

**Inhaled CD86 Antisense Oligonucleotide Suppresses Pulmonary Inflammation and
Airway Hyper-responsiveness in Allergic Mice**

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Abbreviations: ASO, antisense Oligonucleotide; AHR, airway hyper-responsiveness; APCs, antigen presenting cells; MHC, major histocompatibility complex; OVA, ovalbumin; 2'-MOE, 2'-o-methoxyethylribose; IL, interleukin; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff's reagent; ELISA, enzyme-linked immunosorbent assay; Penh, enhanced pause; RL, resistance; CL, compliance;

Abstract

The B7-family molecule CD86, expressed on the surface of pulmonary and thoracic lymph node antigen presenting cells, delivers essential co-stimulatory signals for T cell activation in response to inhaled allergens. CD86-CD28 signaling is involved in priming allergen-specific T cells but it is unclear whether these interactions play a role in coordinating memory Th2 cell responses. In the (OVA)-induced mouse model of asthma, administration of CD86-specific antibody before systemic sensitization suppresses inhaled OVA-induced pulmonary inflammation and airway hyper-responsiveness (AHR). In previously OVA-sensitized mice, systemic and intranasal co-administration of CD86 antibody is required to produce these effects. To directly assess the importance of pulmonary CD86 expression in secondary immune responses to inhaled allergens, mice were sensitized and locally challenged with nebulized OVA before treatment with an inhaled aerosolized CD86 antisense oligonucleotide (ASO). CD86 ASO treatment suppressed OVA-induced up-regulation of CD86 protein expression on pulmonary dendritic cells and macrophages as well as on recruited eosinophils. Suppression of CD86 protein expression correlated with decreased methacholine-induced AHR, airway inflammation, and mucus production following re-challenge with inhaled OVA. CD86 ASO treatment reduced BAL eotaxin levels but did not reduce CD86 protein on cells in the draining lymph nodes of the lung and had no effect on serum IgE levels, suggesting a local and not a systemic effect. These results demonstrate that CD86 expression on pulmonary APCs plays a vital role in regulating pulmonary secondary immune responses

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and suggest that treatment with an inhaled CD86 ASO may have utility in asthma and other chronic inflammatory lung conditions.

Introduction

T cell activation and cytokine production require two distinct signals delivered by cells acting as APC, such as dendritic cells, macrophages, B lymphocytes and eosinophils. The first signal is delivered through interaction of the T cell receptor with the antigen-loaded MHC type II complex. A second or co-stimulatory signal can be provided by various adhesion molecules, CD40, ICOSL, and members of the B7 family of molecules and is essential for full T cell activation and the avoidance of anergy. Interactions between CD80 (B7.1) and CD86 (B7.2) with their cognate ligand CD28 on T cells have been implicated as a pivotal T cell costimulatory event [reviewed in (Sharpe and Freeman, 2002)]. Upon activation, T cells express another ligand, CTLA-4, that binds B7 molecules with higher affinity than CD28 and has been reported to downregulate T cell proliferation (Walunas et al., 1994) and the production of cytokines by both Th2 and Th1 cells (Alegre et al., 1998). More recently, the ICOS/ICOSL pathway and other B7 family members have been implicated in the regulation of recently activated effector T cells [reviewed in (Greenwald et al., 2005)].

Co-stimulation of allergen-loaded T cells by pulmonary mucosal APCs is necessary for the recruitment and activation of airway inflammatory cells and the production of AHR (Nakajima et al., 1994; Krinzman et al., 1996; Keane-Myers et al., 1997; Tsuyuki et al., 1997). Recruited and resident pulmonary inflammatory cells play important roles in asthma pathogenesis through their coordinated control of the release of a variety of protein and lipid mediators that attract additional effector cells and enhance airway

smooth muscle responsiveness to bronchoconstrictive stimuli (Gundel et al., 1991; Garlisi et al., 1997). Despite the demonstrated role of co-stimulatory molecules in mediating experimental asthma, their contribution to the periodicity and persistence of asthma remains poorly understood. For example, regulatory CD4⁺CD25⁺ T cells (T reg) can prevent allergic airway inflammatory responses in experimental settings (Akbari et al., 2002; Zuany-Amorim et al., 2002), suggesting that deficits in T reg may predispose individuals to the development of allergy (Herrick and Bottomly, 2003). Yet the same co-stimulatory pathways that activate allergen-specific T cells may be required for the generation of T reg (Lohr et al., 2003). Furthermore, while it is generally accepted that memory T cells regulate the chronic allergic inflammatory response that underlies persistent asthma, whether memory T cells require co-stimulatory signals from APCs for optimal memory T cell reactivation by pulmonary allergens is not clear. An improved understanding of the relative roles of the co-stimulatory pathways in asthma pathology is therefore still needed to determine whether therapeutic strategies directed at these molecules are warranted.

