IL-12 p80 is an Innate Epithelial Cell Effector that Mediates Chronic Allograft Dysfunction

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Online Data Supplement

Methods

Mice. C57BL/6J (H-2^d), C57BL/6J IL-12 p40 -/-, C57BL/6J IL-12 p35 -/-, BALB/CJ (H-2^b), and BALB/cJ IL-12Rβ1-/- mice were obtained from Jackson Laboratory. Mice were maintained under pathogen-free conditions for study at 7-9 wk of age. Sentinel mice and experimental control mice for Sendai viral infection were handled identically to infected mice and exhibited no serologic or histologic evidence of exposure to 11 rodent pathogens (including Sendai virus). The Animal Studies Committee of Washington University School of Medicine approved all animal studies.

Murine heterotopic subcutaneous tracheal transplantation. The murine heterotopic subcutaneous tracheal transplantation was preformed as originally described (E1). To harvest the donor trachea, intraperitoneal delivery of anesthesia (ketamine 87 mg/kg and xylazine 13 mg/kg), cervical dislocation and disinfection of the anterior neck and chest were performed. The donor trachea was exposed and a tracheal segment from one tracheal ring below the larynx to one tracheal ring above the carina was cleanly dissected, excised and placed in sterile PBS solution for approximately 10 minutes. The recipient mouse received anesthesia and the dorsum was disinfected with betadine. A 0.5 cm skin incision was made and a 1.5 X 1.5 cm subcutaneous pouch was generated by blunt dissection. One donor trachea was inserted into each recipient pouch and the skin incision was closed with veterinary glue. Post-operatively the recipient mice were placed under a warming light and monitored until spontaneous movement returned. To harvest the transplanted graft the recipient mice underwent anesthesia, cervical dislocation and a skin incision that partially circumscribed the graft. The skin was inverted and the graft was

cleanly dissected from surrounding tissue and excised. To assess airway epithelial cell injury, immune cell accumulation, and collagen deposition the grafts were harvested on day 5, 14, and 28 respectively. For all procedures standard aseptic surgical methods were employed to avoid infectious complications.

Procurement and analysis of tracheal graft: To evaluate grafts the tissue was fixed in 10% formalin, embedded in paraffin and sectioned at 5 μm as previously described (E2-E4). To ensure representative sampling of the tracheal graft, the initial 500 μm was discarded because the ends of the graft occasionally contained excessive fibrosis that was not representative of the entire graft. Tissue sections were obtained and then compared to sections from a 300 μm deeper level. Samples from a graft were considered representative when similar pathologic findings were observed between adjacent levels. Specimens were imaged with brightfield microscopy (Olympus Model BX-51) interfaced to a digital photomicrographic system (Optronix camera and MagnaFire v2 software). To quantitate epithelial injury, the percent of basement membrane (BM) covered by epithelium was calculated. The mean values from three representative sections of each graft were recorded as per cent of denuded BM (length of denuded BM/total length of BM multiplied by 100). To quantitate lumen obstruction, the total lumen area within the tracheal rings and the non-obstructed area was measured. The mean value from three representative sections of each graft were recorded as per cent lumen obstruction using the formula: (total lumen area minus non-obstructed area)/total lumen area multiplied by 100. All measurements and calculations were performed in a blinded fashion using Image Pro Plus v4 software (Media Cybernetics) calibrated to the photomicrograph system.

Hydroxyproline assay: To quantitate allograft hydroxyproline concentration the graft was weighed, pulverized, incubated with hydrochloric acid (10 μl of water + 9 μl of 12N hydrochloric acid/50 μg lysate) and heated at 116 ˚C for 18h to form hydrolysate (adjusting volume of hydrochloric acid corrected for differences in graft size). Hydrolysate (10 μl) was incubated with 25 μl of citrate/acetate buffer (0.24M citric acid, 0.89M sodium acetate, 0.85M sodium hydroxide, 0.2M glacial acetic acid, pH solution to 6.0) and 500 μl of chloramine T solution (0.063M N-chloro-p-toluene-sulfonamide sodium salt, 2-propanol 1:10 vol/vol, 64 ml citrate/acetate buffer, 8 ml water) for 20 min at 20 ˚C. Fresh Ehrlich's solution (500 μl of 2-propanol 1:1.4 vol/vol, 1.27M p-dimethylaminobenzaldehyde, 70% perchloric acid) was added and the solution was incubated at 65˚ C for 15 min, cooled at room temperature for 10 min, and absorbance at 550 nm was determined. To quantitate hydroxyproline, duplicate samples were diluted and compared to a cis-4-hydroxyl-L-proline standard curve (0-100 μg/ml, Sigma). Individual values represented the mean of duplicate measurements.

