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T regulatory cells play a significant role in modulating MHC class I Antibody induced obliterative airway disease

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Abstract

The molecular mechanisms leading to the development of chronic lung allograft dysfunction following *de novo* development of antibodies to mismatched donor MHC remain undefined. We demonstrated that intrabronchial administration of antibodies to MHC class I resulted in induction of both innate and adaptive cellular immune responses characterized by a predominance of Th17 specific to lung associated self antigens K α 1-tubulin and Collagen-V leading to the development of obliterative airway lesions (OAD), correlate of chronic rejection following human lung transplantation. To determine the role of regulatory T cells (Treg) in the pathogenesis of OAD, we administered anti-MHC class I to mice, in which Treg were depleted by conditional ablation of FoxP3+cells. Under this condition, we observed a three-fold increase in pulmonary cellular infiltration, luminal occlusion and fibrous deposition when compared anti-MHC class I Ab administered mice maintaining FoxP3. OAD lesions were accompanied with enhanced accumulation of neutrophils along with self-antigen specific Th17 and humoral responses. However, IL-17-blockade or adoptive transfer of Treg abrogated OAD. We conclude that Treg exerts a suppressive effect on anti-MHC induced IL-8 mediated neutrophil infiltration and innate immune responses that leads to inhibition of Th17 immune responses to lung associated self antigens which is critical for development of OAD.

Keywords

OAD; Alloimmunity; Autoimmunity; Treg; Th17

Introduction

Chronic rejection following human lung transplantation (LTx) clinically diagnosed as bronchiolitis obliterans syndrome (BOS) continues to be a significant hindrance for the long term allograft survival (1-4). Studies have suggested that alloimmune responses to mismatched donor HLA plays a major factor in the development of BOS (5). Studies from our laboratory, and others, have demonstrated that development of immune responses to self-antigens (self-Ags) mainly, K- α 1 Tubulin (K α 1T) and Collagen V (ColV), during the post-transplant period also strongly correlates with development of chronic rejection (6-9).

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Disclosures

None of the authors have any conflicts of interest to disclose.

Various animal models (10-14) have been proposed to define the immunopathogenesis of obliterative airway disease (OAD), the correlate of human BOS. However, most of these models either do not fully represent the lesions seen in BOS or are technically challenging. Another complexity in defining the mechanisms for development of BOS stems from the fact that multiple independent risk factors like acute rejection, gastroesophageal reflux disease, respiratory viral infection, have been implicated in its pathogenesis (3). A consensus hypothesis proposed is that these primary insults lead to local inflammatory *milieu* conducive for the development of alloimmune responses including donor specific antibodies (Abs) to HLA (DSA) which leads to the development of immune responses to self-Ags and thus causing chronic lung allograft rejection (15, 16). Therefore, with a goal to specifically define the role of alloimmune responses in the development of OAD, we developed a murine model for OAD, wherein MHC class I antibodies (Abs) were intrabronchially administered into mice (17). In this model, the animals developed OAD lesions. Along with these histological features, we also demonstrated development of both cellular and humoral immune responses to lung-associated self-Ags, $\alpha 1$ T and ColV. However, the role of *de novo* developed Abs to mismatched donor MHC class I molecules in this process remains unclear. Therefore, the goal of this study was to determine the mechanisms by which Abs to mismatched MHC class I molecule result in OAD and to define the role T regulatory cells in the pathogenesis of anti-MHC induced OAD. Using FoxP3-DTR transgenic mice (18, 19) we demonstrate a critical role of regulatory T cells (Treg) in modulating alloimmune mediated innate and autoimmune immune responses in OAD.

Materials and Methods

Animals

We utilized a murine model in which OAD, a correlate of BOS, was induced in the distal airways following intrabronchial administration of specific mAb to MHC class I Ags (17). All experiments were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of Washington University School of Medicine. Murine mAb to H2K^b(IgG2a, endotoxin free, measured by *LAL* assay), was given at a dose of 200 μ g/administration into wild-type C57BL/6 mice or FoxP3-DTR Knockin transgenic C57BL/6 mice (18, 19) (kindly provided by Dr. Alexander Rudensky). Abs (200 μ g) were administered into the lung on days 1, 2, 3, 6, and weekly thereafter. C1.18.4, was given on the days mentioned above as isotype control in Treg depleted group. For depletion of Treg, diphtheria toxin (DT, 1 μ g) was administered intraperitoneally twice, 5 days apart (days-7 and -2). Treg depletion was confirmed using flow cytometry analysis of the cells from spleen and lungs.

