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# Ecto-5′-Nucleotidase (CD73) Attenuates Allograft Airway Rejection through Adenosine 2A Receptor Stimulation

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## Abstract

There are multiple drivers of leukocyte recruitment in lung allografts that contribute to lymphocytic bronchitis (LB) and bronchiolitis obliterans (BO). The innate mechanisms driving (or inhibiting) leukocyte trafficking to allografts remain incompletely understood. This study tested the hypothesis that CD73 (ecto-5′ nucleotidase), an enzyme that catalyzes the conversion of AMP to adenosine, is a critical negative regulator of LB and BO. Implantation of tracheal allografts from wild type (WT) mice into $CD73^{-/-}$ recipients revealed a striking increase in airway luminal obliteration at 7 d (62 ± 4% and 47 ± 5% for $CD73^{-/-}$ and WT allograft recipients, respectively; $p = 0.046$). There was also a concordant increase in $CD3^+$ lymphocytic infiltration (523 ± 41 cells and 313 ± 43 cells for $CD73^{-/-}$ and WT allograft recipients, respectively; $p = 0.013$). Because real-time PCR revealed a 43-fold upregulation of mRNA for the adenosine A2A receptor (A2AR) in WT allografts compared with WT isografts ($p = 0.032$), additional experiments were performed to determine whether the protective effect of CD73 was due to generation of adenosine and its stimulation of the A2AR. Treatment of WT recipients with an A2AR agonist significantly reduced $CD3^+$ lymphocyte infiltration and airway luminal obliteration; similar treatment of $CD73^{-/-}$ recipients rescued them from LB and airway obliteration. These data implicate CD73 acting through adenosine generation and its stimulation of the A2AR as a critical negative modulator of lymphocyte recruitment into airway allografts. The CD73/adenosine axis might be a new therapeutic target to prevent BO.

Lung transplantation is an accepted treatment for end-stage pulmonary diseases. Nevertheless, despite recent advances in lung preservation and immunosuppression, allograft rejection remains a major cause of morbidity and mortality in lung transplant recipients (1,2). Bronchiolitis obliterans (BO) manifests in patients as BO syndrome ascertained by spirometry, affects up to 50–60% of patients who survive 5 y after lung transplantation (1–3). BO develops as a progressive obliteration of small airways within the lung allograft, with pathognomonic features including early lymphocyte infiltration and other signs of inflammation, epithelial cell injury, and fibrosis (4). The pathogenesis of BO, although undoubtedly multifactorial, is recognized to increase with repeated bouts of acute rejection. Aside from infectious complications, BO represents the main factor limiting long-term survival after lung transplantation (5).

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**Disclosures**
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The placement of a solid organ with its associated parenchymal and stromal cells, as well as its integrated vasculature, into an immunologically mismatched recipient initiates a series of downstream processes eventuating in rejection of the transplanted organ. The transplant scenario, including elements of ischemia-reperfusion injury, as well as humoral and cellular rejection, is associated with the release of a wide variety of mediators that can modulate local inflammatory responses. One such mediator, released from activated or injured cells, is the signaling nucleoside adenosine, which is elevated at sites of local tissue damage emanating from ischemia-reperfusion injury or host immune attack on the transplanted organ. Engagement of specific adenosine receptors with released adenosine initiates a cascade of intracellular signaling events that can protect or injure the organ, depending on the degree of activation and contextual milieu. Adenosine is an adenine nucleoside released by injury. Phosphorylated adenine nucleotides, including ATP, ADP, and AMP, are also released. These moieties are phosphohydrolyzed by specific enzymes at the surface of cells by a family of enzymes called ectonucleotidases, so named for their surface localization, as well as their activity. One of these enzymes expressed on endothelial cells and lymphocytes (6), CD73 (extracellular ecto-5′nucleotidase enzyme), serves as an important immunoregulator through the terminal phosphohydrolysis of AMP. Through this action, the proinflammatory adenine nucleotides are dissipated, and anti-inflammatory and immunosuppressive adenosine is generated.

Adenosine is a highly conserved signaling molecule that serves an array of adaptive physiological functions in mammalian cells. It binds through one of four G protein-coupled receptors that have been labeled A1, A2A, A2B, and A3. Among the many places where these receptors are expressed include cells of the vasculature, airways (7), and immune systems (8). All of these receptors have been implicated in tissue protection in lung injury (9). Although only is little known about the role of the A1 and A3 receptors in tissue injury, several studies showed a protective role for the A2A and A2B receptors (10,11). A2B receptor antagonist prevents disease progression in asthma and chronic obstructive pulmonary disease (12), and A2B receptor knockout mice exhibited elevated levels of proinflammatory cytokines (13). Among the adenosine receptor subtypes, the A2A receptors are relatively unique in that they trigger anti-inflammatory signals upon activation (14). Consequently, their stimulation was reported to effectively attenuate ischemia-reperfusion injury of multiple organs, including the heart, lung, and liver (15–18), where inflammatory activation is otherwise believed to contribute to tissue injury. The attenuated injury signal seems to be mediated by a downstream effect of A2A receptor stimulation, including decreased expression of adhesion leukocyte receptors and attendant reduction in endothelial–leukocyte interactions, as well as diminished generation of superoxide, a vasoactive and injurious free radical (15–18). The anti-inflammatory effects of A2A receptor signaling are amplified through downregulated secretion of proinflammatory cytokines (19,20).

When considered from the standpoint of lung transplantation, these data raise the possibility that immunomodulatory effects of CD73 and downstream adenosine could play a tonic inhibitory role on pathological processes of allograft parenchymal and airway rejection. To elucidate a potential mechanistic link between CD73 and graft rejection, a series of experiments were performed using an orthotopic airway transplantation model in which CD73 could be genetically deleted from donor or recipient tissue, and the role of adenosine receptor signaling could be studied pharmacologically. Experimental readouts incorporate observations related to epithelial cell injury, lymphocytic and other inflammatory cell recruitment, and loss of airway lumen, each of which relates to the main pathological features of lung transplant rejection, including early lymphocytic bronchitis (LB) and later stage BO. Because LB often precedes BO and is thought to contribute to BO pathogenesis (21), the experiments performed in this study sought to test the CD73/adenosine axis as an

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early molecular pathway of immune injury that might be susceptible to interruption and, hence, a new target of therapeutic opportunity to prevent BO. Several animal models of BO exist (reviewed in Ref. 22). Although there are scarce reports of reproducible murine models of lung transplantation, our model of orthotropic tracheal transplantation has been validated by other groups as a model with many features similar to BO (23).

## Materials and Methods

### Mice

Male mice aged between 8 and 12 wk old were used in these experiments. C57BL/6 (H-$2^b$) mice and B10.A (H-$2^a$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Isogeneic tracheal transplants were performed using C57BL/6J mice as recipients and donors. Allogeneic (B10.A) tracheal grafts from donor mice were transplanted into wild type (WT) (C57BL/6) or $CD73^{-/-}$ recipient mice, a kind gift from Dr. Linda Thompson (Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, Oklahoma City, OK) (24,25). The $CD73^{-/-}$ mice used for these experiments were backbred for 13 generations onto the C57BL/6 background, so that they had an H-$2^b$ genotype. The genotype of each mouse was confirmed by genomic PCR. To specifically determine the role of epithelial- versus leukocyte-derived CD73, transplantation experiments were performed using reciprocal donor/recipient combination experimental animals. Depending on the experiment, C57BL/6 mice with a $CD73^{+/+}$ or $CD73^{-/-}$ genotype served as the airway allograft donor or the recipient, and B10.A mice (which are $CD73^{+/+}$) served as recipients or a source of airway tissue. In this way, isograft (C57BL/6 - C57BL/6) or allograft (C57BL/6 - B10.A) experiments could be performed, with CD73 present in airway graft and recipient or missing from either. All experiments were performed according to the protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan.

### Tracheal-transplant model

An established tracheal-transplant model was used to study the effect of CD73 on the pathogenesis of allograft rejection. This model consists of the previously described double-lumen airway (orthotopic)-transplant model for studying chronic airway rejection (26–28). Briefly, after anesthesia, donor mice were exsanguinated, and the whole trachea was harvested under sterile conditions by transecting below the cricoid cartilage distal to the bifurcation of the carina (Fig. 1). Recipient mice were subsequently anesthetized, and the whole trachea was exposed to provide a surgical window for anastomosis. Distal and proximal orifices were created on the recipient trachea to enable anastomosis with both ends of the donor tracheal graft. Running sutures permit airway continuity, enabling airflow over the graft epithelial surface. Histologically, this airway-transplant model mimics LB, which was detected as described below. All surgeries were performed with the use of a Leitz-Wild surgical microscope (Urban Engineering, Burbank, CA) under ×16 magnification.