Immune cell and IL-12 p40 immunolabeling: To quantitate immune cell accumulation in the graft lumen the tissue was immunolabeled as previously described (E2-E4). To immunolabel neutrophils, tissue sections were sequentially incubated with nonimmune rabbit serum, rat α-mouse neutrophils IgG (0.66 μg/ml, 7/4 clone, Serotec Inc.) for 18 h at 4 ˚C, biotinylated rabbit α-rat IgG, streptavidin-conjugated alkaline phosphatase complex, and red chromogen (Vector Laboratories). To immunolabel macrophages, tissue sections were sequentially incubated with nonimmune rabbit serum, rat α-mouse

Mac-3 IgG (2 μg/ml, M3/84 clone, BD PharMingen) for 18 h at 4 ˚C, biotinylated rabbit α-rat IgG, streptavidin-conjugated horse radish peroxidase (HRP) complex, and brown chromogen (Vector Laboratories). To immunolabel lymphocytes, tissue sections underwent antigen retrieval using Antigen Retreival Decloaker solution according to manufacturer's protocol (BioCare Medical). Tissue sections were then sequentially incubated with Sniper blocking reagent, rabbit α-CD3 polyclonal antibody (1:50 vol/vol) for 18 h at 4 ˚C, biotinylated goat α-rabbit IgG, streptavidin-conjugated HRP complex, and brown chromogen (BioCare Medical). Tissue sections were counterstained with hematoxylin, dehydrated in graded ethanol, mounted for brightfield microscopy, and imaged as above. To quantitate immune cells in graft lumen, immunolabeled cells were counted in three high power fields (HPF, 400×) from three representative sections of each graft. To immunolabel IL-12 p40, tissue sections were sequentially incubated with nonimmune rabbit serum, goat α-mouse IL-12 p40 polyclonal antibody (2 μg/ml, Santa Cruz Biotechnology, Inc.), biotinylated rabbit α-goat IgG, streptavidin-conjugated alkaline phosphatase complex, and red chromogen as previously described (E2).

Murine IL-12 family member quantification: To quantitate IL-12 family member concentration in grafts, whole cell protein extracts were prepared by sonication using lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 2 mM sodium pyrophosphate, 10 mg/ml leupeptin, and 10 mg/ml aprotinin. Protein concentration of lysate was determined using BCA protein Assay Kit (Pierce). To measure murine IL-12 p80/p40, IL-12 and IL-23 we utilized commercially available

ELISA kits with 250 μg/lysate per well. Sensitivity for the mouse IL-12 p80/p40 ELISA kit (R&D Systems Inc.) is 4 pg/ml. We noted a 20% cross-reactivity to mouse IL-12 at 1000 pg/ml, and we measured 6.4% and 5.3% cross-reactivity to recombinant mouse IL-23 at 250 and 1500 pg/ml respectively. Sensitivity for the mouse IL-12 ELISA kit (R&D Systems Inc.) is 2.5 pg/ml with no cross-reactivity to mouse p80 or IL-23 at 50,000 pg/ml. Sensitivity for the mouse IL-23 ELISA kit (eBioscience) is 15 pg/ml with no cross-reactivity to mouse p80 or IL-12 at 100 ng/ml. Values were adjusted to pg/ml/mg of total protein lysate to correct for varying size of grafts and individual values represent the mean of duplicate measurements.