Histology to determine cellular infiltration, fibrosis and luminal occlusion

Lungs collected at days 7, 15 and 30 were fixed in 10% formaldehyde. Sections were cut at 5 μ m thickness and stained with H&E and Masson's trichrome and analyzed under a Nikon ECLIPSE 55i (Melville, NY) microscope using NIS-Elements BR software (Melville, NY). The presence of OAD lesions in the sections were performed by random sampling and analyzed by two blinded professionals. Morphometric calculations were performed using NIS-Elements BR software (Melville, NY). Percentage of fibrosis was quantified by morphometric analysis by addition of the total area enclosed by basement membrane at 5 different high power of trichrome stained sections (40x) fields and dividing by a factor of 5. Percentage of cellular infiltration and luminal occlusion was calculated at 5 different high power fields in H&E stained sections (40x), respectively. The data was represented as a mean $\pm$ SEM over a 5 different measurements.

ELISpot assay

To enumerate the frequency of specific cytokine secreting T-cells we performed ELISpot, as described previously (20). Briefly, MultiScreen 96-well filtration plates (Millipore) were coated over night at 4°C with 5.0 μ g/ml capture mouse cytokine-specific mAb (BD Biosciences). The plates were blocked with 5% BSA for 2 hrs and washed three times with PBS. Subsequently, 3×10^5 cells were cultured in triplicate in the presence of ColV (20 μ g/ml, Sigma-Aldrich, St. Louis, MO) or recombinant K α 1T (10 μ g/ml, purified in our lab) and irradiated feeder autologous splenocytes (1:1 ratio). After 48-72 hrs, the plates were developed and analyzed by ImmunoSpot Series I analyzer (Cellular Technology, Shaker Heights, OH). The results were expressed as spots per million cells $\pm$ SEM.

ELISA

To analyze the serum concentration of Abs to ColV and K α 1T, ELISA plates (Nunc, Rochester, NY) were coated with ColV or K α 1T (1 μ g/ml) in PBS over night at 4°C (21). Serum samples were collected from mice treated with anti-H2K^b or isotype Abs and tested (1:250 and 1:500) for binding to ColV and K α 1T. A sample was considered as positive if the values were more than an average cutoff value of two SD above the mean obtained from normal sera (n=10). The data was represented as a mean $\pm$ SEM over a 5 different measurements.

Flow cytometry

Expression of individual cell specific markers proteins was analyzed by flow cytometry. Splenocytes and lung infiltrating cells were collected by collagen digestion of harvested spleen and lungs, respectively. The specific cellular infiltration was quantitated using appropriate fluorescent tagged Abs for CD11b, CD4, CD8, and CD19. For FoxP3 and MPO (Santa Cruz Biotech, CA), membrane permeabilization was performed prior to treatment with primary Abs, and then by fluorescent tagged secondary Ab as per manufacturer's protocol (BD Biosciences, Sparks, MD). For ROR γ T expression, CD4 cells were positively selected by MACS beads (Miltenyi Biotec, NY) followed by FACS analysis using appropriate fluorescent tagged Abs (Santa Cruz Biotech, CA). Cells were analyzed on a LSRII flow cytometer (BD Biosciences) using FACSDiva software. All measurements were taken for 5,000 events that are a display of relative cell count. The data was represented as a mean $\pm$ SEM over a 5 different measurements.

Myeloperoxidase (MPO) Assay

Extracts were prepared by sonication of 100 mg of lungs harvested on days 15 and 30. Neutrophil activity was determined by MPO assay as described earlier (21). The data was represented as mean $\pm$ SEM for 5 different measurements.

Gene expression assay

RT-PCR was performed to determine mRNA transcription of chemokines in the lungs harvested from day 7 (17). The expression was analyzed by RT2 Profiler PCR Array for mouse chemokines and receptors and mouse growth factor as per manufacturer's protocol (SuperArray, SA Biosciences, CA). The data was represented as a mean $\pm$ SEM over a 5 different measurements.

Adoptive transfer of enriched CD4+Foxp3+Treg cells

To determine the role of Treg in induction of autoimmunity following ligation of anti-MHC class I Abs, we administered $0.1-10 \times 10^6$ CD4+CD25+ T-cells positively selected from splenocytes of non-DT treated FoxP3-DTR C57BL/6 mice and passively transferred into peritoneal cavity into DT treated FoxP3-DTR C57BL/6 mice on day 1 along with the first

dose of intrabronchial MHC class I Ab. MHC class I Ab was administered intrabronchially on days 1, 2, 3, and 6, and weekly thereafter. The animals were sacrificed and analyzed as described earlier.

IL-17 neutralization

To define the role of IL-17 in induction of autoimmunity following ligation of anti-MHC class I Abs, we administered on days 1, 3, and 5, and weekly thereafter 50 μ g of anti-IL-17 mAb *i.v.* with common epitope specificity for IL-17A and IL-17F (eBioscience, San Diego, CA) into Treg depleted FoxP3DTR C57BL/6 mice treated with anti-MHC class I Ab on days 1, 2, 3, and 6, and weekly thereafter. The animals were sacrificed and analyzed as described earlier.

Statistical analysis

All statistical analyses were performed either using GraphPad5.0 and Origin 6.0 and data represented as a mean $\pm$ SEM over a 5 different measurements. Statistical significance (p-value <0.05) was established using student *t*-test or Mann-Whitney *U*-test (SPSS, version 15.0).