### Histopathological evaluation of tracheal transplants

Grafts were harvested 1 wk after transplantation (27), embedded en bloc in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC), placed in Disposable Base Molds (Richard-Allan Scientific, Kalamazoo, MI), snap-frozen in liquid nitrogen, and stored at −80°C until the time of histological analysis. The degree of graft luminal occlusion was ascertained on 5-μm-thick sections by histochemical staining, using an elastin stain (Accustain; Sigma-Aldrich, St. Louis, MO), to demarcate the epithelial and subepithelial layers. To detect infiltrating T cells, immunostaining was performed using serial adjacent sections from each group, using primary Abs directed against a pan-T cell marker (hamster anti-mouse CD3; BD Pharmingen, San Diego, CA). Morphometric measurements of cross-sectional areas were performed by an investigator blinded to section identity, by tracing

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epithelial and subepithelial areas using a computer-assisted image-analysis system (AxoCamHR; Carl Zeiss Microimaging, Thornwood, NY). Quantitative analysis of recruited T cell numbers was performed by manually counting numbers of $CD3^+$ cells in the epithelial and subepithelial layers in each 5-μm section under high-power magnification.

### mRNA isolation and real-time PCR analysis

Total RNA was extracted from frozen mouse tracheas using RNeasy Mini Protocol for Tissues kits (Qiagen, Valencia, CA) and reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA), using procedures specified by the manufacturer. One microgram of sample RNA was transcribed to cDNA. Real-time PCR was performed on samples of this cDNA using an Applied Biosystems 7000 Real-Time PCR System data-collection system, with data analysis performed using software provided by the manufacturer. Fluorogenic PCR primer sets and probes for all of the specified target genes and endogenous reference housekeeping cDNA (β actin) were purchased as TaqMan Gene Expression Assays (Applied Biosystems).

### Adenosine receptor agonist treatment

Adenosine receptor agonists and antagonists were obtained from Sigma-Aldrich. The following reagents were each used at 2 mg/kg/d: A2A receptor agonist 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS-21680) and A2A receptor antagonist 5-amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine (SCH-58261). This dose of the adenosine receptor agonists/antagonists was chosen based on their previous use in mouse models of T cell transplantation and heterotopic heart transplantation (24,29,30). Adenosine receptor agonists/antagonists or vehicle (0.1% DMSO in PBS) was administered in a sterile 0.1-ml volume by i.p. injection, which was given every 12 h after surgery until the time of graft harvest.

### Quantification of CGS-21680 in mouse plasma by liquid chromatography-tandem mass spectrometry

C57BL/6 mice were dosed with CGS-21680 at the concentrations and time points used in the pharmacological study (i.e., 1 mg/kg twice per day) by i.p. injection. At 0.25, 0.5, 1, 2, 4, and 8 h postdose, three mice were bled by cardiac puncture into EDTA, blood was separated by centrifugation, and plasma was stored at −20°C until analyzed for drug levels. Standards of CGS-21680 and 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purine-9-y1]-N-methyl–D-ribofuranuronamide (internal standard) were purchased from Sigma-Aldrich. Acetonitrile and water were HPLC grade and purchased from Honeywell Burdick and Jackson (Muskegon, MI). Suprapur formic acid was from EMD Chemicals (Darmstadt, Germany). Liquid chromatography-tandem mass spectrometry was performed on an Alliance 2695 HPLC system (Waters, Milford, MA) interfaced directly to the electrospray ionization source of a TSQ Quantum Ultra AM triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). Separation was achieved with a Luna Phenyl-Hexyl column (150 × 2.00 mm, 3 μm, 110 Å; Phenomenex, Torrance, CA) maintained at 40°C using a binary gradient at a flow rate of 0.22 ml/min. The injection volume was 10 μl. Solvent A was 0.1% formic acid in water (v/v), and solvent B was 0.1% formic acid in acetonitrile (v/v). The initial conditions of 15% B were increased linearly to 100% B over 10 min, maintained at 100% B for 1 min, and re-equilibrated to initial conditions. The temperature of the sample compartment was set to 20°C. Positive ions were generated in the electrospray ionization source using nitrogen as the sheath and auxiliary gases under the following conditions: spray voltage, 4000 V; tube lens, 113 V; sheath gas, 50 pounds per square inch; auxiliary gas, 10 U; capillary temperature, 300°C. Mass analysis was performed with single-reaction monitoring using precursor/product ion pairs of (500/327) for CGS-21680 and (511/352) for the internal standard. The scan width was 0.2 mass units, scan

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time was 0.3 s, and the collision gas pressure was 1.5 mTorr. Xcalibur software (v 1.4, ThermoFinnigan) was used for instrument control and data analysis. A simple least-squares regression analysis with 1/x weighting was used to generate the calibration curve, which ranged from 0.2 to 500 ng/ml. Pooled plasma from undosed animals was spiked with a standard solution of CGS-21680 to a concentration of 500 ng/ml, and serial dilutions were used to prepare calibrators for the standard curve. Sixty microliters of the calibration standards and varying aliquots of the mouse samples were transferred to clean polypropylene tubes and then spiked with 20 μl the internal standard working solution. Acetonitrile was added to each tube in a volume ratio of 1.5–2.5 to precipitate the plasma proteins. The samples were vortex mixed and then centrifuged at 14.4 relative centrifugal force for 15 min. The supernatants were transferred to clean tubes and evaporated to dryness in a vacuum concentrator. All samples and standards were reconstituted to a final volume of 60 μl in a solution of $H_2O$/acetronile/formic acid (70:30:0.1, v/v/v), centrifuged at 14.4 relative centrifugal force for 10 min, and then transferred to polypropylene autosampler vials. Reported CGS-21680 concentrations were corrected for all dilutions.

### Statistics

Statistical comparisons were made using a statistical package that is commercially available for the Macintosh personal computer (Stat View-J 5.0; SAS Institute, Cary, NC). Student *t* tests were used to determine *p* values when two groups were compared. The correlations between the graft luminal occlusion and the number of $CD3^+$ cells were studied using the Pearson correlation coefficient. Data are expressed as mean ± SEM; differences were determined to be statistically significant when $p < 0.05$.

## Results

### Effects of CD73 on graft luminal narrowing in orthotopic trachea-transplant model

To determine the influence of CD73 on graft luminal narrowing, morphometric analyses were performed on $CD73^{+/+}$ isografts, $CD73^{+/+}$ allografts, and $CD73^{+/+}$ allografts implanted into $CD73^{-/-}$ recipients (Fig. 1). Compared with $CD73^{+/+}$ isografts, $CD73^{+/+}$ allografts exhibited thickening of the subepithelial and epithelial airway layers, leading to a significant overall narrowing of the airway lumen (luminal narrowing was 31 ± 4% for isografts versus 47 ± 5% for allografts ($p = 0.042$; Fig. 2). $CD73^{+/+}$ allografts placed into $CD73^{-/-}$ recipients demonstrated a significant increase in graft luminal narrowing (62 ± 4%) compared with $CD73^{+/+}$ allografts placed into $CD73^{+/+}$ recipients ($p = 0.046$) or $CD73^{+/+}$ isografts placed into $CD73^{+/+}$ recipients ($p < 0.01$; Fig. 2). These data suggest an important protective role for recipient CD73, because its absence exacerbates the histological equivalent of BO in this mouse model.

An additional cohort of experimental animals was used to define the importance of the cellular site at which CD73 is expressed in the setting of allotransplantation. To determine whether CD73 expression on cells resident within graft tissue was more important in protecting the graft versus CD73 expressed on infiltrating cells emanating from the recipients, CD73 gene-null grafts were placed into gene-competent ($CD73^{+/+}$) recipients; alternatively, CD73 gene-competent grafts were placed into CD73 gene-null hosts. Because the CD73 gene-null mice were bred onto a C57BL/6 background, for those experiments in which reciprocal strain transplantation was anticipated, it was first necessary to obtain a baseline assessment of BO in WT reciprocally transplanted mice (i.e., C57BL/6 onto B10.A versus B.10A onto C57BL/6). This comparison, shown in Fig. 2, illustrated no significant difference in BO when the recipient was B10.A versus when the recipient was C57BL/6 ($p = 0.79$). This result was not entirely unexpected, because in transplant vasculopathy, recipient versus donor strain does not make a difference between forward and reverse transplant

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combinations (24). In our experiments, these WT to WT allografts served as the baseline for comparison for the gene-null grafts implanted into WT ($CD73^{+/+}$) recipients. Although absolute BO was slightly greater when a $CD73^{-/-}$ graft was used, it was not significantly different than from experiments in which a $CD73^{+/+}$ graft was used (i.e., no significant exacerbation of airway rejection was observed when only the donor was CD73 null) (Fig. 2). These data indicate that recipient CD73 status is more critical than donor CD73 status in the pathogenesis of airway rejection.

