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Inhibition of renin angiotensin aldosterone system causes abrogation of obliterative airways disease through inhibition of tumor necrosis factor- α –dependant interleukin-17

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Abstract

BACKGROUND—Alloimmune-induced immune responses to self-antigens are involved in the development of chronic lung allograft rejection. Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) have been shown to modulate autoimmune diseases. This study investigated the effect of modulation of the renin angiotensin aldosterone system (RAAS) a murine model of obliterative airways disease (OAD).

METHODS—Major histocompatibility complex (MHC) class I antibodies were administered intrabronchially to C57Bl/6 mice on Days 1, 2, 3, and 6, and weekly thereafter. ACEI/ARB (10 mg/kg/day) were administered in water 5 days before antibody administration. Antibodies were analyzed by enzyme-linked immunosorbent assay, cytokines by Luminex, Th-frequency by enzyme-linked immunosorbent spot, and transcription factors by Western blotting and real-time polymerase chain reaction.

RESULTS—Significant decreases (50%–70%) in airway lesions and fibrous deposition were noted in lungs at Day 30 in the animals administered ACEI and ARB vs controls. Antibody concentrations to self-antigens also decreased from 14 ± 21 to 62 ± 18 $\mu\text{g/ml}$ for collagen V and from 263 ± 43 to 84 ± 28 $\mu\text{g/ml}$ for K- $\alpha 1$ tubulin. Th-precursor frequency and cytokine analysis showed increased interleukin (IL)-10 (3-fold increase) and decreased levels of IL-6 (3.4-fold) and IL-17 (4-fold decrease; $p < 0.05$) in ACEI and ARB groups. There was also messenger RNA level downregulation of tumor necrosis factor- α (8.6-fold) and p38/mitogen-activated protein (MAP)kinase (3.1-fold) in the treatment groups.

CONCLUSIONS—Our results demonstrate that modulation of RAAS leads to downregulation of IL-17 through tumor necrosis factor- α – dependant IL-6 through p38/MAPKinase pathway and thus abrogation of anti-MHC–induced OAD.

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Disclosure statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

Keywords

renin angiotensin aldosterone system; ACE inhibitors; obliterative airway disease; TNF-alpha; TH-17 pathway

Despite advances in understanding of donor and recipient physiology, operative technique, and immunosuppressive pharmacology, chronic rejection after human lung transplantation (LTx), characterized by bronchiolitis obliterans syndrome (BOS), remains the most important problem for long-term function of the transplanted lung allograft.^{1,2} Chronic rejection is initiated by a host-anti-graft immune response with both antigen-dependent and non-immune (antigen-independent) factors leading to fibroproliferative changes affecting graft function.³ The primary targets of the recipient immune response against the allograft are the donor major histocompatibility (MHC) antigens.⁴⁻⁶ We and others have previously demonstrated that immune responses to donor mismatched human leukocyte antigens (HLA) results in the development of anti-MHC class I antibodies, which precedes the development of BOS.^{5,7}

Studies from our group as well as others have shown that development of immune responses to self-antigens, mainly K- α 1 tubulin (K- α 1T) and collagen V, during the post-LTx period correlated with the development of chronic rejection.^{8,9} Studies have also demonstrated increased frequency of collagen V-reactive T cells in BOS and was associated with the expansion of interferon- γ (INF- γ)- and interleukin (IL)-17-producing collagen V-specific cells with a concurrent reduction in IL-10-secreting T cells.^{10,11}

To determine the mechanism by which antibodies to donor MHC may induce an immune response to self-antigens, which led to chronic rejection, we developed a murine model of obliterative airway disease (OAD).¹² In this model, administration of specific anti-MHC class I antibodies to the native lungs of mice resulted in autoimmunity leading to cellular infiltration, epithelial hyperplasia, endothelitis, fibroproliferation, and luminal occlusion of the small airways, the central events seen during chronic lung allograft rejection.¹²