Results

Kinetics of cellular infiltration following anti-MHC class I administration and OAD development in wild type mice and CD4+Fox p3+ depleted mice

To determine the role of Treg in the modulation of OAD lesions we utilized FoxP3 DTR C57BL/6 mice wherein the DT receptor is driven by the FoxP3 gene promoter. It has been demonstrated that in these animals administration of DT depletes the FoxP3+ T-lymphocytes (18). Similar to previous reports following administration of two doses of DT (1 μ g/dose/mouse), the CD4+ FoxP3+ T-cells were reduced from ~10% to complete ablation in both spleen and lung (Figure 1). This ablation was sustained for 30 days following the two doses of DT administration.

We employed a murine model of OAD in which intrabronchial administration of Abs to MHC class I (anti-H2K^b), but not isotype control (C1.18.4) results in the development of OAD lesions in the lungs of C57BL/6 mice in about 30 days following administration of Abs (17). The two groups, MHC class I Ab and isotype treated groups, were Treg depleted. To determine the role of Treg depletion on cellular infiltration in our murine alloimmune model, we performed flow cytometric analysis of lung infiltrating cells. As shown in Figure 2, temporal correlation from day 7 to 30 demonstrated an increase in the frequency of neutrophils and monocytes as evidenced by increase in CD11b and MPO expressing cells. In addition, increased frequency of CD4+ and CD8+ T cells, and B cells (CD19) were also seen in the Treg depleted animals over the wild type. As expected, no Tregs were seen in Treg depleted animals. However, in the wild type animals the Treg frequency dropped from 15.3 $\pm$ 2.1% at day 15 to 4.5 $\pm$ 2.9% on day 30, while the isotype treated animals had no change in Treg frequency (remained about 9%). The isotype Ab treated FoxP3 depleted animals demonstrated no increase in cellular infiltration which was similar to isotype Ab treated wild type animals (data not shown). These results strongly suggest that depletion of Treg increases lung infiltration with neutrophils, monocytes, CD4 and CD8 T cells, and B cells, thus promoting innate and adaptive immune responses in this murine model of OAD induced by antibodies to MHC class I molecule.

Depletion of CD4⁺Foxp3⁺ conventional Treg increases OAD lesions induced by Abs to MHC class I

To test for the role of Treg in modulating OAD, we administered DT to Foxp3-DTR transgenic mice on days -7 and -2 prior to intrabronchial administration of anti-H2K^b. As shown in Figure 3, histological analysis by H&E staining of the lungs harvested on day 30 following administration of H2K^b Ab demonstrated a significant increase in cellular infiltration around bronchioles resulting in luminal occlusion. Trichrome staining to quantitate fibrosis also demonstrated significant increase in fibrosis in Treg depleted animals. These results demonstrate that depletion of Treg results in markedly increased OAD lesions following administration of anti-MHC and thus strongly suggesting a modulatory role of Treg in OAD.

Conventional Treg significantly modulates immune responses to self-Ags, K α 1T and Col V

We previously demonstrated development of immune responses to self-Ags, ColV and K α 1T following administration of Abs to MHC class I in a murine model of OAD (17) and in LTx recipients diagnosed with chronic rejection (16). To determine the impact of Treg depletion in modulating immune responses to self-Ags in OAD, we performed ELISA for Abs to ColV and K α 1T. As shown in Figure 4A-B, development of Abs to ColV and K α 1T was significantly increased upon depletion of Treg at day 30. We next investigated the role of auto-reactive T-cellular responses associated with the development of OAD. As shown in Figure 4C-D, IL-10 specific T-cellular response to ColV and K α 1T significantly diminished by day 30. However, IL-17 responses (Figure 4G-H) to ColV and K α 1T significantly increased by day 30 in Treg depleted animals. Similarly, IFN- γ response (Figure 4E-F) to ColV and K α 1T significantly increased by day 30 upon Treg depletion. These results indicate that elimination of Treg resulted in increased ColV and K α 1T specific T-cellular and humoral responses.

Since there was marked increase in OAD lesions including cellular infiltration, fibrosis and luminal occlusion upon Treg depletion (Figure 3) we analyzed the gene expression of the pro- inflammatory chemokines and its receptors, and fibrotic growth factors promoting cellular infiltration, hyperplasia and fibrosis. As shown in figure 5A-B, lungs harvested on day 7 following intrabronchial administration of MHC Abs demonstrated a significant increase in the mRNA expression of chemokines, chemokine receptors, and growth factors in Treg depleted over non-Treg depleted groups (individually quantitated over Isotype group). This demonstrates that Treg depletion causes increased expression of pro-inflammatory chemokines and their receptors along with pro-fibrotic growth factor upregulation, thus causing a conducive micro-environment to favor the autoimmune T-cellular response against self-Ags and fibrosis.