Previous pharmacological and genetic studies have indicated a role for the B7/CD28 pathway (Krinzman et al., 1996; Keane-Myers et al., 1997) (Mathur et al., 1999) and particularly CD80 or CD86 (Tsuyuki et al., 1997; Keane-Myers et al., 1998; Mark et al., 1998; Haczku et al., 1999; Mark et al., 2000) in the development of allergic asthma in mice. However, the requirement of CD80 and CD86 for re-stimulation of T cells has been difficult to resolve. Studies using APCs from B7 KO mice have shown that in vitro Th2 recall responses generating IL-4 occur in the absence of B7 molecule expression

(Schweitzer and Sharpe, 1998). Further, CD80 and CD86 expression was not required for the lung inflammatory response to OVA-pulsed dendritic cells in previously sensitized mice (van Rijt et al., 2004). Other reports, however, support a role for B7 molecules in T cell reactivation responses. A co-stimulatory signal provided through CD28 was shown to be required for allergen-induced IL-5 production in bronchial biopsy tissue of atopic mild asthmatic subjects (Jaffar et al., 1999). In a separate study, these investigators found that CD28-B7 co-stimulation was required for IL-5 and IL-13 production from peripheral blood mononuclear cells but not from bronchial tissue of moderate to severe asthmatics (Lordan et al., 2001). Larche et al. found that CD86- but not CD80-specific mAb inhibited the proliferation of airway T cells from atopic asthmatics in response to allergen (Larche et al., 1998). Keane-Myers et al. also showed that CD86 antibody treatment 2 weeks after the initial OVA sensitization effectively suppressed the challenge response in a mouse asthma model (Keane-Myers et al., 1998). In this study, we utilized an aerosolized CD86 ASO to evaluate the role of CD86 in the secondary pulmonary inflammatory response to inhaled allergen. Our results suggest that inhibition of CD86 expression on pulmonary APC may be an effective therapeutic approach for the treatment of allergic or chronic lung disease.

Materials and Methods

Cell Transfection and mRNA Analysis

MH-S mouse alveolar macrophages (American Type Tissue Culture Collection, Manassas, VA) were cultured in RPMI-1640 supplemented with 10% FBS, 1mM sodium pyruvate, 50uM beta-mercaptoethanol and 10mM HEPES, pH 7 (Invitrogen, Carlsbad, CA). 2'-O-methoxyethylribose (MOE)-modified phosphorothioate oligonucleotides were synthesized and purified as described previously (Dean et al., 2001). Chimeric oligonucleotides containing nuclease-resistant 2'-MOE phosphorothioate residues flanking a 2'-deoxyribonucleotide/phosphorothioate region that supports RNase H-based cleavage of the targeted mRNA were used in all experiments. The sequences of the murine CD86 ASO and 7 base pair-mismatch control oligonucleotide are 5'-TCAAGTTTCTCTGTGCCCAA-3' and 5'-TCAAGTCCTTCCACACCCAA-3', respectively. Oligonucleotide transfections were performed. Total RNA was purified 48 hours after ASO transfection by elution into 100 µl of water using the RNeasy 96 Plate kit (Qiagen, Valencia, CA). RNA levels were quantified using the Perkin-Elmer (PE) ABI PRISM 7700 Sequence Detection System by real-time fluorescence PCR detection. The CD86 primers/probe sequences used were: forward primer, 5'-GGCCCTCCTCCTTGTGATG-3', reverse primer, 5'-CTGGGCCTGCTAGGCTGAT-3' and probe, 5'-56-FAM/TGCTCATCATTGTATGTCACAAGAAGCCG/36-TAMTph/-3'. The beta actin primer/probe sequences were: forward primer, 5'-CAAGATCATTGCTCCT CCTGAGCGCA-3', reverse primer, 5'-

GCTGATCCACATCTGCTGGAA-3', and probe, 5'-56-FAM/CAAGATCATTGCTCCTCCTGAGCGCA/36-TAMTph/-3'.