Drugs that modulate the rennin angiotensin aldosterone system (RAAS), specifically angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) have been shown to be beneficial in downregulating inflammatory processes beyond their well-known effect in controlling hypertension.¹³ Studies on experimental autoimmune encephalomyelitis and lupus, have indicated that ACEIs induce a shift in the production of proinflammatory Th1 and Th17 cytokines to the production of Treg/Th2 cytokines.¹⁴⁻¹⁶ Results presented in this report demonstrate that modulation of RAAS can also abrogate the development of OAD by specific downregulation of IL-17 responses. Our data also demonstrate that RAAS modulates its effect through tumor necrosis factor- α (TNF- α)-mediated inhibition of IL-17, leading to inhibition of immune responses to self-antigens and eventual abrogation of OAD after anti-MHC class I administration.

Materials and methods

All experiments in this study were performed in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee of Washington University School of Medicine (protocol 20070121).

Animal studies

We used a novel murine model in which OAD, a correlate of BOS, takes place in the distal airways after ligation of MHC molecules in the lung by a monoclonal antibody (mAb) to

MHC class I antigens.¹² Murine mAb to H2K^b (C57Bl/6, 6 weeks old, male, IgG2a), which has no detectable endotoxin as measured by *Limulus* amoebocyte lysate assay, was given at a dose of 200 μ g/administration into mice. In brief, each mouse was anesthetized, and the tongue was pulled out. A mosquito clamp was inserted into the mouth, and a 20-gauge catheter was placed into the trachea. Antibody (200 μ g) was administered into the lung on Days 1, 2, 3, and 6, and then weekly thereafter. C1.18.4, same isotype, was given on the days mentioned above as controls. In addition, the drug treatment groups were given daily the ACEI lisinopril (10 mg/kg/day) or the ARB candesartan (10 mg/kg/day)¹⁶ in drinking water, starting 5 days before the administration of antibodies.

Histology to determine cellular infiltration, fibrosis and luminal occlusion

Lungs were fixed in 10% formaldehyde. Sections were cut at 5- μ m thickness and stained with hematoxylin and eosin and Masson's trichrome and analyzed under an ECLIPSE 55i microscope (Nikon, Melville, NY) using NIS-Elements BR software (Melville, NY). Specific CD4 and CD8 infiltration was analyzed on frozen sections treated with 3% H₂O₂ and ethanol for 10 minutes to block endogenous peroxidase activity. The sections were blocked with biotin/Avidin blocking reagent kit (Vector Laboratories, Burlingame, CA). Primary antibodies were diluted using antibody dilution solution (BD Biosciences, San Jose, CA). The sections were incubated with purified rat anti-mouse CD4 and CD8 (5 μ g/ml, Santa Cruz Biotech, Santa Cruz, CA). The sections were treated with the appropriate biotin-conjugated secondary antibody and followed with streptavidin-horseradish peroxidase. The presence of positive cells was determined with a diaminobenzidine substrate kit (BD Pharmingen Inc, San Diego, CA).

Lesions that displayed cellular infiltration, epithelial abnormalities, and fibroproliferation were analyzed by random sampling after being individually analyzed by 2 blinded professionals. The percentages of cellular infiltration, luminal occlusion, and fibrosis were morphometrically calculated using NIS-Elements BR software. The percentage of fibrosis was quantified by morphometric addition of the total area enclosed by basement membrane in 5 high-power fields (original magnification $\times 40$) and dividing by a factor of 5. The percentages of cellular infiltration and epithelial abnormalities were calculated in 5 high-power fields (original magnification $\times 40$), respectively. The data are represented as a mean $\pm$ standard error of the mean over a 5 different measurements.

Enzyme linked immunosorbent spot assay

Enzyme-linked immunosorbent spot (ELISpot) assays were performed, as described previously.¹⁷ Briefly, MultiScreen 96-well filtration plates (Millipore Corp, Billerica, MA) were coated overnight (O/N) at 4°C with 5.0 μ g/ml capture mouse cytokine-specific mAb (BD Biosciences) in 0.05 mol/liter carbonate-bicarbonate buffer (pH 9.6). The plates were blocked with 5% bovine serum albumin (BSA) for 2 hours and washed 3 times with phosphate-buffered saline (PBS). Subsequently, 3×10^5 cells were cultured in triplicate in the presence of collagen V (20 μ g/ml) or human K- α 1T (10 μ g/ml) and irradiated feeder autologous splenocytes (1:1 ratio).