Sendai infection and antibody treatment of mice: Mice underwent anesthesia and intranasal inoculation with Sendai Virus (Fushimi Strain, ATCC # VR-105) or UV-inactivated Sendai virus at 50,000 egg infectious dose 50%, diluted in 30 μl of PBS as previously described (E2-E4). Physical inspection and weight loss were performed daily and on post-inoculation day 5 the trachea was harvested for transplantation. For IL-12 p40 blocking experiments recipient mice underwent treatment with control rat IgG (Sigma) or rat α-IL-12 p40 IgG mAb that was purified from athymic nude mouse ascites fluid using protein G affinity chromatography. Mice received a previously established dose of antibody that resulted in p80 blockade (1.0 mg in 0.66 ml PBS every four days) beginning one day prior to transplantation (E2).

Generation and characterization of CCSP-p40 transgenic C57BL/6J mice: To achieve airway epithelial cell overexpression of mouse p40 we generated a C57BL/6J transgenic

mouse strain using 2.3 kb of the rat Clara cell secretory protein (CCSP) promoter (kindly provided by Dr. Jeffrey Whitsett, University of Cincinnati College of Medicine) (E5). The mouse p40 cDNA (ATCC catalogue number #87595) was inserted downstream of the CCSP promoter and upstream of the SV40 polyadenylation sequence using standard molecular cloning techniques. Following sequence verification, the entire CCSP promoter-p40 cDNA-SV40 polyadenylation fragment was excised with restriction enzyme digest, gel purified, resuspended in standard microinjection buffer, and microinjected into the pronuclei of fertilized C57BL/6J embryos. The microinjected embryos were implanted into the oviducts of pseudopregnant Swiss Webster foster mothers. Germline transmission was verified in 2 of 83 offspring and IL-12 p40 protein overexpression was identified in the bronchoalveolar lavage (BAL) of one founder line that was used in this study. Expression of the CCSP-p40 transgene was quantitated in the BAL by ELISA and we detected a selective increase in IL-12 p80/p40 but not IL-12 or IL-23. Comparison of transgene negative and positive littermates demonstrated a 91-fold increase in p80/p40 (9.9 vs 876 pg/ml), a 1.4 fold increase in IL-12 (4.8 vs 6.8 pg/ml) and a 1.9 fold increase in IL-23 (45 vs 87 pg/ml). Western analysis of the BAL from the transgene positive strain demonstrated expression of p80 and p40 but not IL-12 or IL-23.

Mixed lymphocyte reaction: To test the proliferative response of alloreactive T cells we performed the mixed lymphocyte reaction as described previously (E6). Spleen cells from naïve or post-transplant BALB/cJ or BALB/cJ IL-12 Rβ1 -/- recipient mice were seeded at a concentration of 3×10^5 cell/well in triplicate cultures in a round bottom 96-

well plate in the presence of irradiated (3×10^3 Rad) BALB/cJ, C57BL/6J or C3H spleen cells at a concentration of 2×10^5 cells/well. The cells were cultured in RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES buffer, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 125 ng/ml Fungizone, and 50 μM 2-mercaptoethanol. After 3 days the cells were treated with 1 μCi/well [^{3}H]thymidine for 24 h. [^{3}H]thymidine incorporation into DNA was then determined using a liquid scintillation counter and the results were expressed as net counts per minute.

Lung transplant recipient care and cohort assembly: The Institutional Review Board of Washington University School of Medicine approved all human studies. All recipients underwent pre- and post-transplant care by the Lung Transplant group at Washington University School of Medicine as previously described (E7). To analyze IL-12 p40 expression in lung transplant recipients we performed three separate experiments.

First, to analyze IL-12 p40 expression in allografts from recipients with a respiratory viral infection, we identified 17 recipients that had transbronchial biopsies obtained at the time of respiratory viral infection (E7). Of these 17 recipients, 10 contained sufficient epithelium for adequate IL-12 p40 immunolabeling and all 5 of the recipients with a histologic diagnosis of transplant bronchitis demonstrated epithelial IL-12 p40 expression. IL-12 p40 immunolabeling of human specimens was performed as described above.

Second, to further evaluate IL-12 p40 expression in bronchitis from allografts that contained a larger number of airways with better preserved epithelium, we identified 5

open lung biopsy specimens using a computerized search of pathology reports that contained the keywords: transplant, open lung biopsy and bronchitis. IL-12 p40 immunolabeling of human specimens was performed as described above.

