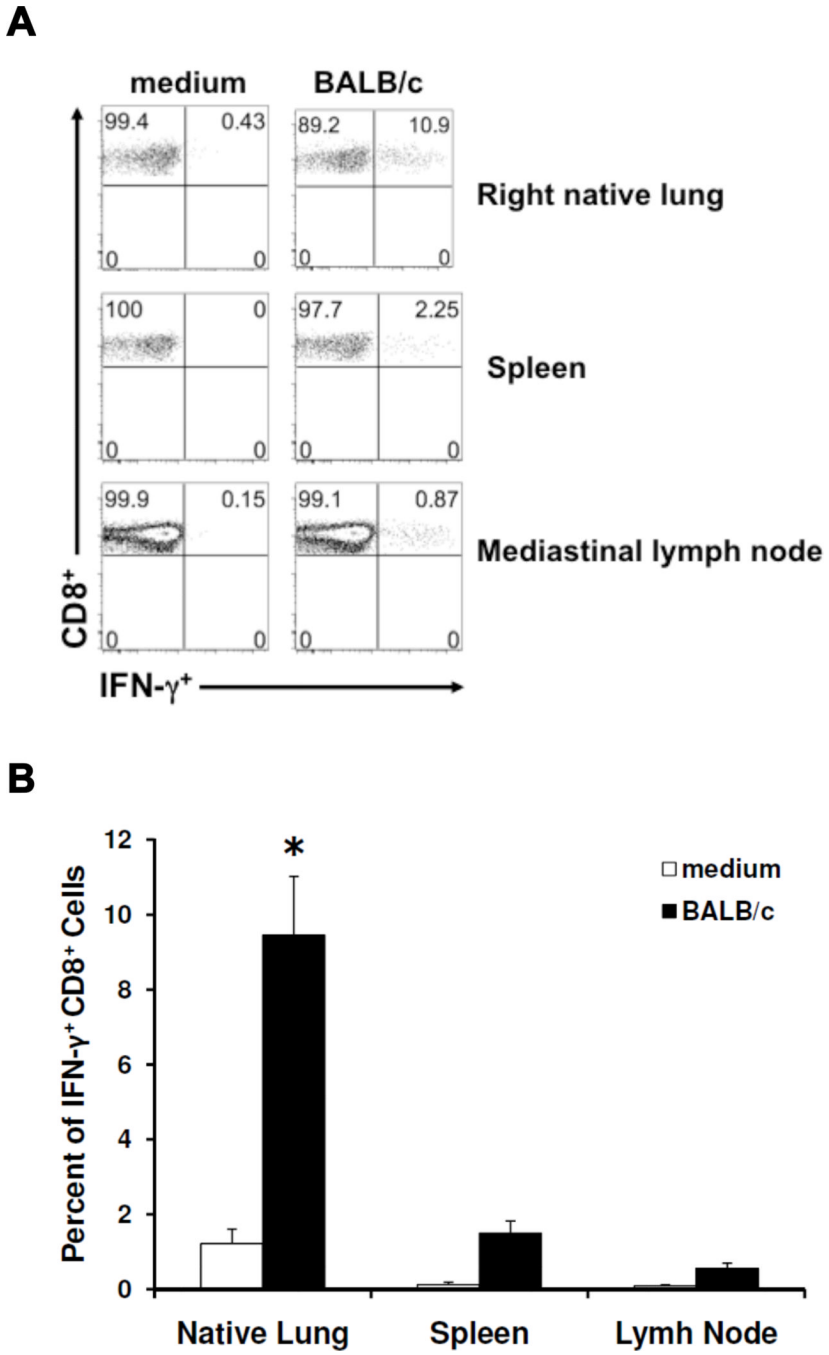

Figure 4. Systemic allospecific effector responses are detectable following orthotopic lung transplant

(A) Representative flow plots of $CD8^+$ IFN-γ responses at day 10 in mononuclear cells isolated from the native lung, spleen, and mediastinal lymph node of transplanted mice, cultured for 5h in the presence or absence of BALB/c splenocytes (1:1 ratio); gating performed on *H-2*^{d} negative, $CD8^+$ T cells in mixed cultures. (B) Cumulative day 10 mean allospecific $CD8^+$ IFN-γ responses (expressed as percent of $CD8^+$ cells positive for IFN-γ) from the native lung, spleen, and mediastinal lymph node of mice receiving allografts (n=4–6 mice per group). (p=0.0006 when comparing means of native lung and spleen, and p=0.002 when comparing means of native lung and lymph node.)