Adoptive transfer of CD4⁺CD25⁺Treg decrease the OAD lesions

To confirm the role of Tregs in modulating OAD lesions, we performed adoptive transfer with an individual serial titration from 0.1, 1 and 10 million CD4⁺CD25⁺ T-cells, into DT treated Treg depleted FoxP3-DTR-C57BL/6 mice. CD4⁺CD25⁺ T-cells were positively selected using MACS beads (Miltenyi Biotec, NY) from splenocytes of non-DT treated FoxP3-DTR-C57BL/6 animals. As shown in Figure 6A-D, H&E and trichrome analysis of the lungs harvested from adoptively transferred animals on day 30 demonstrated decreased OAD lesions compared to wild-type animals receiving Abs to MHC. As expected, adoptive transfer of Tregs into non-Treg depleted animals also abrogated OAD lesions (Fig 6E-H). Flow cytometric analysis to determine the frequency of Treg in the lungs demonstrated active expression of FoxP3 in the CD4 gated T cells on lung infiltrating lymphocytes collected on day 30 harvested lung from animals which were adoptive transferred with Treg (Figure 6 I-K). Furthermore, there was a significant dose-dependent decrease (Figure 6L) in

histologic markers of OAD with decreased Abs to self-Ags ColV and K α 1T (Figure 6M). Similarly, there was a decrease in OAD lesions (Figure 6N) and auto-antibodies (Figure 6O) in non-Treg depleted group. These results confirm that Treg exerts a strong modulating effect towards decreasing both immune responses to self-Ags and the severity of OAD lesions.

Neutralization of IL-17 in Treg depleted animals also abrogates development of OAD lesions

To demonstrate the role of IL-17 in OAD, we performed IL-17 neutralization by administration of 50 μ g of mouse-IL-17mAb (with common epitope specificity for IL-17A and IL-17F) to DT treated Treg depleted FoxP3-DTR-C57BL/6 mice. As shown in Figure 7A-D, analysis of the lungs harvested on day 30 from IL-17 neutralized animals demonstrated complete abrogation of OAD lesions. Furthermore, there was a significant decrease (Figure 7E) in histologic markers of OAD and decrease in Abs to self-Ags (Figure 7F). These results establish that IL-17 exerts a potent pro-inflammatory effect towards causing immune responses to self-Ags leading to OAD.

ROR γ T and IL-17 play a crucial role in Th17 mediated immune responses to self-Ags leading to OAD in murine model

The orphan nuclear receptor ROR γ T has been implicated in the activation and differentiation of Th17 cells (22). To determine the role of ROR γ T in OAD, we performed flow cytometric analysis to determine the expression of ROR γ T in lung infiltrating lymphocytes. As shown in Figure 8A, there was significantly increased expression of ROR γ T (p -value <0.05) upon Treg depletion. However, adoptive transfer of Treg caused diminished expression of ROR γ T ($6.2 \pm 4.5\%$). This strongly suggests that Treg inhibit ROR γ T expression and thus decrease Th17 activation (22).

Analysis of the serum cytokine levels demonstrated that adoptive transfer of Treg and IL-17 neutralization both caused significant elevation of serum IL-10 concentration (Figure 8B). Adoptive transfer of Tregs also caused suppression of serum IL-17 and IFN γ concentrations (Figure 8C-D). Similarly, the neutrophil marker IL-8 and myeloperoxidase activity in the harvested lung were also decreased upon adoptive Treg transfer and IL-17 neutralization. Taken together, these results support the conclusion that Tregs exert an inhibitory effect on Th17 responses and development of OAD.

Discussion

Previous studies from our laboratory demonstrated an important role for alloimmunity in chronic lung allograft rejection in both human LTx and animal model of OAD (16, 17, 23). In particular our studies have shown that Abs to mismatched donor HLA develop much before the onset of chronic rejection following human LTx making it a viable marker for identifying patients at risk of development of BOS (16, 24). Based on this, we also instituted pre-emptive antibody directed therapy of patients who developed DSA with normal pulmonary function following LTx (23). The results demonstrated significant improvement in graft function and survival especially freedom from BOS in LTx recipients who cleared DSA in comparison to those who did not clear DSA (23).

Although the underlying molecular mechanisms by which Abs to MHC induce chronic rejection following human lung transplantation and OAD in animal models are still unclear, evidence strongly supports a cross talk between alloimmune and autoimmune responses which leads to chronic rejection (8, 16, 17). It has been shown that LTx recipients who develop Abs to mismatched donor HLA also develop Abs to lung associated self-Ags (16)

and patients awaiting LTx with pre-existing Abs to self-Ags have increased risk of developing primary graft dysfunction as well as development of Abs to mismatched donor HLA (25), both of which are well known risk factors for development of BOS following LTx (26). Further, longitudinal analysis of LTx recipients have demonstrated that development of autoimmune Th17 responses to lung associated self-Ags (ColV and K α 1T) correlate significantly with decreased suppressive Treg/IL-10 responses in BOS (7, 16, 27). These results strongly support an important role for Treg in the development of BOS following human LTx and the goal of this study was to define the mechanisms by which Abs to MHC can contribute towards the development of BOS and the role of Treg in this process.