## Quantification of graft T cell infiltration in orthotopic trachea-transplant model

To study the effect of CD73 on T cell infiltration into airway grafts, pan-T cell–labeled $CD3^+$ cells were quantified using immunohistochemically stained frozen sections. Comparisons were made between $CD73^{-/-}$ and WT allografts and WT isografts. Total $CD3^+$ cell counts were obtained from an entire section taken from the middle third of the tracheal grafts. Not surprisingly, WT airway allografts exhibited more T cell infiltration than did WT isografts (Fig. 3). The largest degree of T cell infiltration was observed in allografts implanted into CD73 gene-null recipients. There was a significant increase (66%) in the number of T cells infiltrating allografts in $CD73^{-/-}$ recipients compared with $CD73^{+/+}$ recipients. These quantitative differences closely match the degree of luminal obliteration observed in histological sections shown in Fig. 2. In fact, when individual data points were plotted for degree of luminal obliteration and quantitative T cell infiltration, there was a striking concordance, with the numbers of $CD3^+$ cells correlating directly and linearly with the exacerbation of airway luminal narrowing (Fig. 4; $p < 0.01$; $r = +0.75$).

## Intragraft expression of Th cytokines

Studies were performed to investigate the relationship between presence/absence of CD73 and expression of Th1 cytokines, which are known to upregulate intragraft inflammatory-immune events. We examined the expression of IFN-γ and IL-2 mRNA in orthotopic tracheal-transplant tissue of WT isografts and allografts, as well as $CD73^{-/-}$ allografts. IFN-γ mRNA was markedly increased (7,677-fold; $p < 0.01$) in WT allografts compared with isografts, and it was also increased (5.5-fold; $p = 0.04$) in $CD73^{-/-}$ allografts compared with WT allografts (i.e., it was increased 42,224-fold when $CD73^{-/-}$ allografts were compared with $CD73^{+/+}$ isografts; Fig. 5*A*). Similarly, the expression of IL-2 mRNA was strongly upregulated in WT allografts compared with isografts (173-fold; $p < 0.01$), and it was increased even further when $CD73^{-/-}$ allografts were compared with WT allografts or isografts (Fig. 5*B*). When a prototypical Th2 cytokine (IL-10) was examined, no difference was found between allograft recipients when CD73 was present or not (Fig. 5*C*).

## mRNA expression of adenosine receptors

The end result of CD73 enzymatic activity is the cleavage of a terminal phosphate group from AMP to form adenosine. Because adenosine binding to its own discrete receptors initiates important signaling events that can modify inflammation, we next looked at how the transplant process and milieu might affect the expression of these adenosine receptors in the transplanted airways. Quantitative PCR analysis of mRNA encoding the four known adenosine receptors was performed on isografts and WT allografts in orthotopic trachea-transplant tissue. There were no significant differences detected in the expression of A1, A2B, and A3 receptors among naive tracheas, tracheal allografts, and tracheal isografts (Fig. 6). In contrast, there was a significant upregulation (43-fold) of the A2A receptor in the WT allografts compared with isografts ($p = 0.032$).

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### Effect of adenosine receptor stimulation and inhibition

Because A2A receptor was upregulated in allografts compared with isografts, we next examined whether stimulating or blocking A2A receptor signaling would affect graft cytokine production or luminal compromise. The hypothesis driving these experiments is that adenosine/A2A receptor interaction would suppress inflammation, especially given the known anti-inflammatory role of A2A receptor (19,31). These experiments showed that expression of IFN-γ mRNA was downregulated in WT allografts treated twice daily with a potent and specific A2A receptor agonist (CGS-21680) (32,33) compared with WT allografts treated with vehicle (73% suppression of IFN-γ for CGS-21680 compared with vehicle; $p < 0.05$; Fig. 7). However, a specific A2A receptor antagonist (32,33) also given twice daily led only to a mild, but statistically insignificant, increase in IFN-γ mRNA expression (150% compared with vehicle; $p = 0.33$) (Fig. 7*A*). Similarly, and even more strikingly, the expression of IL-2 mRNA was also downregulated in WT allografts treated with CGS-21680 compared with WT allografts treated with vehicle (80% compared with vehicle; $p < 0.01$). As for the IFN-γ studies, A2A receptor antagonist treatment led to a nonsignificant increase in IL-2 mRNA expression (163% compared with vehicle; $p = 0.16$) (Fig. 7*B*).

These particular two measured cytokines represent only a small facet of the complex milieu in which an allograft resides, an environment rich with a plethora of other biological response modifiers and immune cells. To biologically assess positive and negative immune effector mechanisms in vivo, A2A stimulation and inhibition were performed in allografts, and luminopathy was assessed quantitatively. WT allografts treated with an A2A agonist exhibited significantly reduced luminal obliteration ($p = 0.035$; Fig. 8*A*) and less $CD3^+$ cell infiltration compared with allografts treated with vehicle ($p = 0.018$; Fig. 8*B*). However, A2A receptor antagonist treatment did not significantly alter graft luminal occlusion or $CD3^+$ cell infiltration (Fig. 8*B*).

To determine whether the increased luminal obliteration and T cell infiltration observed in $CD73^{-/-}$ allografts were due to a lack of adenosine formation and its downstream actions on the A2A receptor in the orthotopic model of chronic airway rejection, experiments were set up to determine whether the A2A agonist could rescue mice null for CD73. $CD73^{-/-}$ allografts treated with the A2A receptor agonist exhibited significantly less $CD3^+$ cell infiltration (79 ± 28 cells/slice) compared with allografts treated with vehicle (552 ± 58 cells/slice, $p < 0.01$; Fig. 9*A*). Furthermore, $CD73^{-/-}$ allografts treated with the A2A receptor agonist exhibited significantly reduced luminal obliteration (36 ± 3%) compared with allografts treated with vehicle (57 ± 3%) ($p = 0.009$; Fig. 9*B*). These data indicate that an A2A agonist can rescue airways implanted into $CD73^{-/-}$ mice from luminal obliteration.

### Quantification of CGS-21680 in mouse plasma by liquid chromatography-tandem mass spectrometry

Plasma levels of CGS-21680 dosed i.p. in normal mice were examined (Fig. 10). Our results indicated that plasma exposures were greater than the inhibition constant (Ki) of CGS-21680 for the A3 receptor (67 nM or 37 ng/ml) for <1 h after each dose. In contrast, plasma exposures were greater than the Ki for A2A (27 nM or 15 ng/ml) for ~2 h postdose (34). Although these data do not take into account CGS-21680 plasma-protein binding, nor do they consider the duration of the pharmacodynamic affect of receptor agonism, they are consistent with the primary effect of drug treatment being agonism of the A2A receptor. These data do not rule out a minor contribution of agonism at the A3 receptor. However, it would be very unlikely that these levels of drug exposure could affect the A1 or A2B receptors, because the Ki values for CGS-21680 are much higher (165 and 50,000 ng/ml, respectively).

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## Discussion

Lung transplantation remains the only effective therapy for a large number of patients with end-stage lung disease (5,35). Despite significant advances in immunosuppressive therapies, as well as organ preservation and surgical techniques, the overall 5-y survival rate remains at 50% (36). Outside of infections, mortality after lung transplantation results mainly from the development of chronic graft dysfunction (BO), which develops in >60% of lung transplant recipients (1–3). Although the pathogenesis of BO is multifactorial, clinical studies implicated acute rejection as one of the major causative factors leading to the development of BO (5,37), with a pro minent role for T lymphocytes in its pathogenesis (38,39). The present study examined the role of a specific purine nucleotide degradative pathway in the immune and fibrotic events that culminate in BO. These data demonstrated that disruption or absence of CD73, the extracellular enzyme that phosphohydrolyzes AMP, leads to increased airway graft luminal occlusion and T cell infiltration. These data indicated a strong protective role for native CD73 against BO.

CD73 has been implicated as an immune effector molecule, although not to our knowledge in the setting of lung or airway allotransplantation. One possible explanation for the immunosuppressive role for CD73 in the setting of airway allotransplantation is that byproducts of its catalytic action modulate immune regulatory cell function. Kobie et al. (40) showed that CD73, expressed by regulatory T (Treg) cells, suppresses proliferation of activated T effector cells by converting 5′-AMP to adenosine. It is generally agreed that Treg cells, which are $CD4^+CD25^+$ T lymphocytes, function to suppress auto- and alloimmune responses. It is interesting to note that, although defined by the presence of CD4 and CD25 on their cell surface, Treg cells themselves are known to abundantly express CD73, suggesting that its presence there is of functional significance. The development of an immune response to self- and alloantigens, as occurs in acute or chronic lung-transplant rejection, suggests that Treg cell dysfunction occurs during lung-allograft rejection. Although data are limited regarding the role of Treg cells in lung-transplant rejection, one report in humans indicated that the rejection response and BO were associated with fewer numbers of $CD4^+CD25^+$ T cells (41,42).