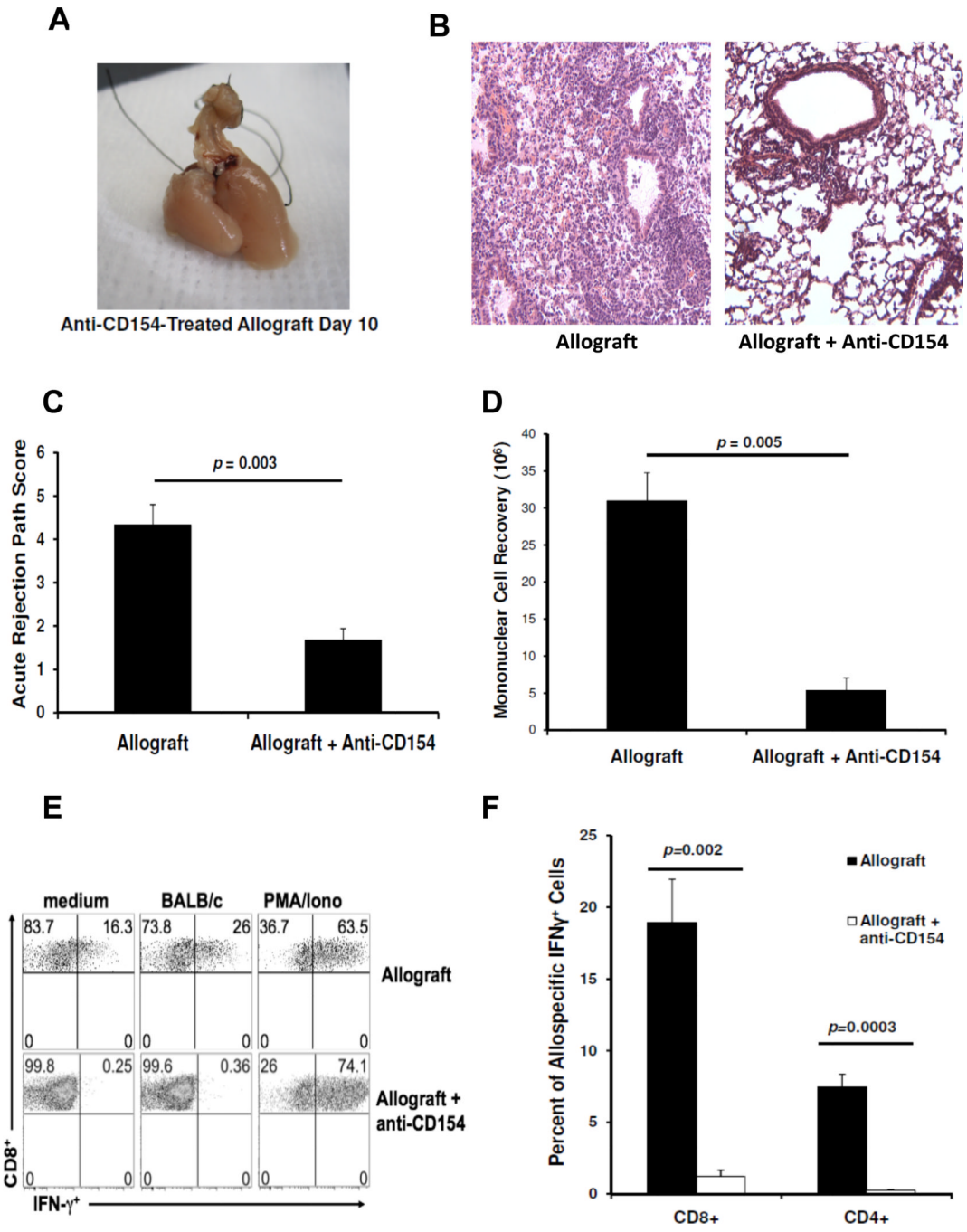

Figure 5. Anti-CD154 Ab treatment attenuates acute cellular rejection and abrogates CD8$^+$ and CD4$^+$ allospecific effector responses following mouse orthotopic lung transplant

(A) Representative day 10 gross pathology of an allograft from an anti-CD154 Ab-treated mouse and (B) lung allograft histology of untreated and anti-CD154 Ab-treated mice. (C) Acute rejection pathology score means in allografts of untreated versus anti-CD154 treated mice (n=4 mice per group). (D) Comparison of mean mononuclear cell recoveries at day 10 in untreated versus anti-CD154 Ab-treated mice (n=4–6 per group). (E) Representative flow plots of day 10 CD8$^+$ IFN-γ responses in lung mononuclear cells from allograft of untreated mouse versus anti-CD154 Ab -treated mouse after 5h culture conditions of medium alone, in the presence of BALB/c splenocytes (1:1 ratio), or with PMA/ionomycin; gating on *H-2^d*

negative, $CD8^+$ T cells in mixed cultures. (F) Cumulative mean allospecific $CD8^+$ and $CD4^+$ IFN-γ responses (expressed as percent of cells positive for IFN-γ) from lung allografts of both groups after 5h culture of lung mononuclear cells with BALB/c splenocytes (n=4–6 mice per group).