While the role of Treg in systemic autoimmune diseases is well established, their role in solid organ transplantation is still being debated (28). It is well understood that Tregs expressing the transcription factor FoxP3 dictate suppressive response on diverse immune-dependent and independent inflammatory and autoimmune responses, thus acting as watch dogs in immune homeostasis (29-31). We have previously reported the increased Treg frequency in patients with stable lung allograft (27, 32) and human and animal studies have demonstrated that following viral infections Treg frequency decreases which then skews the immune responses to Th17 leading to lung allograft dysfunction (33). Further, IL-17 has been shown to activate IL-8 mediated neutrophil accumulation and thus innate immunity (34). The role for neutrophil accumulation in the transplanted lungs has also been strongly associated with development of BOS, although the precise role of Abs in this process is not established (35).

In this report, we employed a murine model of alloimmune mediated OAD to investigate the interplay between antibodies to MHC and the role of Treg in the development of autoimmune responses leading to OAD (17). We used FoxP3-DTR knockin transgenic C57BL/6 animals with human DTR expression in whom Treg can be ablated by administering DT (18). We demonstrated that the Treg depleted animals upon administration of specific Abs to MHC developed significantly increased OAD lesions and heightened Th1/IFN- γ and Th17/IL-17 to the self-Ags, ColV and K α 1T (Figure 4). This was accompanied by significant decrease in suppressive IL-10 response. This is in agreement with other studies on autoimmune diseases including experimental autoimmune encephalitis (36, 37) and collagen-arthritis (38, 39) wherein a dynamic balance between Tregs and Th17 have been demonstrated to play a crucial role in the development of immune responses to self-Ags.

The increased gene expression of chemokines and soluble mediators along with their receptors which we have noted following administration of Abs to MHC (Figure 5) also points out a critical role by which Abs to MHC facilitates the recruitment and activation of various effector cells to the site. It is significant that CCR6 is a signature cytokine for Th17 activation and recruitment (40) and this was significantly increased in our model of anti-MHC class I induced OAD. Activation of Th17 cells has been shown to correlate with the expression of ROR γ T (22). In our study, upon Treg depletion we demonstrated increased ROR γ T expression in the lung infiltrating lymphocytes and further upon adoptive transfer of Tregs there was a significant (5.5 fold) reduction in ROR γ T expression. (Figure 8). However there was no statistical decrease in ROR γ T expression ($33.4 \pm 7.5\%$ to $27.8 \pm 5.7\%$) following IL-17 neutralization in Treg depleted animals. Although further studies are needed in this direction, it could be possibly due to the lack of positive feedback following IL-17 neutralization, as noticed by Hwang et al in arthritis disease models (41). Further, CCL9 and CCR2 chemokines, known attractants of both dendritic cells and macrophages (42), were also up regulated. Similarly increased expression of CXCL12 (Figure 5A), a known chemokine for activation of leukocytes and lymphocytes through TNF- α signaling

(43) was also noted in our study following administration of anti-MHC in Treg depleted animals, thus favoring the development of autoimmune responses in Treg depleted animals following MHC class I Abs administration (44).

In vitro studies demonstrated that HLA class I Abs can induce airway epithelial cell proliferation, the release of fibrogenic growth factors, and epithelial cell apoptosis (45). Our data demonstrating increased expression of pro-fibrotic growth factors, BMP8a, FGF6 and IGF2 in Treg depleted animals following administration of anti-MHC also supports our previous studies that these Abs can lead to lipid-raft and HIF-1 α mediated activation of fibrosis resulting in OAD (46, 47). To confirm an essential role for Treg in these processes, we performed adoptive transfer of Treg as well as neutralization of IL-17 which clearly demonstrated that modulation of Th17 cellular immune response (Figure 6 and 7) leads to diminished humoral immune responses against self-Ags, ColV and α 1T, along with significantly reduced OAD lesions. These results strongly establish that Treg play an important role in attenuating the autoimmune responses to self-Ags and OAD lesions induced by Abs to MHC class I.

The decrease in Th17 responses noted either when Treg were passively transferred or by IL-17 neutralization also results in decreased B-cell activation as evidenced by diminished serum concentration of Abs to self-Ags (Figure 6M and 7F) which we consider to be necessary for the development of OAD in this model as well as chronic rejection following human lung transplantation. Our results demonstrate that alloimmunity especially Abs to MHC binding to epithelial and endothelial cells induces neutrophil accumulation (Figure 2) and activation of Th17 pathway and adoptive transfer of Treg or IL-17 neutralization diminishes neutrophil accumulation along with diminished IL-8 serum cytokine levels (Figure 8). Therefore, ligation of MHC class I on airway epithelial or endothelial cells by its specific Abs causes accentuation of a series of inflammatory cascades of innate, cellular and humoral immune responses, all of which are involved in the development of OAD.