After 48 to 72 hours, the plates were washed 3 times with PBS and 3 times with 0.05% PBS/Tween 20. Then, 2.0 μ g/ml biotinylated mouse cytokine-specific mAb (BD Biosciences) in PBS/BSA/Tween 20 was added to the wells. After an O/N incubation at 4°C, the plates were washed 3 times, and horseradish peroxidase (HRP)-labeled streptavidin (BD Biosciences), diluted 1:100 in PBS/BSA/Tween 20, was added to the wells. After 2 hours, the plates were developed and spots were analyzed by ImmunoSpot Series I analyzer (Cellular Technology, Shaker Heights, OH). The results were expressed as spots per million cells $\times$ standard error.

Any spots obtained by culturing T cell lines with antigen-presenting cells alone, without collagen V or human K- α 1T, were subtracted from the number of spots in the test cultures.

Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) plate (Nunc, Rochester, NY) was coated with collagen V or K- α 1T (1 μ g/ml) in PBS overnight at 4°C.¹⁸ Serum samples were collected from mice treated with anti-H-2K^d antibody or isotype antibody and non-treated mice. Serum samples were tested (1:250 and 1:500) for binding to collagen V. Detection was done with anti-mouse immunoglobulin Ig (IgG), IgM-HRP, and development using tetramethylbenzidine substrate and read at 450 nm. A sample was considered positive if the values exceeded the average cutoff values obtained from normal sera from 10 different mice. Concentration of antibodies was calculated based on a standard curve using the binding of known concentration of anti-collagen V antibodies (Santa Cruz Biotechnology).

Gene expression assay

Real-time polymerase chain reaction (RT-PCR) was performed to determine chemokines mRNA transcription in the splenocytes. Total RNA was extracted from 10⁶ cells using TRIzol reagent (Sigma-Aldrich, St. Louis, MO). RNA samples were quantified by absorbance at 260 nm. The RNA was reverse-transcribed and RT-PCR was performed in a final reaction volume of 50 μ L using iCycler 480 Probes Master (Roche Diagnostics, Indianapolis, IN). Each sample was analyzed in triplicate. Cycling conditions consisted of an initial denaturation of 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, followed by 61°C for 1 minute.

Western blot

The protein levels of various transcription factors were analyzed using Western blot. The retrieved cells from the spleen on day 15 were lysed using 4% sodium dodecylsulfate cell lysis buffer supplemented with protease inhibitor cocktail and ethylenediaminetetraacetic acid. The lysates were boiled for 20 minutes in sample buffer, centrifuged, and run on 4% to 12% gradient Bis-Tris denaturing gel (Nupage, Invitrogen). The gel was transferred onto nitrocellulose membrane and blocked overnight with 5% non-fat milk in PBS-T (0.1% Tween 20). Thereafter, the membrane was incubated for 1 hour at room temperature with the appropriate antibodies and probed by HRP-labelled secondary antibody (1: 10,000 dilution, R&D Systems). The membrane was developed using the chemiluminescence kit (Millipore) and analyzed on using Bio-Rad Universal Hood II (Hercules, CA). Morphometric analysis was done using the software provided by the company.