Third, to analyze and quantitate IL-12 family member expression and determine if specific family members were selectively increased in transplant recipients with a single type of allograft dysfunction, we prospectively collected BAL and transbronchial biopsies. All BAL specimens underwent microbiologic analysis for the presence of bacteria, fungus, mycobacteria, and virus according to standard microbiologic techniques and all transbronchial biopsies underwent pathologic analysis for acute rejection and transplant bronchitis according to the Lung Rejection Study Group (E8). We collected BAL and transbronchial biopsies on 86 recipients. Of these 86 specimens, we excluded 39 because identification of: bacterial, fungal, or mycobacterial infectious pathogens (n=19), infectious pathogens and a concurrent pathologic abnormality (n=14), two concurrent pathologic abnormalities (n=4), reperfusion injury (n=1), and recurrent sarcoid (n=1). The remaining 47 recipients with a single abnormality were segregated into 4 cohorts. Recipients without infection, acute rejection, or bronchitis constituted the Normal Transplant cohort (A0B0 without infection, n=16). Recipients with only acute rejection constituted the Acute Rejection cohort (A1B0=1, A2B0=8 and A3B0=1, total n=10). Recipients with only CMV detected in the BAL constituted the CMV cohort (A0B0 with CMV pneumonitis=4 and A0B0 with BAL positive for CMV by shell vial assay=5, total n=9). Recipients with only transplant bronchitis constituted the Bronchitis cohort (A0B1=7 and A0B2=5, total n=12). Normal non-transplant subjects had no clinical history of lung disease, normal spirometry and normal airway reactivity and

constituted the Non-transplant Normal cohort (n=10). The exclusion and inclusion of recipients into specific cohorts was done prior to the measurement of BAL cytokines and our recruitment goal for each cohort was approximately 10 recipients since this sample size allowed for the detection of significant differences in IL-12 family member expression previously (E2).

Human BAL analysis: BAL fluid was obtained from the combination of the second and third 50 ml normal saline aliquots and total cell count was determined using a hemocytometer chamber as previously described (E2). The BAL fluid cell pellet was subjected to cytocentrifugation, methanol fixation, Wright-Giemsa staining, and manual characterization of 300 immune cells using standard histologic criteria. Individual values represent mean of two blinded readers, or three readers if original two values differed by more than 5% for any cell type. To quantitate BAL fluid IL-12 family member concentration the fluid was concentrated approximately 10 fold using a Centriprep YM-3 concentrator (Millipore) and then used to determine levels of IL-12 p80/p40 and IL-12 with ELISA kits (R & D Systems Inc.) (E2). The sensitivity for the High Sensitivity human IL-12 ELISA kit is 0.5 pg/ml with no cross-reactivity to human p40 or IL-23 at 10 ng/ml. Sensitivity for the human IL-12 p80/p40 ELISA kit is 15 pg/ml with 2.7% cross-reactivity to human IL-12 at 5,000 pg/ml and we verified a 0% cross-reactivity to human IL-23 at 125 pg/ml, a 6.8% cross-reactivity at 500 pg/ml. and a 6.6% cross-reactivity at 2000 pg/ml. To measure human IL-23 we performed a Receptor Binding Assay utilizing reagents according to manufacturer's recommendations (R & D Systems Inc.). We incubated 96 well plates with recombinant human IL-23 receptor/FC chimera protein (1

μg/ml for 18 h at 25°C). Non-specific protein binding was prevented by incubation with blocking buffer (300 μl/well of 0.5% BSA in PBS for 1 h at 37˚C). BAL fluid or recombinant human IL-23 (100 μl/well at 0-2,000 pg/ml, R & D Systems Inc) was then incubated for 2 h at 25°C. Detection of IL-23 occurred by sequential incubation with biotinylated anti-human IL-12 p40 (0.1 μg/ml for 2 h at 25°C, clone 8.6, Endogen), streptavidin-conjugated HRP, substrate solution and stop solution. Values represent mean of duplicate measurements of optical density at 450 nm (with wavelength correction set to 540 nm). We determined the sensitivity for this human IL-23 detection assay was 31 pg/ml and found no cross-reactivity to recombinant human IL-12 or human p40 at 2000 pg/ml.