T cell Co-stimulation Assay

The effects of ASO or control oligonucleotide treatment on the T cell co-stimulatory activity of MH-S cells were assessed by co-culturing ASO-transfected MH-S cells with anti-CD3 activated primary mouse CD4⁺ T cells. MH-S cells were seeded at 6500 cells/well the day before oligonucleotide treatment (approximately 50% confluency). MH-S cells were then transfected with ASO or control oligonucleotide as described above, washed, and cultured in growth medium for forty-eight hours before Fc blockade (2 ug/mL for 10 minutes) and concomitant addition of soluble anti-CD3 ϵ antibody (Pharmingen, 100 ug/mL) and CD4⁺ primary T cells. CD4⁺ T cells were purified from the spleens of female BALB/c mice (2 to 4 months of age) by immunomagnetic bead selection as per the manufacturer (Dyna, Lake Success, NY). The cells were then separated from the beads by immunomagnetics, washed and counted. Twenty thousand CD4⁺ T cells were added per well and co-culture supernatants were evaluated for IL-2 levels by ELISA (Pharmingen, San Diego, CA) 16 hrs later.

Asthma Models and Oligonucleotide Administration

All mice used in this study were covered by a protocol approved by our Institutional Animal Care and Use Committee. A modification of the secondary challenge mouse OVA model of experimentally-induced asthma was utilized in these studies, as previously described (Taube et al., 2002). Male Balb/c mice (6-8 wks of age; Charles

River Laboratories, Wilmington, MA.) were sensitized by intraperitoneal (ip) injection (100ul) of 20ug chicken OVA (Sigma, St. Louis, MO.) emulsified in 2mg of Imject Alum (Pierce, Rockford, IL). Mice were sensitized on Day 0 and Day 14. The mice were subsequently challenged locally by inhalation (20 minutes, daily) of an aerosol generated from 1% OVA in normal saline by ultrasonic nebulization on Days 24-26. Mice were re-exposed to 1% nebulized OVA on days 66 and 67. Endpoints were measured on day 68. ASOs dissolved in saline were administered by aerosol delivery on Days 56, 59, 61, 63 and 66. ASOs were suspended in 0.9% sodium chloride (Baxter Healthcare corporation, Deerfield, IL) and delivered via inhalation using a nose-only delivery system as described previously (Silbaugh et al., 1987; Karras et al., 2007). A Lovelace nebulizer (model 01-100), was used to deliver the ASO at a flow rate of 1 L/min. The total flow rate of the delivery system was 10 L/min. The exposure chamber was equilibrated with the oligonucleotide aerosol suspension for 5 minutes before mice were placed in restraint tubes and attached to the chamber. Restrained mice were treated for a total of 10 minutes.

Determination of Inflammatory Cells in Bronchial Alveolar Lavage (BAL) Fluid

Mouse lungs were lavaged two times with 0.5 ml of PBS containing 2% FCS. BAL fluid samples were centrifuged to generate a cell pellet and a cell-free supernatant. The recovered airway cells were resuspended in PBS-2% FCS, and a cytopspin was performed. Cells were stained with Diff-Quik stain (Dade Behring, Newark, DE). Data is presented as the percent of eosinophils present in the total recovered BAL cell population. Cell-free lavage fluid was frozen and stored at -80°C until analyzed. Murine cytokine and

chemokine levels were measured using an ELISA assay as described by the supplier (Pharmingen, San Diego, CA).

Measurement of Airway Hyperresponsiveness

AHR was determined by inducing bronchoconstriction with methacholine aerosol at escalating doses (Hamelmann et al., 1997). Total pulmonary airflow in unrestrained mice was estimated using a whole body plethysmograph (Buxco Electronics). Pressure differences between a chamber containing an individual mouse and a reference chamber were used to extrapolate the enhanced pause (Penh). Penh is a dimensionless parameter that is a function of total pulmonary airflow in mice during each respiratory cycle. This parameter closely correlates with airway resistance as measured by traditional invasive techniques using ventilated mice (Hamelmann et al., 1997). For invasive lung measurements, mice were first weighed and anesthetized with ketamine (150 mg/kg) mixed with xylazine (10 mg/kg). A tracheostomy was performed and the mice were ventilated using the Flexivent system (SCIREQ, Montreal, Canada) using traditional mouse parameters. Increasing concentrations of methacholine were aerosolized using the Flexivent system with an Aeroneb lab nebulizer system, and resistance (RL) and compliance (CL) were measured. Values for all measurements were obtained using Excel software and are expressed as the mean $\pm$ SEM.