To compare the importance of CD73 expression on graft epithelium versus that on recipient-derived graft-infiltrating leukocytes, experiments were performed in which donor (airway graft) tissue or recipient cells alone expressed CD73. This type of experiment is readily achievable by transplanting tissue null for CD73 into $CD73^{+/+}$ WT animals or conversely, by transplanting tissue expressing CD73 into CD73-null recipients. The results demonstrated that recipient-derived $CD73^+$ lymphocytes are critical for mitigation of airway rejection, with a minor role for donor-derived CD73 epithelium. In addition to a role for transiting cells that bear CD73, there is the very likely possibility that the cytokines that they or their neighboring cells secrete have a bearing on the development of obstructive airway disease. In acute allograft rejection, depending on the cytokine environment during initial Ag encounter, T cells can differentiate into Th1 cells, which secrete primarily IFN-γ and IL-2, or Th2 cells, which secrete primarily IL-4, IL-5, and IL-10 (43). In the current study, depletion of CD73 in recipients caused an increase in Th1-dominated cytokine (IFN-γ and IL-2) mRNA expression compared with WT recipients, although no differences were observed in the type 2 cytokine (IL-10). Previous studies demonstrated upregulation of Th1 cytokines (IFN-γ and IL-2) in a rat trachea-transplant model (44,45) especially in the setting of chronic rejection. In a clinical report of a lung-transplant patient, increasing levels of IL-2 and IFN-γ transcripts were detected in transbronchial lung biopsy specimens as the patient progressed from normal histologic status to BO (46). These data are concordant with our own and suggest that the increment in these Th1 cytokines marks or mediates the effects of a lack of CD73 with respect to chronic airway rejection. Specifically, loss of CD73 is

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associated with an increase in Th1 cytokines, further implicating CD73 as a modulator of transplant rejection.

Previous data from our laboratory and from other investigators suggested that deficiency in CD73 results in diminished levels of extracellular adenosine, which reduces activation through the four adenosine receptors: A1, A2A, A2B, and A3 (47). Our initial scan of expression levels of the four adenosine receptor subtypes led us to focus on the A2A receptor, because its expression was greatly stimulated (at the mRNA level) by airway allograft transplantation. Additional experimentation suggested that its expression was functionally important to suppress the development of rejection in our airway-allotransplantation model. In other work performed in the setting of ventilator-induced lung injury, it was shown that adenosine, acting through stimulation of the A2B adenosine receptor, attenuated the pulmonary inflammatory response, edema, and overall ventilator-induced lung injury (48). Together, these data suggest that adenosine can play a critical anti-inflammatory and protective role, acting through adenosine receptors whose subtypes may vary, depending on the experimental model and the tissue under study. T lymphocytes mainly express the high-affinity A2A receptor and the low-affinity A2B receptor (47), which regulate diverse lymphocytic functions (49,50). Support for the concept that reduced adenosine contributes to the pathologic airway remodeling observed in $CD73^{-/-}$ mice comes from our studies with a specific agonist of the A2A receptor (CGS-21680). Figs. 8 and 9 show that administration of this A2A receptor agonist protects the graft from luminal occlusion and T cell infiltration in WT and $CD73^{-/-}$ recipients. Similarly, this agonist attenuated the production of IFN-γ and IL-2, as measured by quantitative PCR. These data highlight a critical role for A2A receptor activation in limiting the rejection of the airway graft, because this interferes with lymphocytic function during acute rejection and may exert anti-inflammatory effects, in part by suppressing cytokine secretion and cellular migration. These data are consistent with recent studies showing that adenosine acts via the A2A receptor to exert anti-inflammatory effects, suppressing the production of IL-12, TNF-α, and IFN-γ by monocytes and lymphocytes (19). Sevigny et al. (31) showed that A2A receptor agonist attenuates alloantigen recognition through T cells and APCs expressing A2A receptor. Thus, the effects of A2A receptor stimulation could limit direct and indirect alloimmunity and, hence, potently reduce airway rejection.

To determine the pharmacokinetics of the agonist (CGS-21680), mice were dosed identically to the transplant studies. Our results indicated that plasma exposures were greater than the $IC_{50}$ for the A3 receptor (67 nM or 37 ng/ml) for <1 h after each dose. In contrast, plasma exposures were greater than the Ki for A2A receptor (27 nM or 15 ng/ml) for ~2 h postdose. These data do not take into account CGS-21680 plasma protein binding, nor do they consider the duration of the pharmacodynamic effect of receptor agonism. With those caveats, we believe that the data are still consistent with the idea that agonism of the A2A receptor is primarily responsible for the in vivo effect we describe, with the acknowledged possibility that agonism at the A3 receptor may have contributed modestly to our results. It seems very unlikely that effects on the A2B or A1 receptors are involved in our results, because their Ki were ~50,000 and 165 ng/ml, respectively.

The data make a compelling case for adenosine generation by circulating cells being of prime importance in mitigating rejection, because CD73 deletion from bone marrow-derived cells caused a marked increase in rejection. This is in contrast to experiments in which CD73 deletion from graft tissue was without apparent affect. These data do not speak directly to whether A2A receptors on circulating or resident cells are most important in the A2A signaling response. However, one could speculate that there would likely be an important contribution from the circulating cell population if adenosine acts in proximity to its site of generation, a likely assumption given its relatively short half-life in the circulation.

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Indeed, CD73 and A2A receptor were shown to reside in lipid rafts (51,52). A recent publication confirmed that A2A receptor activation on circulating $CD4^+$ cells, rather than resident cells, mediates an anti-inflammatory phenotype in lung ischemia-reperfusion injury (53).

In this study, we showed that deletion of CD73 in transplant recipients resulted in an exacerbated immune response toward a tracheal allograft. This was manifested by increased graft luminal occlusion, T cell infiltration, and proinflammatory cytokine production. These effects are reversible with a specific agonist of the A2A adenosine receptor, consistent with an anti-inflammatory role for adenosine produced by CD73 cleavage of AMP. Although further studies are necessary to elucidate the detailed mechanistic link between CD73 and pathogenesis of acute airway rejection, the current data suggest CD73 and A2A receptor as additional therapeutic targets for lung transplantation.

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## Abbreviations used in this paper

| | |
|---|---|
| **BO** | bronchiolitis obliterans |
| **CGS-21680** | 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamidoadenosine |
| **Ki** | inhibition constant |
| **LB** | lymphocytic bronchitis |
| **Treg** | regulatory T |

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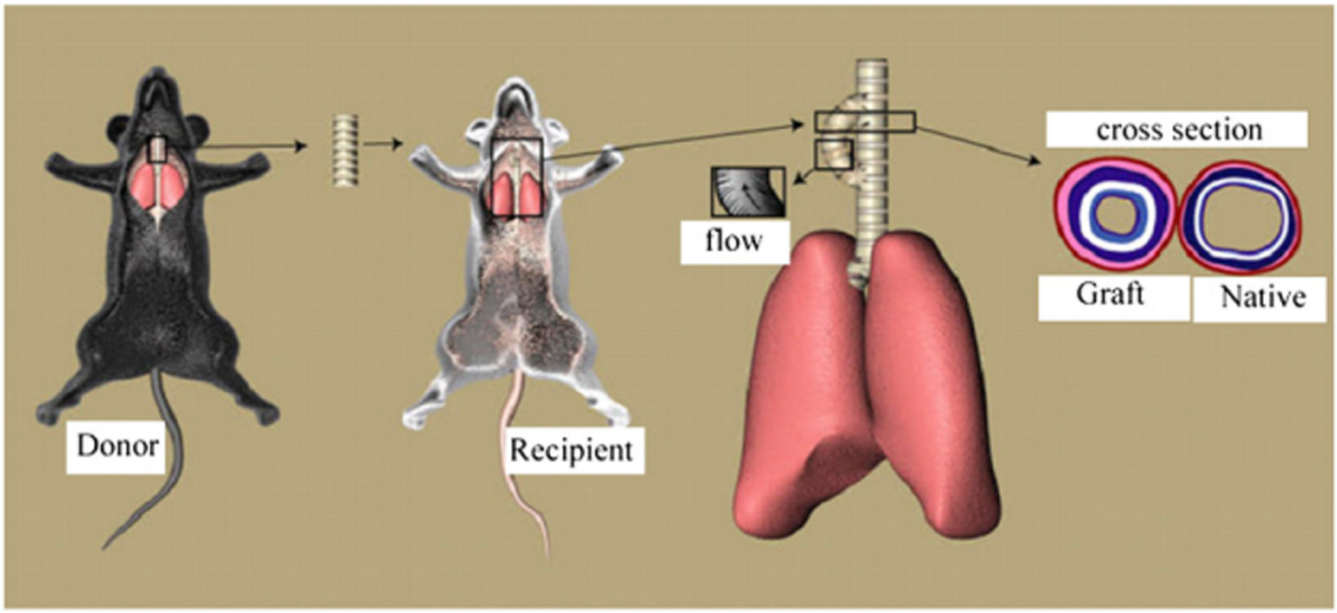


**FIGURE 1.**
Orthotopic tracheal transplantation. The tracheal graft can fulfill its function as an airway by placing the proximal and distal graft anastomoses in the native trachea. This environment allows epithelium in the graft to be exposed to air and drain mucus through the anastomosis.