Results

ACEI and ARB abrogate the development of OAD lesions after administration of antibodies to MHC class I

To determine the role of alloimmunity in development of OAD, we previously developed a murine model for OAD. At 30 days after intrabronchial administration of anti-MHC class I antibody (anti-H-2K^b), classic OAD lesions developed in the lungs of C57Bl/6 mice, as evidenced by the cardinal features, namely, cellular infiltration around the vessels, bronchioles, epithelial hyperplasia, and fibrosis. However, similar administration of C1.18.4 control antibody of the same isotype in the control animals did not produce lesions.¹²

To test for the immunomodulatory role of ACEI and ARBs, we administered these drugs orally in drinking water daily beginning 5 days before anti-MHC administration. As shown in Figure 1, histologic analysis of the lungs harvested on 30 days after administration of H-2K^b antibody along with administration of ACEI or ARB caused a significant reduction

in the ACEI, ARB, and H-2K^b groups ($p < 0.01$ for all variables), respectively, in cellular infiltration around vessels ($23\% \pm 7\%$, $22\% \pm 10\%$, and $54\% \pm 14\%$), cellular infiltration around bronchioles ($4\% \pm 2\%$, $5\% \pm 2\%$, and $40\% \pm 7\%$), luminal occlusion ($2\% \pm 1\%$, $2\% \pm 1\%$, $14\% \pm 4\%$), and fibrosis ($5\% \pm 2\%$, $4\% \pm 2\%$, and $32\% \pm 7\%$). Also reduced was the specific CD4 ($5.7\% \pm 1.6\%$, $6.4\% \pm 2.4\%$, and $17.6\% \pm 6.9\%$) and CD8 ($11.7\% \pm 2.3\%$, $10.3\% \pm 2.5\%$, and $24.1\% \pm 3.6\%$; $p < 0.01$ for each group for both variables) cellular infiltration. This demonstrates that the administration of ACEI and ARB markedly reduces OAD lesions in the murine model of anti-MHC-induced OAD.

ACEI and ARB inhibit the development of humoral and cellular responses to self-antigens

Alloimmune-mediated development of immune responses to self-antigens mainly collagen V and K- α 1T have been well documented in human LTx with BOS and in this murine OAD model.^{12,19} To analyze for the changes in immune responses to self-antigens upon coadministration of ACEI or ARBs with MHC antibodies in the murine model of OAD, we used ELISA to determine the serum concentrations of antibodies to collagen V and K- α 1T. The development of antibodies to K- α 1T by Day 30 (ACEI, $54\% \pm 20\%$; ARB, $52\% \pm 17\%$; and H2K^b antibody, $234\% \pm 56\%$; $p < 0.01$ for each group compared with H2K^d group) was significantly inhibited upon administration of ACEI and ARBs (Figure 2A). Similarly, development of antibodies to collagen V (Figure 2B) were also significantly inhibited with ACEI ($35\% \pm 12\%$) and ARBs ($18\% \pm 11\%$) vs H2K^b antibody ($154\% \pm 44\%$; $p < 0.01$ for each group vs H2K^d group).

To determine the cellular immune responses to collagen V and K- α 1T we performed ELISpot on the lung infiltrating lymphocytes. As shown in Figure 2C, cellular responses to K- α 1T by Day 30 diminished for IL-17 (ACEI, 18 ± 5 ; ARB, 17 ± 9 ; and H2K^d antibody, 92 ± 23 ; $p < 0.01$ for each group compared with H2K^d group) and INF- γ (ACEI, 64 ± 19 ; ARB, 69 ± 22 ; and H2K^b antibody, 178 ± 41 ; $p < 0.01$ for each group vs H2K^d group in spots per million) were inhibited upon administration of ACEI or ARBs. Similarly, development of cellular responses to collagen V (Figure 2D) specific to IL-17 (ACEI, $20\% \pm 9\%$; ARB, $22\% \pm 9\%$; and H2K^b antibody, $134\% \pm 44\%$; $p < 0.01$ for each group vs H2K^d group in spots per million) and INF- γ (ACEI, 31 ± 11 ; ARB, 32 ± 12 ; and H2K^b antibody, 102 ± 31 ; $p < 0.01$ for each group vs H2K^b group in spots per million) were also inhibited with ACEI or ARBs.