In conclusion, we demonstrate a pivotal role for T regulatory cells (CD4+CD25+Foxp3+) in the control of Th17 mediated immune response to self-Ags and development of OAD induced by Abs to MHC class I molecules. It has been demonstrated in all solid organs (heart, kidney, pancreas and lung) that *de novo* development of Abs to MHC have been correlated with development of not only acute rejection but also chronic rejection (15). Early inflammatory cascade induced by the Abs to MHC play a crucial role in the development of Th17 cellular and humoral immune responses against self-antigens leading to chronic rejection, which could potentially be diminished by Treg. Therefore, novel therapeutic targets aimed at reduction in the Th17 activation or IL-17 neutralization as well as development of immunosuppressive regimens which will not affect Treg (48) will be of value in reducing the incidence of chronic rejection following human LTx.

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Abbreviations

Abs	antibodies
BOS	bronchiolitis obliterans syndrome

ColV	collagen V
DT	diphtheria toxin
Kα1T	K- α 1 Tubulin
LTx	lung transplantation
MPO	myeloperoxidase
OAD	obliterative airway disease
Treg	regulatory T cells
self-Ags	self-antigens

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Figure 1.

Depletion of FoxP3⁺ Tregs following administration of DT. FACS analysis done on splenocytes (A) and lungs (B) collected from FoxP3-DTR C57BL/6 mice 24 hrs and 30 days following the administration of DT.

Figure 2.

Kinetic analysis of the cellular infiltration following Treg depletion in the alloimmune murine OAD model. The infiltration with neutrophils and monocytes by CD11b (A), myeloperoxidase (MPO) enzyme expression as a specific marker of neutrophils (B), CD4+T cells (C), FoxP3+ cells of CD4 sorted T cells as a marker of Treg (D), CD8+T cells (E), and B cells quantitated by CD19 (F). Temporal analysis was performed on day 7, 15, and 30 on isotype Ab treated, anti-MHC class I treated wild type and Treg depleted animals. The data is represented as mean fluorescence shift (Δ MFI) in arbitrary units. The data is represented as a mean $\pm$ SEM over a 5 different measurements. (squares) represent isotype administered Treg depleted group, (circles) represent non-Treg depleted MHC class I Ab treated group, and (triangles) represent Treg-depleted MHC class I Ab treated group.

Figure 3.

Histology from lungs harvested on day 15 and 30 following intrabronchial administration of H2K^b Abs to FoxP3-DTR C57BL/6 mice on day 1,2,3,6 and weekly. (A-D) represent isotype treated Treg-depleted FoxP3-DTR C57BL/6 with two doses of DT on day -7 and -2, (E-H) represent Treg depleted FoxP3-DTR C57BL/6 with two doses of DT on day -7 and -2; (I-L) represent non-Treg depleted FoxP3-DTR C57BL/6 without DT treatment; (A,E,I) represent H&E staining on lungs harvested on day 0; (B,F,J) represent H&E staining on lungs harvested on day 15; (C,G,K) represent H&E staining on lungs harvested on day 30; (D,H,L) represent trichrome staining on lungs harvested on day 30 (I-K); (M-O) represents morphometric analysis performed using NIS-Elements BR software of the cellular infiltration (M), luminal occlusion (N) and fibrosis (O); The histological analysis by H&E staining of the lungs harvested on day 30 following administration of H2K^b Ab demonstrated a significant increase in cellular infiltration around bronchioles (Isotype= $2.1 \pm 1.8\%$; non-Treg depleted/ H2K^b Ab = $34.1 \pm 18.6\%$; and Treg depleted/ H2K^b Ab = $76.4 \pm 22.6\%$) resulting in luminal occlusion (Isotype= 0% ; non-Treg depleted/ H2K^b Ab = $11.8 \pm 3.3\%$; and Treg depleted/ H2K^b Ab = $43.4 \pm 18.9\%$). Trichrome staining to quantitate the fibrosis also demonstrated significant increase in fibrosis (Isotype= $3.9 \pm 2.9\%$; non-Treg depleted/ H2K^b Ab = $32.8 \pm 8.9\%$; and Treg depleted/ H2K^b Ab = $56.7 \pm 13.8\%$) in Treg depleted animals. The data is represented as a mean $\pm$ SEM over a 5 different measurements, (**) *p*-value <0.01.

Figure 4.