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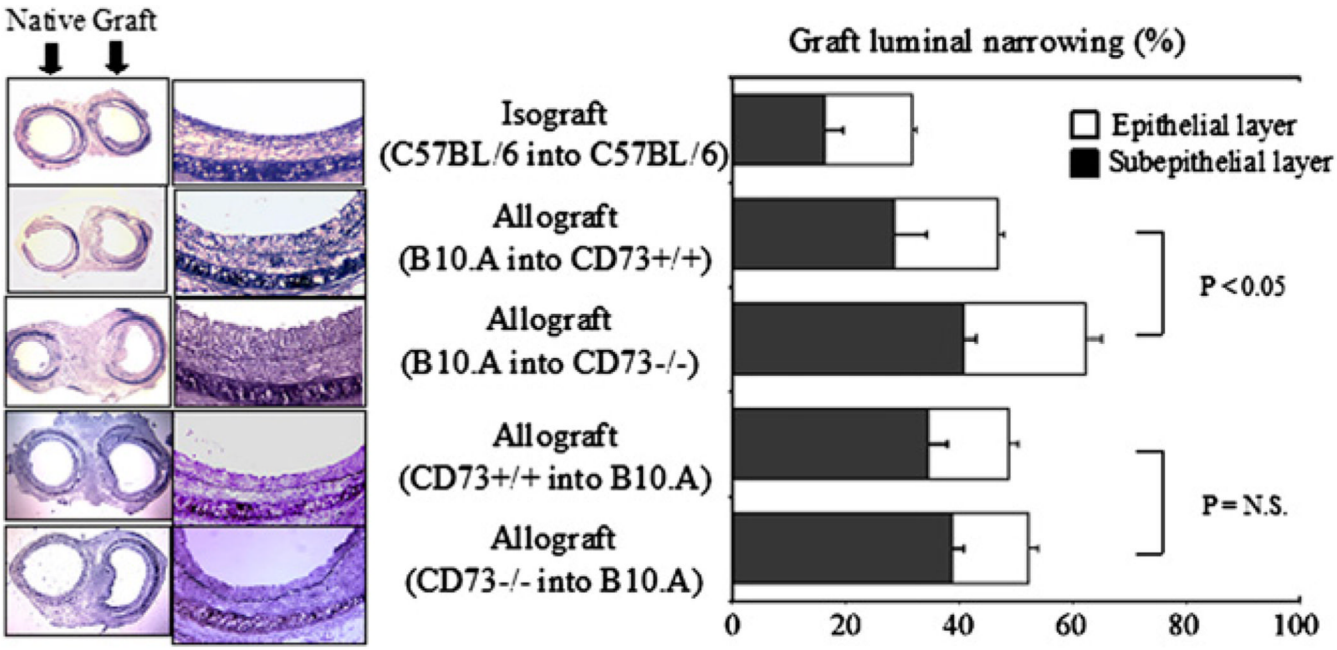


**FIGURE 2.**

Effect of CD73 on graft luminal narrowing 1 wk after surgery. *Left panels*, Representative van Gieson staining of tracheal sections for the indicated conditions 1 wk after transplantation (low magnification of graft and native trachea, original magnification ×100; high magnification of graft, original magnification ×400). *Right panel*, Graft luminal occlusion was assessed by histomorphometry 1 wk after implantation ($n$ = 4–6 each). Genotype of donors and recipients were $CD73^{+/+}$ or $CD73^{-/-}$, as indicated. C57BL/6 and B10.A are $CD73^{+/+}$.

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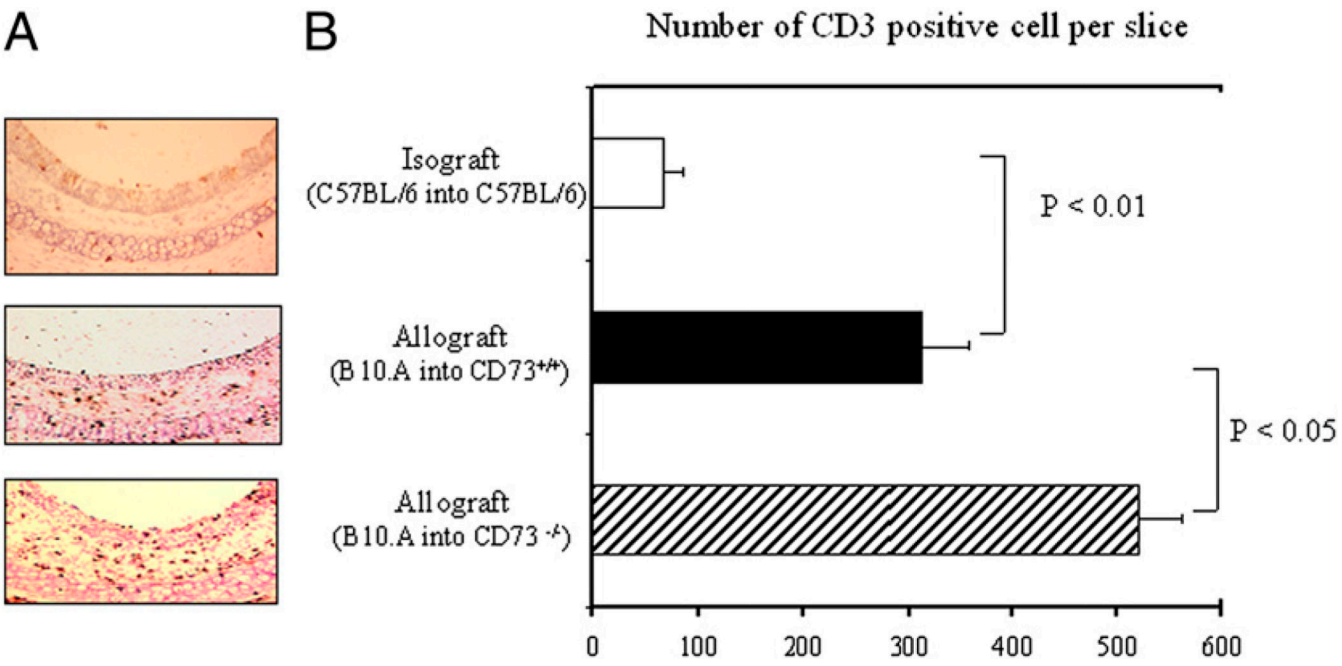


**FIGURE 3.**

Quantification of graft $CD3^+$ T cell infiltration. *A*, Representative immunohistochemical staining for the pan-T cell marker CD3 in sections of airway grafts (original magnification ×400). *B*, Quantitative analysis of T cell infiltration by counting the number of$CD3^+$ cells per slice under high-power magnification. Total $CD3^+$ cell counts were obtained for an entire section taken from the middle third of the tracheal graft. Each bar represents mean ± SEM. ($n$ = 5–6).

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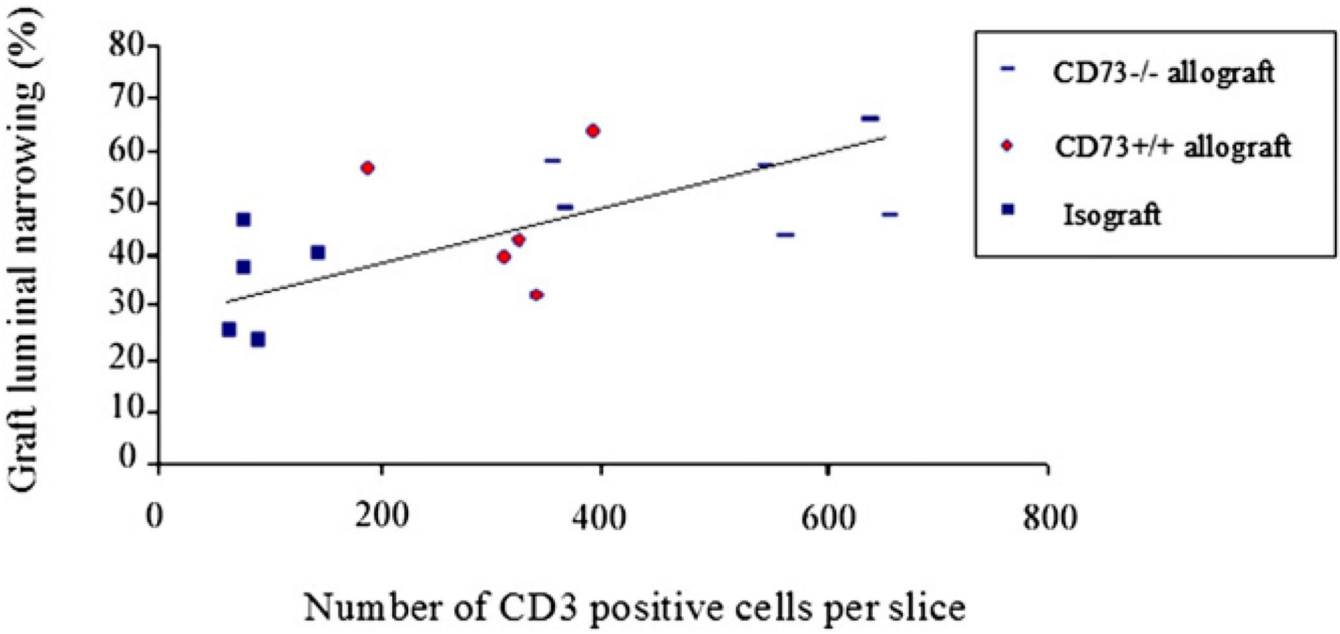


**FIGURE 4.**

Correlation between graft luminal occlusion and number of $CD3^+$ cells. The percentage of graft luminal occlusion was plotted against the number of $CD3^+$ cells per slice, counted under high-power magnification as in Fig. 3. $r = +0.75$; $p < 0.01$.