Decreased p38 mitogen-activated protein kinase, IL-6, IL-17, and transforming growth factor- β gene expression

To determine the nuclear factors mediating the downstream effects of ACEI and ARBs we analyzed the mitogen-activated protein (MAP) kinases (MAPKinases) pathway after administration of lisinopril and candesartan. As shown in Figure 3A, coadministration of ACEI or ARB with MHC antibodies specifically inhibited the gene expression of p38/MAPKinase in splenocytes by Day 15, but not other nuclear factors, including extracellular signal-regulated kinase 1/2 nuclear factor- κ B, and PI3k. Furthermore, coadministration of ACEI or ARB with MHC antibodies caused decreased splenocyte gene expression for ACEI, ARB and H2K^b, respectively, of TNF- α (0.2 ± 0.15 ; 0.25 ± 0.15 ; and 7.2 ± 2.1), IL-6 (0.2 ± 0.1 ; 0.3 ± 0.1 ; and 12.1 ± 4.3), IL-17 (0.2 ± 0.1 ; 0.2 ± 0.1 ; and 9.1 ± 3.1), and transforming growth factor- β (TGF- β ; 2.2 ± 1.1 ; 2.9 ± 1.4 ; and 5.6 ± 1.8), but increased expression of IL-10 (10.2 ± 2.4 ; 12.1 ± 4.1 ; and 0.5 ± 0.2 ; $p < 0.01$ for each group for each variable compared with H2K^b group). This specifically demonstrates that ACEI as well as ARBs act by inhibiting p38 MAPkinases, leading to downregulation of TNF- α , IL-6, IL-17, and TGF- β production.

TNF- α addition to splenocytes upregulates p38, IL-6, IL-17, and TGF- β with no change in IL-10

To further demonstrate the role of TNF- α in ACEI or ARBs, we cultured splenocytes from various groups in the presence or absence of TNF- α . As shown in Figure 4A, culturing splenocytes of ACEI or ARB groups in TNF- α caused upregulation of the previously inhibited (Figure 3A) gene expression of p38/MAPKinase. Furthermore, this also resulted in increases in gene expression, in ACEI, ARB, and H2K^b antibody groups, respectively, of IL-6 (10.2 ± 3.1 ; 9.2 ± 3.5 ; and 12.1 ± 4.3), IL-17 (11.2 ± 3.1 ; 7.4 ± 2.9 ; and 9.1 ± 3.1), and TGF- β (5.1 ± 1.4 ; 4.8 ± 1.7 ; and 5.6 ± 1.8 ; $p \pm 0.01$ for each group compared with H2K^d group for all variables), with no changes in the expression pattern of IL-10 (Figure 4B). This demonstrates that ACEI and ARBs act by specifically inhibiting p38/MAPKinases, leading to down regulation of TNF- α .

IL-6 addition to splenocytes also increases IL-17 induction

To further demonstrate the role of IL-6 in ACEI and ARBs we cultured splenocytes in IL-6 from various groups to see if this caused changes in the gene expression pattern. Culturing splenocytes of ACEI or ARB groups in IL-6 did not show any change in the gene expression of p38 (Figure 5A) but resulted in increases in gene expression of IL-17 (ACEI, 12.4 ± 2.1 ; ARB, 13.6 ± 3.9 ; and H2K^b antibody, 9.1 ± 3.1 ; $p < 0.01$ for each group compared with the H2K^b group), with no change in the expression pattern of IL-6, IL-10, and TGF- β (Figure 5B). This demonstrates that ACEI and ARB act by inhibiting p38 and TNF- α , which is in the upstream of IL-6 and IL-17.

Discussion

Although the pathogenesis of BOS is not fully defined, several risk factors have been associated with the development of chronic rejection, including acute rejection, cytomegalovirus, gastroesophageal reflux disease, and HLA mismatches.²⁰ Previous studies from our laboratory and others have proposed a role for antibodies to self-antigens collagen V and K- α 1T in the pathogenesis of BOS after LTx.^{9,19,21} Alloimmune-mediated development of de novo autoimmunity to self-antigens appears to have a central role in the development of chronic rejection after solid organ transplantation.²² Tissue inflammation and remodeling provides a congenial microenvironment for the exposure of self-antigens and their antigenic determinants and thus leading to the activation of both cell-mediated and humoral effector arms.²³