Assessment of Mucus Production

Lungs were inflated with 10% formalin overnight prior to imbedding in paraffin. Mucus cell development along the airway epithelium was assessed in paraffin imbedded tissue sections (4um) stained with periodic acid-Schiff's reagent (PAS). The number of PAS-positive airways present in each lung section was determined. Parasagittal tissue sections were analyzed by bright field microscopy, and images were collected. Images were analyzed using ImagePro Plus (Media Cybernetics, Silver Spring, MD) to derive an airway mucus index (MI) reflective of both the amount of mucus per airway and the number of airways affected (Karras et al., 2007). The mucus content of all the airways per section (proximal to distal) was measured from groups of five animals. Image ProPlus was used to quantify the area and intensity of PAS staining per airway. The data were quantified as follows: $MI = (\text{average PAS staining intensity of airway epithelium}) \times (\text{area of airway epithelium staining with PAS}) / (\text{total area of conducting airway epithelium})$. This value is determined for each airway positive for mucus and the sum of values for all positive airways is divided by the total number of airways in the lung.

Flow Cytometric Analysis of Cell Surface CD80 and CD86 Protein

Following transfection of MH-S cells with CD86 ASO or control oligonucleotides, protein expression was analyzed by immunostaining and flow cytometry. Cell surface Fc receptors were masked with Fc blocking antibody (Pharmingen, San Diego, CA) at a final concentration of 2 µg/ml for 10 minutes at room temperature. CD86 immunostaining was analyzed following addition of CD86-specific monoclonal antibody

(Pharmingen, San Diego, CA) at a final concentration of 2 μ g/mL. *Ex vivo* flow cytometric analyses of lung cells was performed following digestion with collagenase as previously reported (Karras et al., 2007). Eighty microliter aliquots of lung cells were stained with combinations of pre-mixed antibodies. Antibody combinations utilized were: 1. CD11b (FITC), GR-1 (PerCP), CD80 or CD86 (PE) 2. CD19 (FITC), CD3 (PerCP), CD80 or CD86 (PE) 3. CD83 (FITC), Mac-3 (PE), CD80 or CD86 (Biotin) 4. MHC class II (FITC), CD11c (Biotin), CD80 or CD86 (PE). The secondary antibody used was SAv-PE-Cy5. All antibodies were obtained from Pharmingen (BD Biosciences, San Jose, CA) with the exception of the CD83 antibody which was obtained from BioCarta (San Diego, CA). Samples were incubated at 4 $^{\circ}$ C for 45 min, and then washed once with PBS before secondary antibody was added at 1:200. Cells were incubated with the secondary antibody for 45 min at 4 $^{\circ}$ C, washed twice with PBS, and then resuspended in 2% paraformaldehyde in PBS and held at 4 $^{\circ}$ C until time of analysis. Samples not requiring secondary antibodies were incubated at 4 $^{\circ}$ C for 45 min, washed twice with PBS, resuspended in 2% paraformaldehyde in PBS, and kept at 4 $^{\circ}$ C until time of analysis. CD86 protein expression on total and gated populations of lung cells was analyzed using a FACScan (Becton Dickinson, Palo Alto, CA) and macrophages (CD11b $^{+}$ GR-1 $^{-}$ SSClo) and eosinophils (CD11b $^{+}$ GR-1lo) were grouped together, as described previously (Karras et al., 2007).

Statistics

Analysis of differences between entire dose response curves in Penh response and the invasive resistance and compliance measurements, were performed using two-way repeated measures ANOVA using JMP statistical discovery software (SAS, NC, USA). Analysis of group differences in all other endpoints was performed using either a Student's T-test or Dunnett's test, as indicated in each figure legend.