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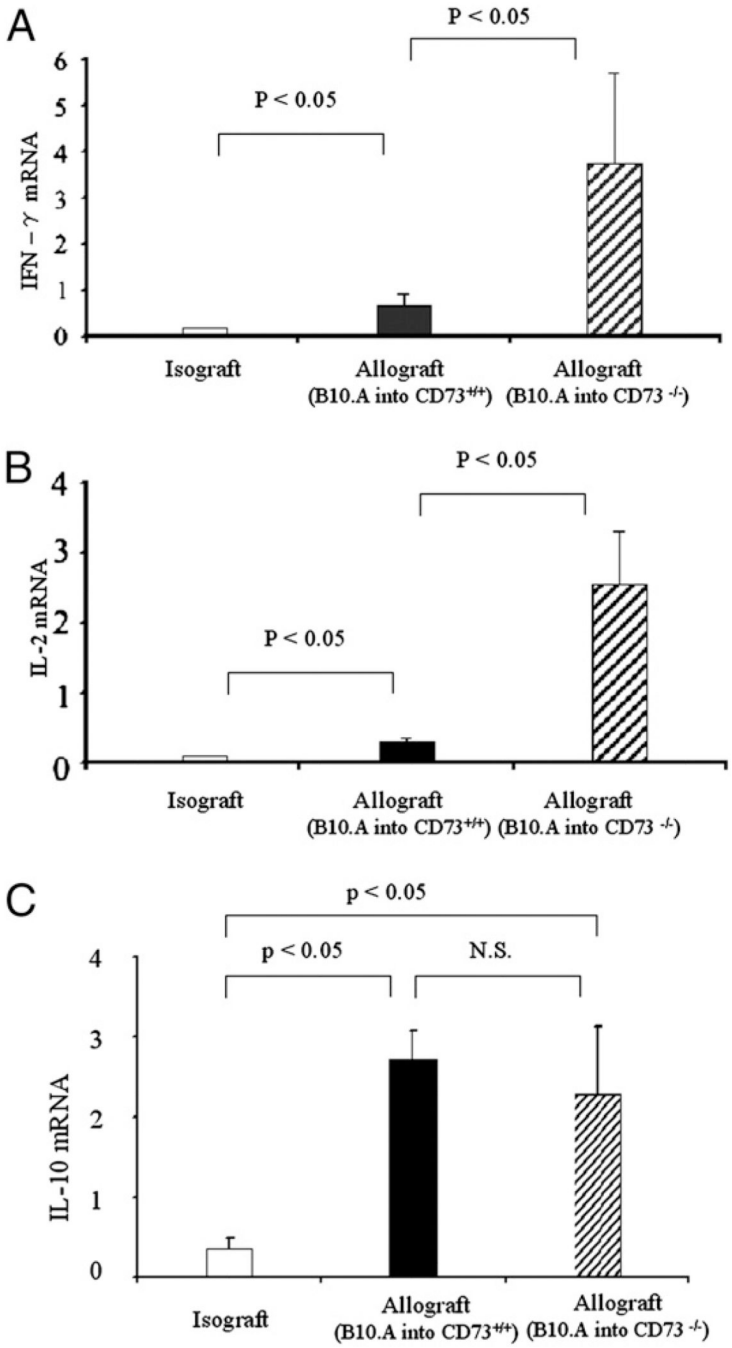


**FIGURE 5.**

Quantitative analysis of Th1- and Th2-type cytokine mRNA. IFN-γ (*A*), IL-2 (*B*), and IL-10 (*C*) extracted from trachea of isografts, WT allografts, and $CD73^{-/-}$ allografts in which the recipient lacked CD73. Data are presented as the fold induction of mRNA to β-actin; $n = 4$ transplants for each group, with mean ± SEM shown.

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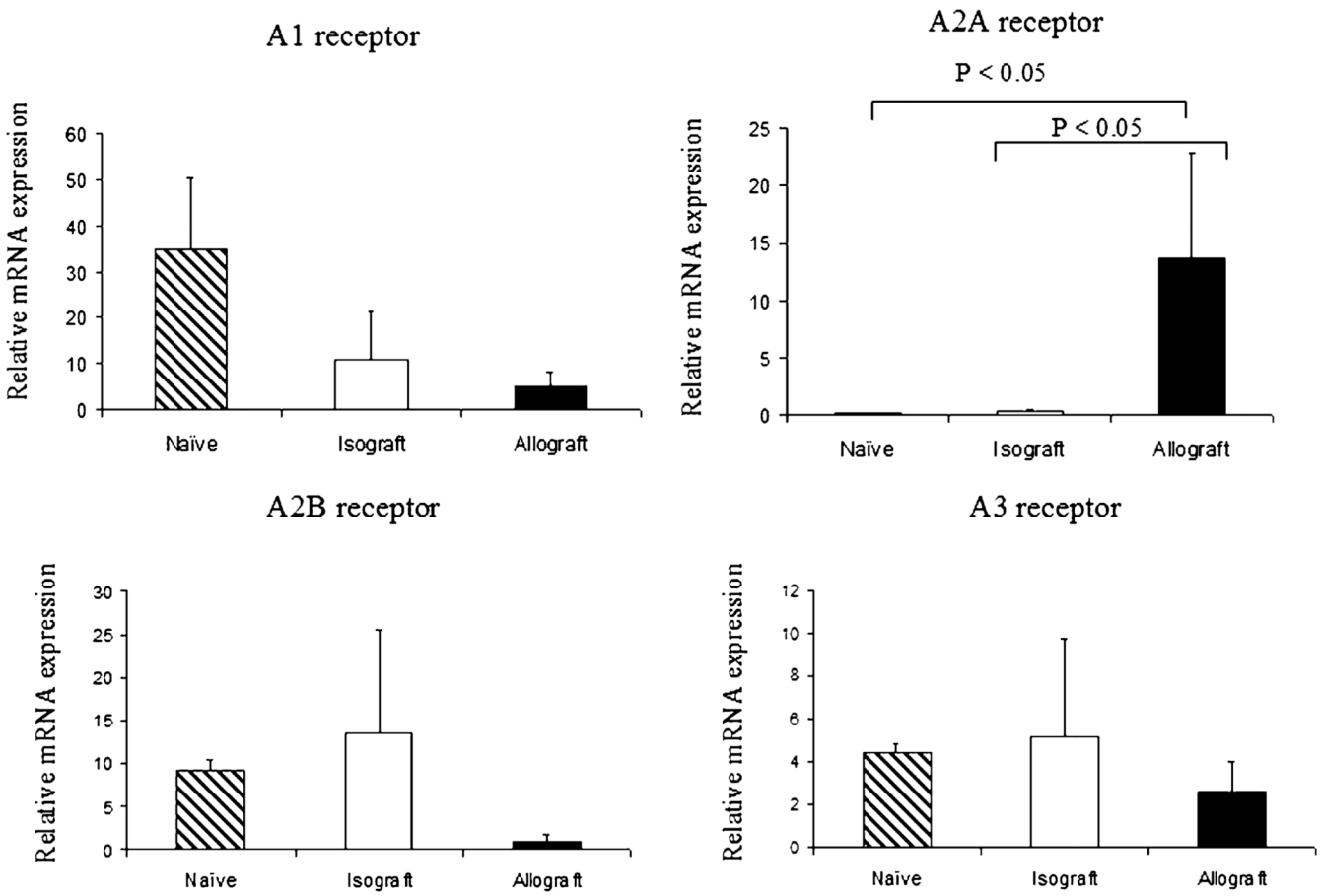


**FIGURE 6.**

Adenosine receptor subtype expression in tracheal grafts. Quantitative PCR analysis of RNA extracted from naive trachea, isografts, and WT allografts in the orthotopic trachea-transplant model. The four main adenosine receptor subtypes were examined, including A1, A2A, A2B, and A3 ($n$ = 4 transplants for each group, with mean ± SEM shown). Where groups differed significantly between allograft and isograft values, a $p$ value is indicated in the figure.