Analysis of the humoral and cellular responses on day 15 and 30 following administration of MHC class I Abs to Treg depleted and non-Treg depleted animals. (A,B) represent the serum concentration of Abs to self-Ags ColV (A) and Kα1T (B) analyzed by ELISA. The Abs to ColV (Isotype= $34 \pm 15 \mu\text{g/mL}$; non-Treg depleted/ H2K^b Ab = $161 \pm 32 \mu\text{g/mL}$; and Treg depleted/ H2K^b Ab = $221 \pm 41 \mu\text{g/mL}$; p-value<0.05 for each group) and Kα1T (Isotype= $23 \pm 11 \mu\text{g/mL}$; non-Treg depleted/ H2K^b Ab = $219 \pm 39 \mu\text{g/mL}$; and Treg depleted/ H2K^b Ab = $394 \pm 44 \mu\text{g/mL}$; p-value<0.05 for each group). (C-H) ELISpot analysis to quantitate the cellular responses determined from splenocytes; (C,D) represent the IL-10 cellular responses to self-Ags ColV (C) and Kα1T (D), IL-10 specific T-cellular response to ColV (Isotype= $81 \pm 28 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $41 \pm 19 \text{ spm}$; and Treg depleted/ H2K^b Ab = $19 \pm 7 \text{ spm}$; p-value<0.05) and Kα1T (Isotype= $78 \pm 25 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $36 \pm 12 \text{ spm}$; and Treg depleted/ H2K^b Ab = $15 \pm 5 \text{ spm}$; p-value<0.05 for each group); (E,F) represent the IFN-γ cellular responses to self-Ags ColV (E) and Kα1T (F), IFN-γ response to ColV (Isotype= $38 \pm 21 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $104 \pm 23 \text{ spm}$; and Treg depleted/ H2K^b Ab = $171 \pm 39 \text{ spm}$; p-value<0.05 for each group) and Kα1T (Isotype= $35 \pm 20 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $98 \pm 21 \text{ spm}$; and Treg depleted/ H2K^b Ab = $154 \pm 28 \text{ spm}$; p-value<0.05 for each group with respect to Isotype); (G,H) represent the IL-17 cellular responses to self-Ags ColV (G) and Kα1T (H), IL-17 responses (figure G-H) to ColV (Isotype= $16 \pm 7 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $113 \pm 26 \text{ spm}$; and Treg depleted/ H2K^b Ab = $192 \pm 34 \text{ spm}$; p-value<0.05 for each group with respect to Isotype) and Kα1T (Isotype= $17 \pm 8 \text{ spm}$; non-Treg depleted/ H2K^b Ab = $134 \pm 37 \text{ spm}$; and Treg depleted/ H2K^b Ab = $253 \pm 42 \text{ spm}$; p-value<0.05 for each group with respect to Isotype). The bars in grey shade represent day 15 and bars with crossed lines shade represent day 30. The data is represented as a mean $\pm$ SEM over 5 different measurements.

Figure 5.

Gene expression analysis of chemokines, their receptors and growth factors on the mRNA collected from the lungs harvested on day 7. Bars in grey shade represent non-Treg depleted animals and bars in dark shade represent Treg depleted animals. Increased expression of CCL9 (2.2 fold, $p < 0.05$), CCR2 (2.6 fold, $p < 0.05$), CXCL12 (3.1 fold, $p < 0.05$) and CCR6 (4 fold, $p < 0.05$), in Treg depleted over non-Treg depleted groups (individually quantitated over Isotype group) was observed. Expression of the growth factors (Figure 4B), BMP8a (3.8 fold, $p < 0.05$), FGF6 (4.5 fold, $p < 0.05$) and IGF2 (1.4 fold, $p < 0.05$) were also significantly increased in Treg depleted vs. non-Treg depleted group (individually quantitated over Isotype group). Other CXC and CC chemokines (CXCL13, CCL2,7,8, MIP-2 etc) showed marginal or no increase at all and did not achieve statistical significance. The data is represented as a mean $\pm$ SEM over 5 different measurements. Bars in grey shade represent non-Treg depleted group and those in dark shade represent Treg depleted group.

Figure 6.