Results

In vitro Characterization of CD86 ASO and Inhibition of CD86-mediated T cell Co-stimulatory Activity by CD86 ASO in MH-S Alveolar Macrophage Cells

The CD86 ASO used in these studies was characterized in transfected mouse MH-S cells, including evaluation of potency, specificity, and mechanism of action. The data presented in Fig 1A and 1B demonstrate dose-dependent reduction of CD86 mRNA and protein expression in MH-S cells following transfection with the CD86 ASO, as determined by TaqMan quantitative RT-PCR and immunostaining followed by flow cytometry, respectively. The IC₅₀ for CD86 protein reduction in MH-S cells was approximately 1 nM. CD86 RNA and protein were maximally reduced at the highest ASO concentration tested (10 nM). MH-S cells were also transfected with a series of oligonucleotides containing either one, three, five, or seven base mismatches to the CD86 ASO sequence, and target mRNA reduction was evaluated in these treated cells (Fig. 1A; see Table 1 for sequences). Only the one base mismatched oligonucleotide reduced CD86 mRNA expression, and no effect of the 3 base mismatch oligonucleotide was confirmed in the CD86 protein analysis (Fig 1B). These results provide evidence that a hybridization-based effect was responsible for the activity of the parent CD86 oligonucleotide, consistent with an antisense mechanism of action. The CD86 ASO was specific for CD86, as no effect on the related CD80 mRNA or protein was observed (data not shown and Fig. 1B).

The effect of the CD86 ASO on inhibition of APC co-stimulatory activity was also evaluated. IL-2 production in co-cultures of ASO-transfected MH-S cells with anti-CD3 activated primary CD4⁺ T lymphocytes was used as a surrogate of a functional co-stimulatory signal. T cells stimulated with anti-CD3 antibody produced a high level of IL-2 (300-800 pg/ml culture supernatant) following incubation for 16 hours with plate-bound MH-S cells. In the absence of anti-CD3 or MH-S cells, no IL-2 was detected (Fig. 1C and data not shown). Treatment of the cells with a CD86 blocking antibody inhibited IL-2 production in a concentration-dependent manner, confirming the importance of CD86 signaling in the co-stimulation of primary CD4⁺ T cells (Fig. 1C). Transfection of MH-S cells with the CD86 ASO prior to the addition of T cells suppressed IL-2 production in a dose-dependent manner while transfection with a three base mismatch control oligonucleotide had no effect (Fig. 1C). The magnitude of functional inhibition correlated with the level of reduction of CD86 protein expression on the MH-S cell surface (compare Fig. 1B and C), indicating that the CD86 ASO effect was antisense-mediated.

Pharmacokinetic Characterization of Aerosolized ASO Exposures in Mice

Nebulization of ASO in simple saline formulations produced aerosol particles in the respirable range (Karras et al., 2007). Following nose-only inhalation of the CD86 ASO, the concentration of CD86 oligonucleotide in mouse lung tissue was quantified using a hybridization-based ELISA. Concentrations of the CD86 ASO in lungs increased with dose following aerosol inhalation; this increase was proportional to dose as dose

increased from 0.6 to 5 mcg/kg but was smaller than proportional to dose as dose increased from 5 to 60 mcg/kg, suggesting possible saturation in tissue uptake. Specifically, lung concentrations of 15, 131, and 425 ng oligonucleotide/g tissue were detected on Day 10 for doses of 0.6, 5, and 60 mcg/kg, respectively (5 doses administered over 10 days).

Kinetic analyses indicated that the inhaled mouse CD86 MOE-modified oligonucleotide was cleared slowly from mouse lung. Tissue half-life was determined to be 4.1 days following the administration of a single dose of 100 mcg oligonucleotide/kg (Karras et al., 2007), supporting an infrequent treatment regimen in mouse pharmacology studies. Based on these observations, we exposed mice to CD86 ASO inhalation three times per week for a total of five doses and evaluated target protein reduction and pharmacological responses to allergen challenge.

Inhalation of aerosolized CD86 ASO reduces CD86 protein expression on alveolar macrophages, dendritic cells, and eosinophils in allergen-challenged mice

CD86 is expressed constitutively at low levels on pulmonary macrophages, dendritic cells, eosinophils and T cells in mice (Inaba et al., 1994). In response to inhaled allergen exposure, resident pulmonary dendritic cells (Huh et al., 2003), as well as recruited eosinophils (MacKenzie et al., 2001), transiently up-regulate CD86 expression. To characterize the kinetics of CD86 expression in response to OVA challenge, lungs were removed on various days and the level of CD86 expression on individual cell types was