To determine the mechanism by which antibodies to donor MHC induces immune responses to self-antigens leading to chronic rejection, we developed a murine model of OAD after administration of anti-MHC class I antibodies.¹² In this model, administration of specific anti-MHC class I antibodies resulted in development of immune responses to self-antigens and classic OAD lesions characterized by cellular infiltration, epithelial hyperplasia, endothelitis, fibroproliferation, collagen deposition, and luminal occlusion of the small airways—the central events seen during chronic lung allograft rejection. We also demonstrated that IL-17, a potent proinflammatory cytokine, is crucial in the induction of humoral immune responses to self-antigens.¹²

ACEIs have been shown to modulate immune responses in various animal models of autoimmune diseases.²⁴ Therefore, we postulated that modulation of RAAS will have an effect on abrogating anti-MHC class I-induced OAD. In this murine model of OAD, we demonstrate that administration of ACEI causes attenuation of humoral immune responses to self-antigens as evidenced by diminished serum concentrations of antibodies to K- α 1T and collagen V. This diminished humoral response is also associated with significantly

reduced cellular responses to self-antigens as evidenced by ELISpot analysis of decreased IFN- γ and IL-17. To further analyze the effect of blockade of AT1 directly by the angiotensin receptor and its ability to inhibit TNF- α , we administered ARB to specifically block the receptor and to determine its downstream effect. Unlike ACEI, ARB can selectively block the angiotensin II type 1 (AT1) receptor without increasing bradykinin levels.²⁵ Direct inhibition of AT1 angiotensin receptor by ARB also caused inhibition of gene expression of TNF- α and p38 leading to a downstream downregulation of IL-6 and IL-17. These results are in complete agreement with studies reported by Tsutamoto et al,²⁶ who demonstrated that specific AT1 receptor blocker caused decreased plasma levels of the immune markers TNF- α , IL-6, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 and leading to the improved biologic compensatory action of endogenous cardiac natriuretic peptides in patients with mild to moderate congestive heart failure. These reports, along with our current studies, clearly demonstrate the immunomodulatory effect of ACEI and ARB is through TNF- α .

Webb et al²⁷ demonstrated that p38/MAPK activation by TNF- α enhanced IL-6 gene expression in the MG-63 osteosarcoma cell line. Similarly, TNF- α is shown to autoregulate IL-6 production through various phospholipase pathways.²⁸ Our studies demonstrated that modulation of RAAS through ACEI and ARB causes decrease in TNF- α , and addition of TNF- α to splenocytes of ACEI or ARB treated OAD murine model caused upregulation of p38 and IL-6 gene transcription. Furthermore, RAAS modulation has also been shown to affect TGF- β expression renal injury.²⁹ This is in agreement with our current studies in which we noticed a reduction of TGF- β expression in both ACEI and ARB cohorts. However, when the splenocytes from these cohorts were treated with TNF- α there was upregulation of TGF- β expression, but no change was noticed when treated with IL-6, thus strongly suggesting that TGF- β is downstream of TNF- α signaling. Taken together, our data along with published literature strongly indicate that TNF- α is upstream and modulates IL-6 production. This TNF- α is downregulated by inhibition of the RAAS system.

The role of IL-6 in induction of Th17 autoimmune response is well recognized. Various in vitro and knockout models have established that IL-6 stimulates the production of IL-17 in the autoimmune setting.^{30,31} In our OAD model, we show that ACEI and ARB downregulate TNF- α -dependent IL-6 production, which in turn causes diminished IL-6 – dependent IL-17 responses. However, given the small sample size of our cohorts ($n = 5$) and non-physiologic nature of this model for BOS after human LTx, further studies to validate the efficiency of ACEI and ARBs, possibly by retrospective analysis in the patients who receive RAAS modulation therapy, are warranted. Use of an appropriate TNF- α knockout model will also shed important mechanistic information in addition to confirming results presented in this report.