Histology from lungs harvested on day 30 following adoptive transfer of 0.1 , 1 and 10×10^6 Tregs peritoneally, and intrabronchial administration of $H2K^b$ Abs to FoxP3-DTR C57BL/6 mice on day 1,2,3,6 and weekly. (A,C) H&E and trichrome staining of the lungs collected from Treg depleted FoxP3-DTR C57BL/6 mice without adoptive transfer of Tregs; (B,D) H&E and trichrome staining of the lungs collected from Treg depleted FoxP3-DTR C57BL/6 mice with adoptive transfer of 10×10^6 Tregs; (E,G) H&E and trichrome staining of the lungs collected from non-Treg depleted FoxP3-DTR C57BL/6 mice without adoptive transfer of Tregs; (F,H) H&E and trichrome staining of the lungs collected from non-Treg depleted FoxP3-DTR C57BL/6 mice with adoptive transfer of 10×10^6 Tregs; (I) frequency of FoxP3+ cells in CD4+ gated lung infiltrating cells in Treg depleted FoxP3-DTR C57BL/6 mice harvested from lungs on day 30; (J) frequency of FoxP3+ cells in CD4+ gated lung infiltrating cells in non-Treg depleted FoxP3-DTR C57BL/6 mice harvested from lungs on day 30; (K) frequency of FoxP3+ cells in CD4+ gated lung infiltrating cells in Treg depleted FoxP3-DTR C57BL/6 mice adoptively transferred with 10×10^6 Treg and harvested from lungs on day 30; (L) morphometric analysis to quantitate the cellular infiltration, luminal occlusion and fibrosis following adoptive transfer to Treg depleted group at various serial titration of 0.1 , 1 and 10×10^6 Tregs; (M) serum concentration of Abs to self-Ags ColV and K α 1T in adoptive transfer to Treg depleted group; (N) morphometric analysis to quantitate the cellular infiltration, luminal occlusion and fibrosis following adoptive transfer (1×10^6 Treg) to non-Treg depleted group; (O) serum concentration of Abs to self-Ags ColV and K α 1T in adoptive transfer (1×10^6 Treg) to non-Treg depleted group; Cellular infiltration around bronchioles (Treg depleted/ $H2K^b$ Ab = $76.4 \pm 22.6\%$ vs. adoptively transferred Treg depleted/ $H2K^b$ Ab = $29.3 \pm 16\%$; p-value<0.05), luminal occlusion (Treg depleted/ $H2K^b$ Ab = $43.4 \pm 18.9\%$ vs. adoptively transferred Treg depleted/ $H2K^b$ Ab = $18.4 \pm 6.7\%$; p-value<0.05) and fibrosis (Treg depleted/ $H2K^b$ Ab = $56.8 \pm 13.8\%$ vs. adoptively transferred Treg depleted/ $H2K^b$ Ab = $22.4 \pm 7.6\%$; p-value<0.05) was decreased. Similarly, Abs to self-Ags, ColV (Treg depleted/ $H2K^b$ Ab = $221 \pm 42 \mu\text{g/mL}$ vs. adoptively transferred Treg depleted/ $H2K^b$ Ab = $121 \pm 19 \mu\text{g/mL}$; p-value<0.05) and K α 1T (Treg depleted/ $H2K^b$ Ab

= $394 \pm 65 \mu\text{g/mL}$ vs. adoptively transferred Treg depleted/ H2K^b Ab = $132 \pm 45 \mu\text{g/mL}$; p-value<0.05) were also decreased. The data is represented as a mean $\pm$ SEM over 5 different measurements.

Figure 7.

Histology from lungs harvested on day 30 following IL-17 neutralization peritoneally, and intrabronchial administration of H2K^b Abs to FoxP3-DTR C57BL/6 mice on day 1,2,3,6 and weekly. (A,C) H&E and trichrome staining of the lungs collected from Treg depleted FoxP3-DTR C57BL/6 mice without IL-17 neutralization ; (B,D) H&E and trichrome staining of the lungs collected from Treg depleted FoxP3-DTR C57BL/6 mice with IL-17 neutralization; (E) morphometric analysis to quantitate the cellular infiltration, luminal occlusion and fibrosis following IL-17 neutralization; (F) serum concentration of Abs to self-Ags, ColV and K α 1T. The cellular infiltration around bronchioles (Treg depleted/ H2K^b Ab = 76.4 $\pm$ 22.6% vs. IL-17 neutralized Treg depleted/ H2K^b Ab = 11.3 $\pm$ 3.4% ; p-value<0.05), luminal occlusion (Treg depleted/ H2K^b Ab = 43.4 $\pm$ 18.9% vs. IL-17 neutralized Treg depleted/ H2K^b Ab = 5.6 $\pm$ 2.1% ; p-value<0.05) and fibrosis (Treg depleted/ H2K^b Ab = 56.8 $\pm$ 13.8% vs. IL-17 neutralized Treg depleted/ H2K^b Ab = 7.8 $\pm$ 3.4% ; p-value<0.05). The Abs to self-Ags, ColV (Treg depleted/ H2K^b Ab = 221 $\pm$ 42 μ g/mL vs. IL-17 neutralized Treg depleted/ H2K^b Ab = 84 $\pm$ 32 μ g/mL; p-value<0.05) and K α 1T (Treg depleted/ H2K^b Ab = 394 $\pm$ 65 μ g/mL vs. IL-17 neutralized Treg depleted/ H2K^b Ab = 64 $\pm$ 31 μ g/mL; p-value<0.05). The data is represented as a mean $\pm$ SEM over 5 different measurements.

Figure 8.

(A) RORγ T expression on CD4⁺ lung infiltrating lymphocytes in lungs harvested on day 15 from isotype treated, MHC class I Abs treated non-Treg depleted, MHC class I Abs +Treg depleted, MHC class I Abs +Treg depleted +Adoptive transfer of Treg, and MHC class I Abs +Treg depleted +IL-17 neutralization groups. (B-E) serum concentration of cytokines IL-10 (B), IFN-γ (C), IL-17 (D), and IL-8 (E) from all five cohorts on day 30. (F) Myeloperoxidase (MPO) activity from the lungs harvested on day 30. The data is represented as a mean ± SEM over 5 different measurements.