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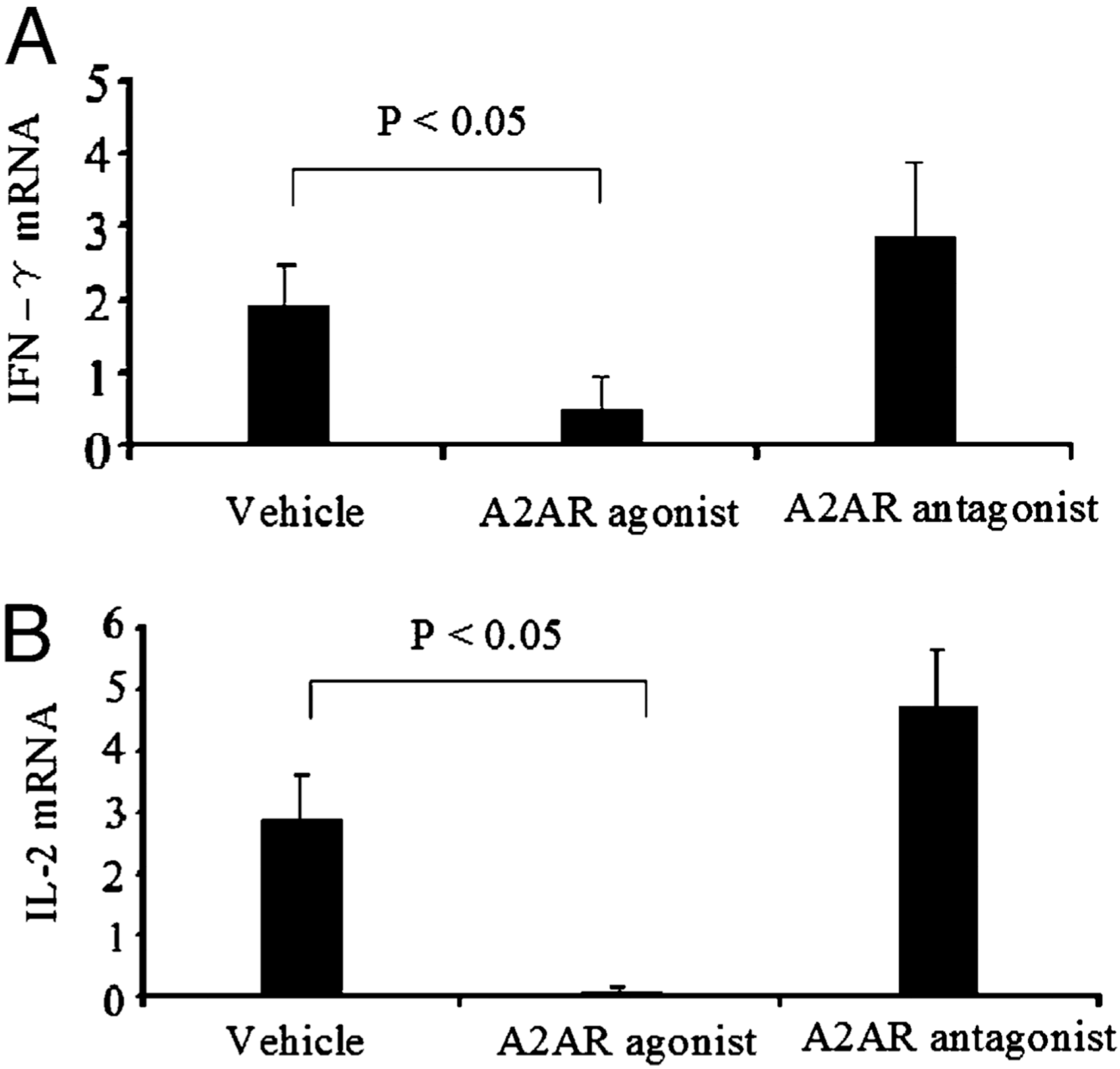


**FIGURE 7.**

Effect of A2A receptor stimulation or inhibition on expression of Th1 cytokines in tracheal grafts. The expression of IFN-γ (*A*) and IL-2 (*B*) mRNA was measured in tracheal graft tissue following twice-daily treatment with vehicle, the A2A receptor agonist CGS-21680, or the A2A receptor antagonist 5-amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo (4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine, each given at 2 mg/kg/d. $n = 4–6$ in each group; $p < 0.05$ versus vehicle.

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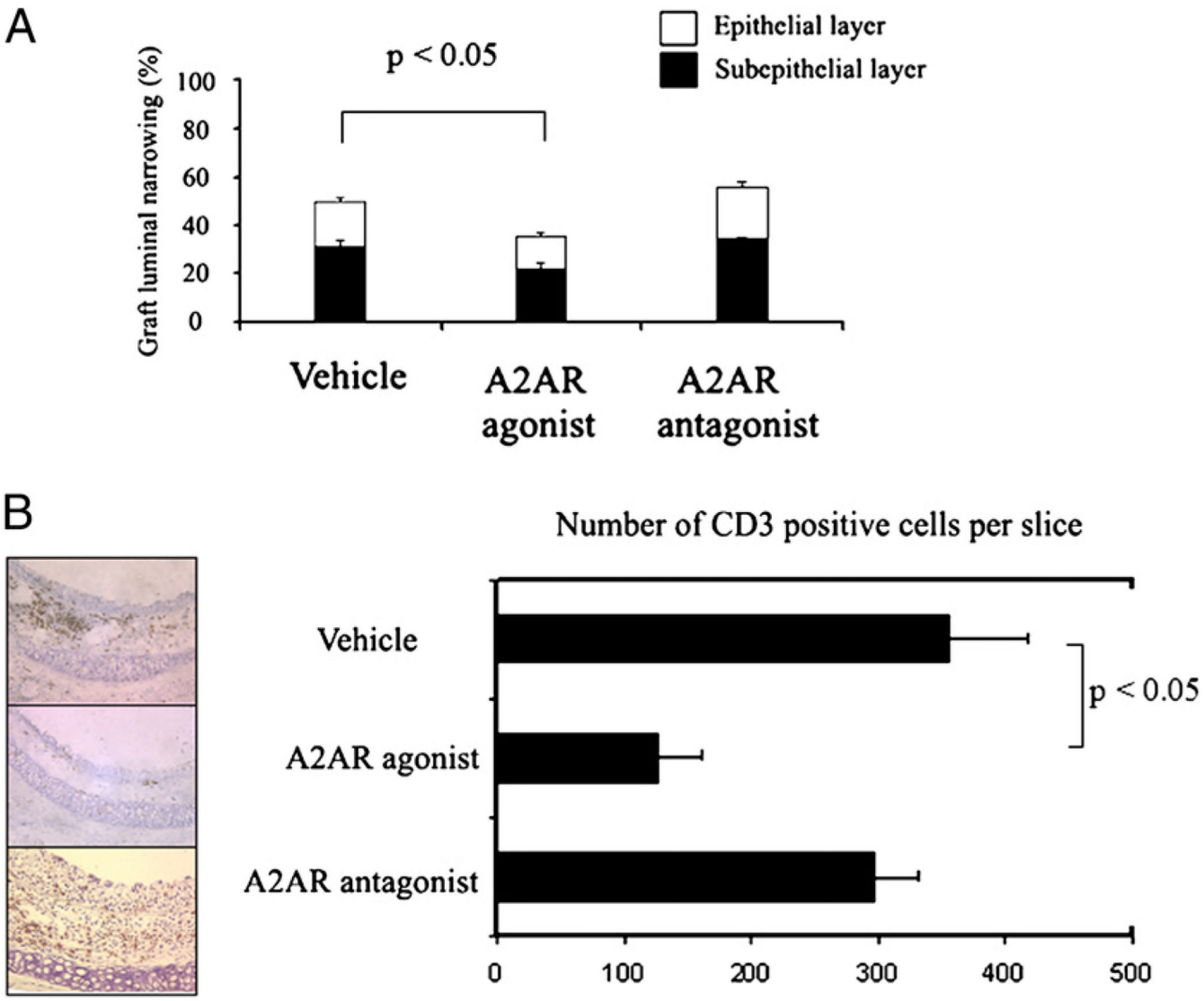


**FIGURE 8.**

Effect of A2A receptor stimulation or inhibition on graft luminal narrowing and T cell infiltration into tracheal grafts implanted into WT recipients. *A*, Graft luminal occlusion was measured after treatment with vehicle, an A2A receptor agonist, or an A2A receptor antagonist, as indicated in the legend for Fig. 7. *B*, Quantitative analysis of T cell infiltration was assessed by counting the number of $CD3^+$ cells per slice under high-power magnification. Each bar represents the mean of six experiments ± SEM. Representative immunohistochemical staining for CD3 in sections of graft for the indicated conditions (original magnification ×400) is also shown.