Supplement References:

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E6. Smith MA, Jaramillo A, SivaSai KS, Naziruddin B, Kaleem Z, Patterson GA, and Mohanakumar T. Indirect recognition and antibody production against a single mismatched HLA-A2-transgenic molecule precede the development of obliterative airway disease in murine heterotopic tracheal allografts. *Transplantation* 2002;73:186-93.

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E8. Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ohori NP, Ritter J, Stewart S, et al. Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: Lung Rejection Study Group. *J Heart Lung Transplant* 1996;15:1-15.

Figure Legends

Figure E1. **Transplantation and respiratory viral infection combine to enhance epithelial cell injury**. C57BL/6J donor tracheas underwent heterotopic tracheal transplantation into C57BL/6J recipient mice (C57 → C57) or BALB/cJ recipient mice (C57 → BALB). C57BL/6J donor mice underwent intranasal inoculation with Sendai virus or UV-inactivated Sendai virus and five days later the tracheas were harvested for heterotopic tracheal transplantation into BALB/cJ recipient mice (Virus + C57 → BALB and UV Virus + C57 → BALB respectively). Five days post-transplant the grafts were harvested and underwent hematoxylin and eosin (H & E) staining. Arrows in Virus + C57 → BALB photomicrograph indicate denuded basement membrane (BM).

Figure E2. **Blockade of p80 function attenuates viral-driven allograft dysfunction.** Given that we had demonstrated a synergistic increase in p80 expression in viral-driven allograft dysfunction, we next reasoned that this phenotype could be attenuated in recipient mice that could not respond to p80 (that is IL-12 receptor β1 deficient mice). Compared to a wild-type recipient, viral-driven allogeneic dysfunction in an IL-12 receptor β1 recipient resulted in less epithelial cell injury at day 5, macrophage accumulation at day 14, and collagen deposition at day 28.

Figure E3**. Inhibition or blockade of p80 function attenuates lumen obliteration**. To test the possibility that epithelial cell p80 may be critical in mediating allograft lumen obliteration we employed a loss-of-function experiments using IL-12 p40 -/- allografts,

IL-12 Rβ1 -/- recipients and recipients treated with IgG control or IL-12 p40 blocking antibodies. Compared to allogeneic transplantation with wild-type recipients, there was a significant decrease in lumen obstruction in IL-12 Rβ1 -/- recipients and recipients treated with IL-12 p40 blocking antibodies and a trend toward a decrease in IL-12 p40 -/- donors ($p = 0.07$).

Figure E4. **Recipient alloreactivity in transplant conditions that enhance or block p80.** To assess T-cell alloreactivity following transplant conditions that either enhanced allograft p80 expression (viral infection, Tg-p40 and IL-12 p35 deficient donors) or blocked p80 function (IL-12 Rβ1 deficient recipient) we performed a mixed lymphocyte reaction on recipient splenocytes harvested following transplantation. Compared to alloreactivity measured in naïve spleen cells, we observed a significant increase in alloreactivity from splenocytes in wild-type recipients that had undergone allogeneic heterotopic tracheal transplantation. However, this response was not different in conditions that either enhanced p80 expression or blocked its function. (Figure E4).