determined using multi-color flow cytometry. Peak expression of CD86 was observed on lung dendritic cells, macrophages, and eosinophils 24 hours after the first local OVA challenge (data not shown). This time point was selected for evaluation of CD86 ASO pharmacodynamic activity in lung cells. CD86 ASO was administered to BALB/c mice previously sensitized to OVA at a dose of 10 or 0.3 mcg oligonucleotide/kg/day (estimated inhaled dose) for a total of five treatments over a period of 10 days. Lungs were removed and treated with collagenase to generate single cell suspensions. Subsequent FACS analysis demonstrated that the mixed eosinophil and macrophage population had the highest percentage of CD86-positive cells following allergen challenge (Fig. 2A). Treatment with the CD86 ASO but not the mismatched control oligonucleotide reduced the percentage of CD86-positive cells in this mixed macrophage/eosinophil cell population. Similarly, the percentage of CD86-positive dendritic cells was also reduced following inhalation of aerosolized CD86 ASO. The mean fluorescence intensity (MFI) of CD86 expression in eosinophils/macrophages and dendritic cells was also significantly reduced in CD86 ASO- but not mismatch control oligonucleotide-treated mice (Fig. 2B). CD80 expression on lung cells was determined in parallel. Neither CD86 ASO nor mismatched control oligonucleotide treatment significantly altered the percentage of CD80 positive cells in any of dose groups (Fig. 2C) or the MFI (Fig. 2D), indicating that CD86 ASO treatment specifically reduced CD86 levels on relevant APCs in the mouse lung. These results are similar to previously published results using an inhaled IL-4R α ASO in a mouse asthma model (Karras et al., 2007). Although CD86 can be expressed on activated T Cells, we have not observed

downregulation of T cell-specific target genes following ASO inhalation in mice, suggesting that lymphocyte CD86 expression is not likely to be affected.

Inhaled CD86 ASO Suppresses Airway Hyperresponsiveness, Airway Eosinophilia and Mucus Production in Previously Allergen Sensitized and Challenged Mice

To determine the pharmacological effects of pulmonary CD86 inhibition, we treated mice previously rendered allergic to nebulized OVA with aerosolized CD86 ASO. Vehicle treated, allergen re-challenged mice demonstrated significant methacholine (Mch)-induced enhanced pause (Penh) compared to naïve mice (Figure 3A). Treatment with 10 or 100ug/kg CD86 ASO abrogated the Penh response to Mch in OVA re-challenged mice. Aerosol delivery of a seven base mismatch control oligonucleotide at doses identical to those of the CD86 ASO had no effect on the Penh response. In separate experiments in OVA re-challenged mice, inhaled CD86 ASO reduced airway resistance (RL) and improved lung compliance (CL) in anaesthetized, tracheotomized, intubated, and mechanically ventilated mice, in the absence of similar effects by the mismatched control oligonucleotide (Fig. 3B & C). These results demonstrate that CD86 signaling is necessary for airway hyper-reactivity following allergen re-exposure.

Administration of aerosolized CD86 ASO reduced airway eosinophilia (100 mcg/kg dose) (Fig. 4) and demonstrated a non-statistically significant trend toward reduction of lymphocytes and neutrophils (Data not shown). Treatment with the mismatched control

oligonucleotide did not reduce the percentage of eosinophils, lymphocytes or neutrophils recovered from the airways. A second CD86 ASO of similar *in vitro* potency targeted to a different region of the CD86 mRNA was found to be a potent inhibitor of both OVA-induced Penh and airway eosinophilia responses following aerosol exposure in mice (data not shown). These results support a target-directed antisense mechanism of action.

BALF levels of IL-5 and IL-13 in OVA re-challenged mice were increased compared to the levels observed in mice exposed to a first set of local allergen challenges (Table 2), indicative of a secondary or recall T lymphocyte response. CD86 ASO treatment, however, did not decrease the levels of IL-5 or IL-13 in the BALF in re-challenged mice (Table 2). In contrast, BALF levels of Eotaxin (CCL11) and RANTES (CCL5) were significantly reduced by aerosol delivery of CD86 ASO while the MM control showed a slight, but not statistically significant, reduction on Eotaxin, but not RANTES levels (Table 2). This result suggests that down-regulation of CD86 expression is not sufficient to suppress elaboration of Th2 cytokines in the airways of allergic mice and implies that chemokine expression is regulated by CD86 during allergic pulmonary inflammation.

Local OVA challenge in allergic mice induces inflammatory cell recruitment to the lung parenchyma, goblet cell metaplasia and the production of mucus (Fig. 5, upper left panel). Bronchus-associated lymphoid tissue (BALT) was also apparent after allergen challenge. Treatment with inhaled CD86 ASO (10 and 100 ug/kg) but not the mismatched control oligonucleotide significantly reduced the PAS-positive staining

compared to the vehicle-treated group (Fig. 5; compare middle and lower panels with upper left panel). CD86 treated mice also displayed reduced amounts of airway mucus in compared to mismatch oligonucleotide treated counterparts, as determined by digital image quantitation of PAS-stained sections (Fig. 5B). These data suggest that CD86-induced T cell activation contributes to mucus overproduction in OVA-challenged allergic mice.