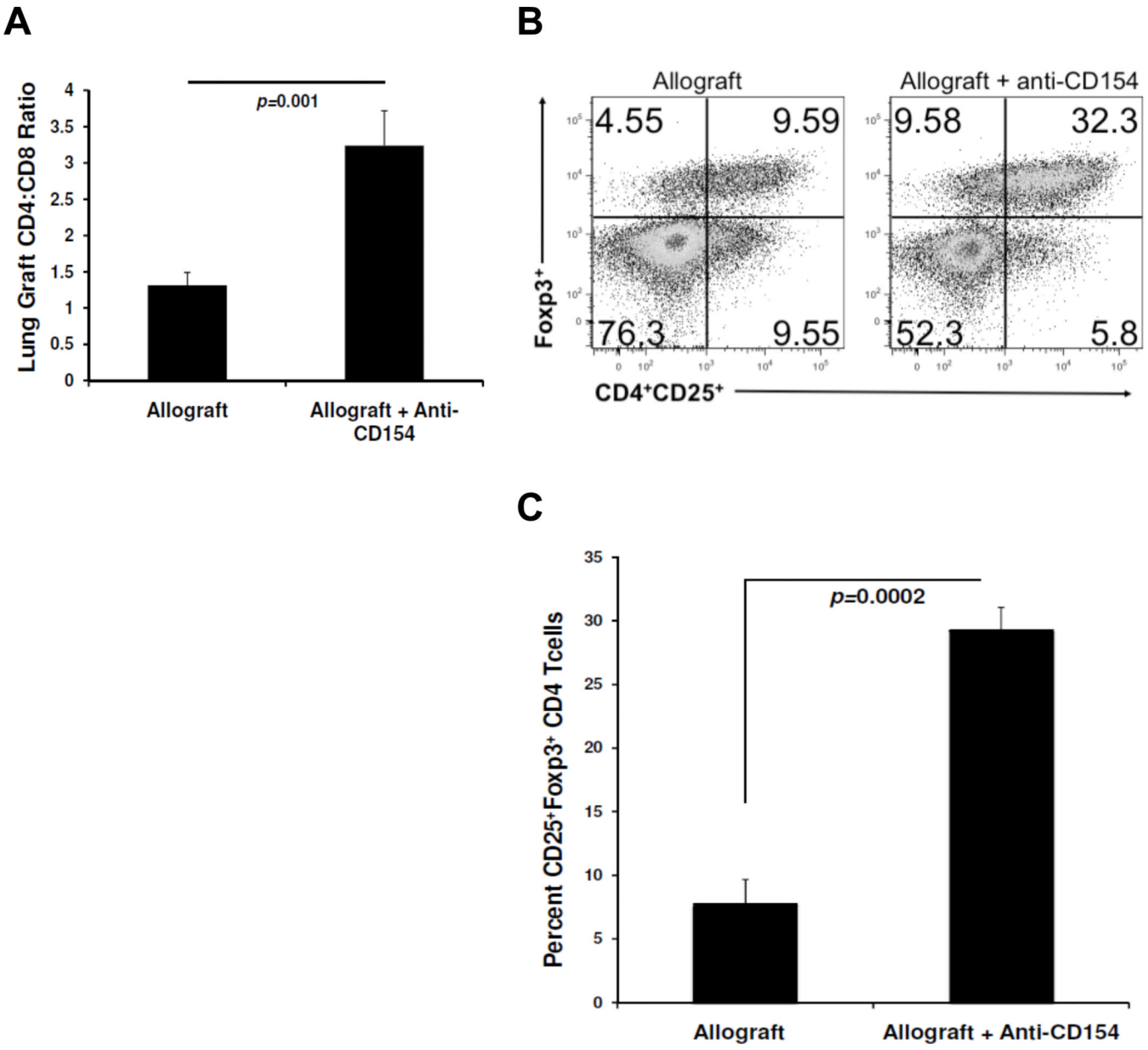

Figure 6. Anti-CD154 Ab treatment increases the lung allograft CD4:CD8 ratio and enhances $CD4^+CD25^+Foxp3^+$ T regulatory cells

(A) Mean lung allograft CD4:CD8 ratio determined by flow cytometry in anti-CD154-treated mice versus untreated mice (n=4 mice per group). (B) Representative flow plot of $CD4^+CD25^+Foxp3^+$ T cells in lung allografts from anti-CD154-treated mice versus untreated mice; gating on $CD4^+$ cells (n=4 mice per group). (C) Cumulative mean frequency of $CD25^+Foxp3^+$cells as a percent of $CD4^+$ T cells in lung allografts from anti-CD154-treated versus untreated mice (n=4 mice per group).