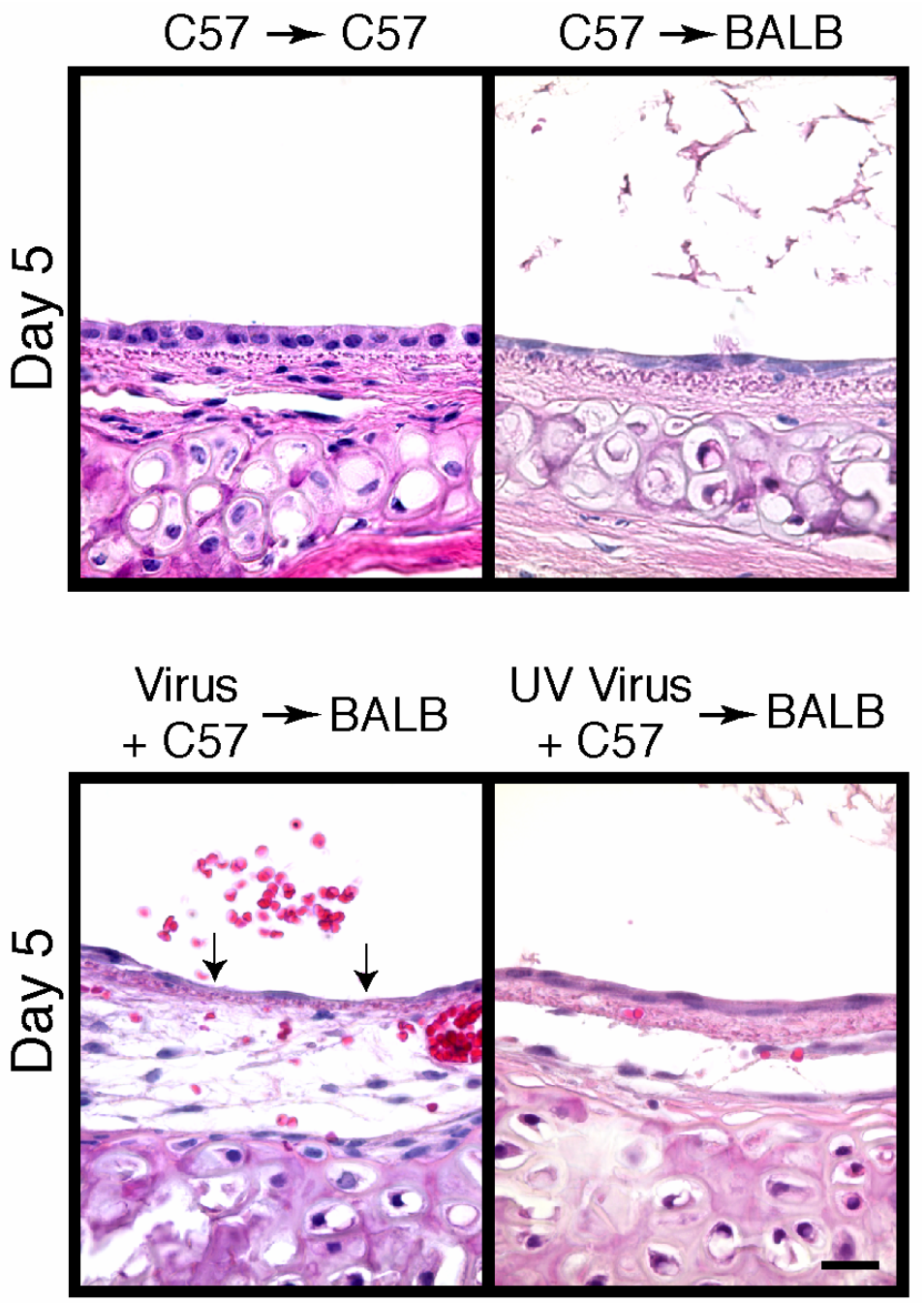

Figure E1.