In conclusion, we demonstrate that modulation of RAAS through ACEI or ARB causes diminished TNF- α -dependent IL-6 responses through inhibition of p38/MAPK kinase. This in turn leads to inhibition of IL-6-dependent IL-17 responses and thus down regulating the Th17 autoimmune cellular responses. Along with cellular responses ACEI and ARB also lead to diminished humoral responses against self-antigens. Our data strongly suggest that ACEI and ARB can be a useful strategy towards immunomodulation that has the potential to prevent the development of BOS.

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Figure 1.

Abrogation of obstructive airway disease lesions by angiotensin-converting enzyme inhibitor (ACEi) and angiotensin-receptor blocker (ARB). C57Bl/6 mice received an intrabronchial administration of 200 μ g of antibodies (Abs) on Days 1, 2, 3, and 6, and weekly thereafter. Representative of the sections taken from Day 30: (A–D) hematoxylin and eosin stain; (E–H) trichrome stain; (A and E) C1.18.4 Ab-treated mice; (B and F) lisinopril (ACEi, 10 mg/kg/day) fed in drinking water to C57Bl/6, which were treated with H2K^b Abs; (C and G), losartan (ARB, 10 mg/kg/day) fed in drinking water to C57Bl/6, which were treated with H2K^b Abs; (D and H) C57Bl/6 treated with H2K^b Abs (original magnification $\times 40$). (I) Graphs show morphometric analysis on the sections to measure cellular infiltration, epithelial hyperplasia, luminal occlusion, fibrosis, and CD4 and CD8 cellular infiltration, representative of 5 different sections and presented as mean $\pm$ standard error of the mean.

Figure 2.

Analysis of antibodies (Abs) to self-antigens and cellular responses to self-antigens: (A) serum concentration of K α 1T Abs; (B) serum concentration of ColV Abs; (C) frequency of interleukin (IL)-10, interferon (IFN)- γ , and IL-17-secreting memory CD4⁺ T cells specific to K α 1T; and (D) frequency of IL-10, IFN- γ and IL-17-secreting memory CD4⁺ T-cells specific to collagen V (ColV). ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker. Data are presented as mean $\pm$ standard error of the mean.

Figure 3.

Analysis of the nuclear factors and chemokines. (A) Western blot analysis is shown for the nuclear proteins extracted from spleens and probed to analyze protein expression of nuclear factors phosphorylated p38, phosphorylated extracellular signal-regulated kinase 1/2 (ERK-1/2), p65 nuclear factor- κ B (NF- κ B), and phosphorylated PI3K. (B) Real-time polymerase chain reaction analysis is shown for messenger RNA of the splenocytes for tumor necrosis factor (TNF)- β , interleukin (IL)-6, IL-17, IL-10, and transforming growth factor (TGF)- α . Representative of 5 animals and presented as mean $\pm$ standard error of the mean. Abs, antibodies; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.

Figure 4.

Analysis of the nuclear factors and chemokines in the splenocytes after treatment of the extracted splenocyte cultures with tumor necrosis factor- α . (A) Western blot analysis is shown for the nuclear proteins extracted from lungs and probed to analyze protein expression of nuclear factors phosphorylated p38 and phosphorylated extracellular signal-regulated kinase 1/2 (ERK-1/2). (B) Real-time polymerase chain reaction analysis is shown for messenger RNA of the splenocytes for interleukin (IL)-6, IL-17, IL-10, and transforming growth factor (TGF)- β . Representative of 5 animals and presented as mean $\pm$ standard error of the mean. Abs, antibodies; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.

Figure 5.

Analysis of the nuclear factors and chemokines in the splenocytes after treatment of the extracted splenocyte cultures with interleukin (IL)-6. (A) Western blot analysis shows the nuclear proteins extracted from lung and probed to analysis protein expression of nuclear factors phosphorylated p38 and phosphorylated extracellular signal-regulated kinase 1/2 (ERK-1/2). (B) Real-time polymerase chain reaction analysis on messenger RNA of the splenocytes for tumor necrosis factor (TNF)- α , IL-17, IL-10, and transforming growth factor (TGF)- β . Representative of 5 different animals and presented as mean $\pm$ standard error of the mean. Abs, antibodies; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.