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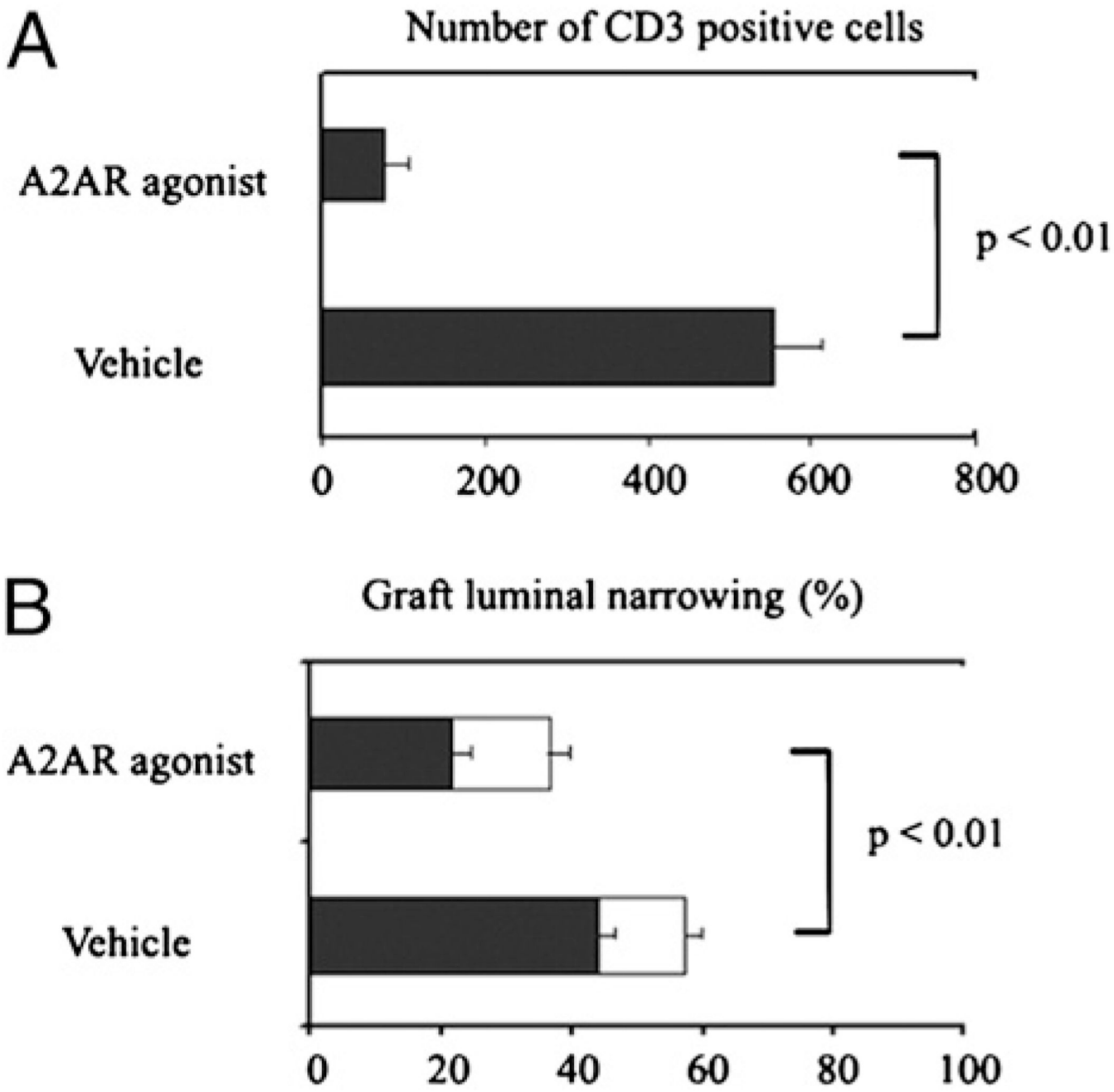


**FIGURE 9.**

Effect of A2A receptor stimulation on graft luminal narrowing and T cell infiltration into tracheal grafts implanted into $CD73^{-/-}$ recipients. *A*, Quantitative analysis of T cell infiltration was assessed by counting the number of $CD3^{+}$ cells per slice under high-power magnification. Each bar represents the mean of six experiments ± SEM. $CD73^{-/-}$ allografts treated with the A2A receptor agonist exhibited significantly less $CD3^{+}$ cell infiltration (79 ± 28 cells/slice) compared with allografts treated with vehicle (552 ± 58 cells/slice; $p < 0.01$). *B*, Graft luminal occlusion was measured after treatment with vehicle or an A2A receptor agonist, as indicated in the legend for Fig. 7. $CD73^{-/-}$ allografts treated with the

A2A receptor agonist exhibited significantly reduced luminal obliteration (36 ± 3%) compared with allografts treated with vehicle (57 ± 3%; $p = 0.009$)

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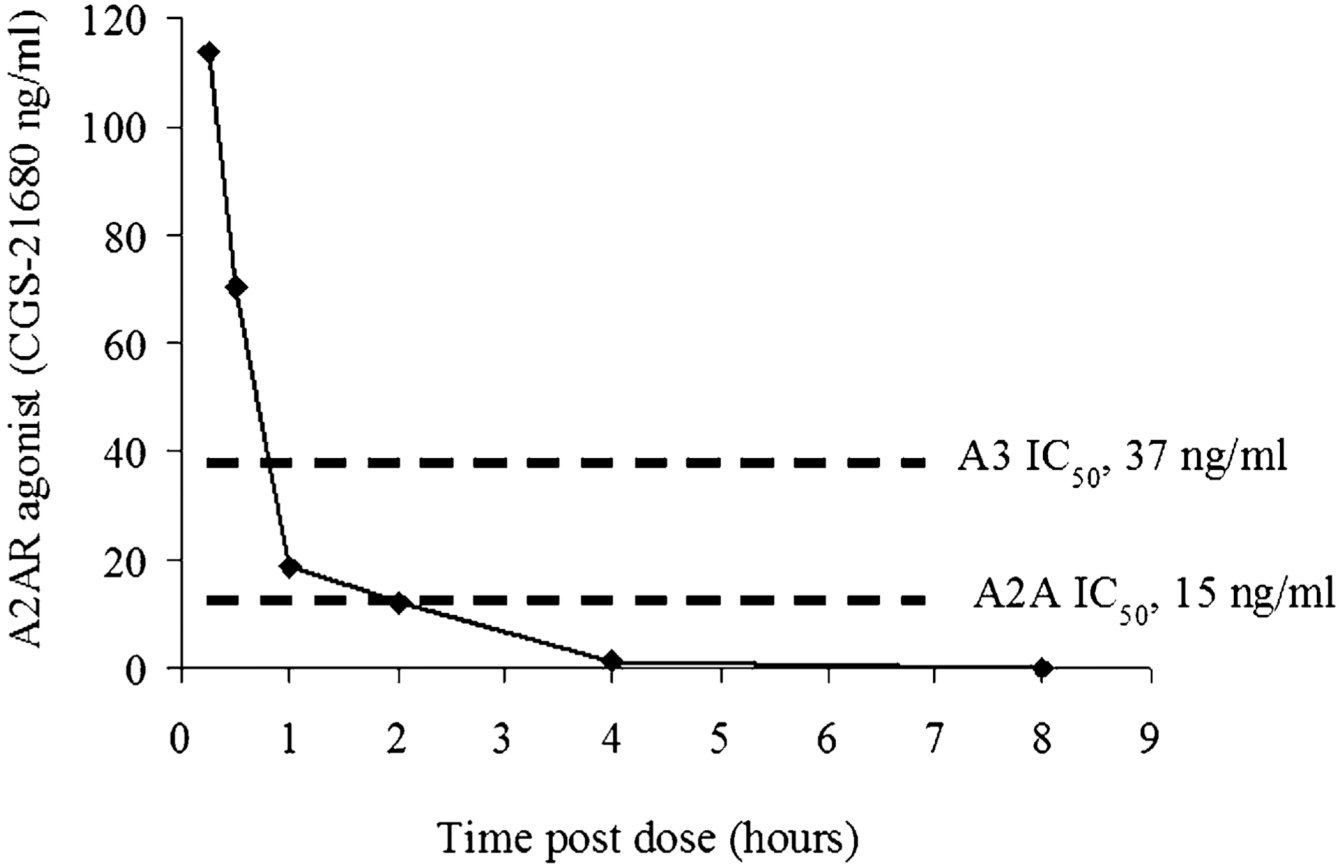


**FIGURE 10.**

Quantification of A2A receptor agonist (CGS-21680) in mouse plasma. Plasma levels of CGS-21680 were examined after i.p. dosing. Ki of CGS-21680 for the A3 and A2A receptors were 37 and 15 ng/ml, respectively. These data show that drug levels decreased to less than the Ki for the A3 receptor between 30 and 60 min postdose but remained greater than the Ki for the A2A receptor for almost 2 h.