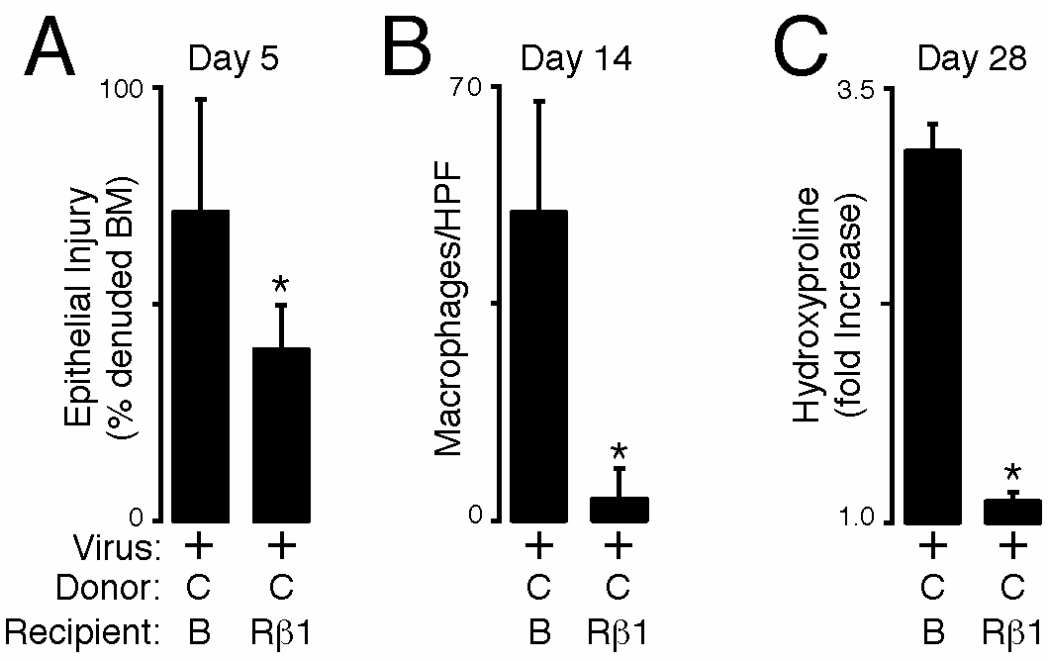
A
Day 5
100
0
Epithelial Injury
(% denuded BM)
*
B
Day 14
70
0
Macrophages/HPF
*
C
Day 28
3.5
1.0
Hydroxyproline
(fold Increase)
*
Virus: + + + + +
Donor: C C C C
Recipient: B Rβ1 B Rβ1

Figure E2.

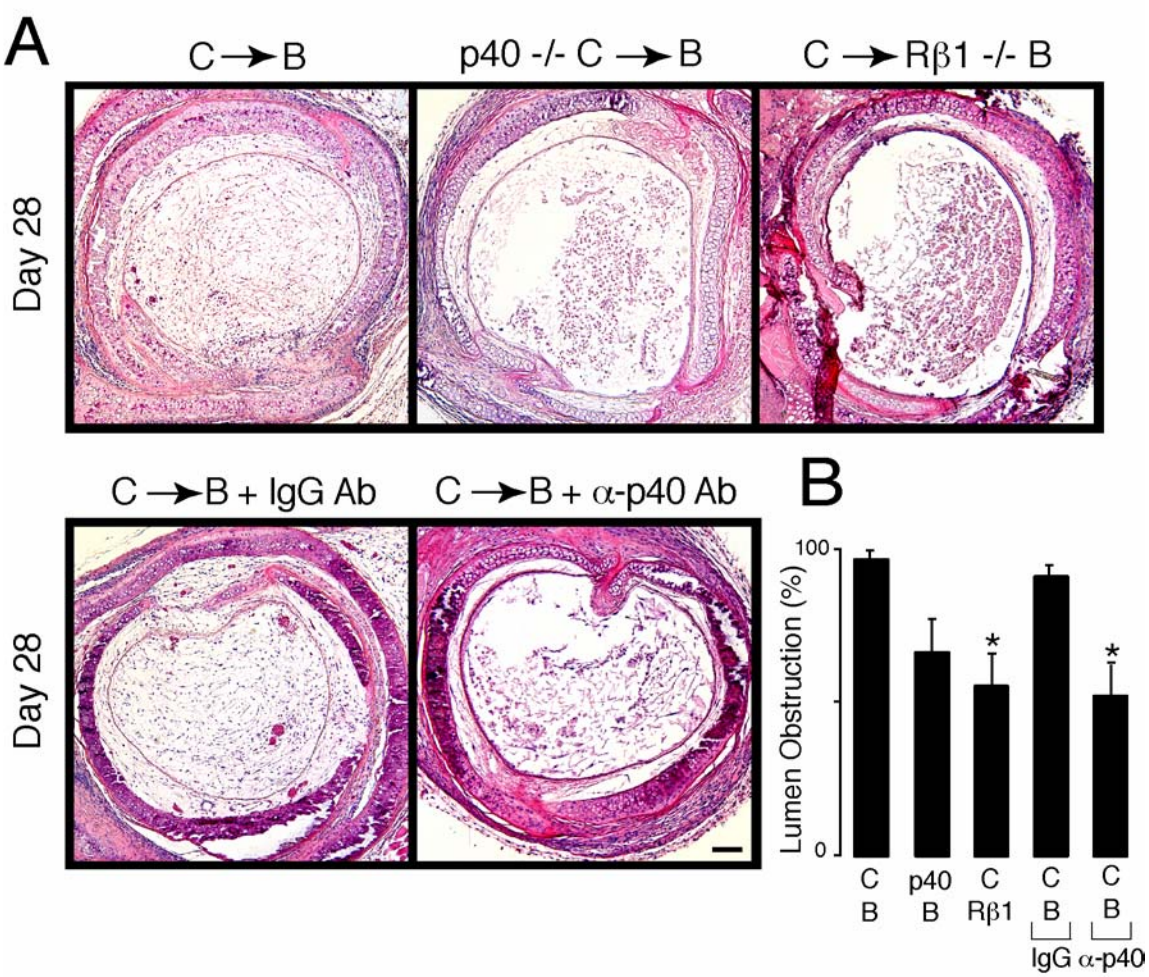
A
C → B
p40 -/- C → B
C → Rβ1 -/- B
Day 28
C → B + IgG Ab
C → B + α-p40 Ab
Day 28
B
Lumen Obstruction (%)
100
0
C B
p40 B
C Rβ1
C B
C B
IgG
α-p40

Figure E3.

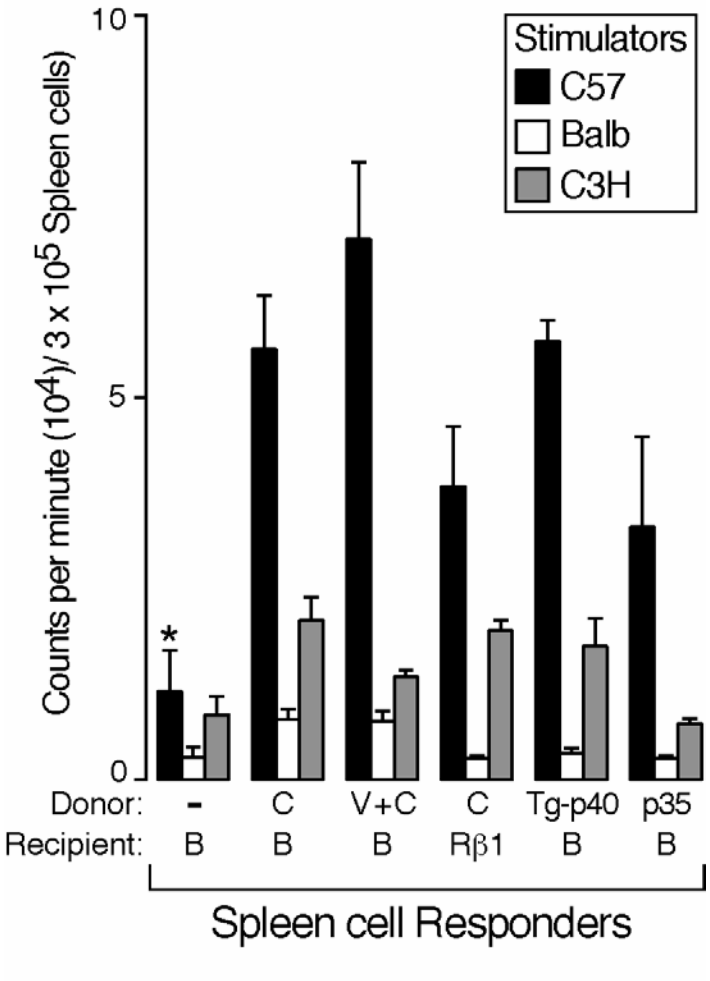
Stimulators
C57
Balb
C3H
Counts per minute (10^4)/ 3 x 10^5 Spleen cells)
10
5
0
*
Donor: - C V+C C Tg-p40 p35
Recipient: B B B Rβ1 B B
Spleen cell Responders

Figure E4.