Discussion

In this study, we show that pulmonary CD86 expression is necessary to support the secondary immune response to pulmonary allergen exposure, manifested as increased inflammation and airway hyperresponsiveness. Previous studies have employed systemic administration of CD80- and CD86-antibodies, CTLA4-Ig, and B7- or CD28-deficient mice to examine the role of CD86 in the pulmonary response to inhaled allergens. Each of these approaches verified that systemic inhibition or deletion of CD86 attenuated T cell priming by allergen (Krinzman et al., 1996, Keane-Myers, 1997 #5958; Mark et al., 1998; Haczku et al., 1999; Mathur et al., 1999)]. Intranasal administration of CD86 antibody also blocked the induction of the primary lung mucosal immune response (Tsuyuki et al., 1997). Other investigators studying systemically administered CD80 or CD86 antibodies in experimental models have either failed to find an effect (Haczku et al., 1999) or have only observed a partial reduction (Mathur et al., 1999) of the secondary response to allergen. In contrast, a study using combined systemic and intranasal CD86 mAb exposure demonstrated marked suppression of the recall immune response to inhaled allergen (Keane-Myers et al., 1998). Here we demonstrate that inhaled CD86 ASO specifically inhibits the expression of CD86 cell-surface protein in multiple APCs in the mouse lung, including dendritic cells, macrophages, and eosinophils. Local inhibition of CD86 expression in OVA-sensitized and previously challenged mice suppressed allergen-induced airway inflammatory cell influx, AHR and the overproduction of mucus. Our data demonstrate that pulmonary expression of CD86 is necessary for co-stimulation of secondary immune responses to inhaled allergens and

suggest that targeting pulmonary CD86 is sufficient to attenuate allergen-induced experimental asthma.

Inhalation of an aerosolized CD86 ASO results in potent and specific downregulation of CD86 protein expression on lung APCs. Pharmacokinetic analyses of CD86 ASO levels in mice following inhalation of aerosolized oligonucleotide solution showed that systemic absorption is low, on the order of 1% of the tissue concentration in the lung (unpublished observations). In *Cynomolgus* monkeys, after three administrations of aerosolized CD86 ASO via intubation directly into the airways at the top of the bronchial tree, 0.02% of the delivered dose was recovered in peribronchial lymph nodes and 0.2% was recovered in kidney, with no measurable oligonucleotide detected in liver. Immunohistochemical staining of oligonucleotide following either intratracheal instillation or aerosol inhalation revealed oligonucleotide distributed throughout the large and small airways and associated with alveolar macrophages and lung alveolar epithelium (Templin et al., 2000). An increase in the numbers of CD86⁺ DCs in the mediastinal lymph nodes was noted following successive OVA challenges. Inhalation of CD86 ASO prior to OVA challenge had no effect on the level of CD86 protein expressed on DCs from the mediastinal lymph node (unpublished observations). These data suggest that inhibition of CD86 in lung APCs is sufficient to suppress the secondary pulmonary immune response to allergen.

We observed pharmacological effects on allergen-induced airway cell infiltration, mucus production, and AHR in allergic mice despite no measurable decrease in Th2 cytokines in

the BALF following inhaled CD86 ASO treatment. Other studies support B7-dependent immunosuppression in the absence of Th2 cytokine reduction. Schweitzer and Sharpe showed that re-stimulation of previously activated T cells with CD80/CD86 -/- APCs inhibited proliferation and IL-2 production without affecting IL-4 secretion (Schweitzer and Sharpe, 1998). *In vivo* studies in an intestinal nematode parasite model demonstrated that worm-induced increases in effector and memory Th2 cell IL-4 production are unabated in B7-deficient mice while humoral immunity is inhibited (Ekkens et al., 2002). Lordan et al. showed that CD28/B7 blockade inhibited allergen-induced production of IL-5 and IL-13 by PBMC collected from asthma patients. However in bronchial explants, allergen re-stimulation only induced IL-5 expression, which was not affected by CD28/B7 inhibition (Lordan et al., 2001). The authors suggest these divergent results demonstrate the importance of the tissue microenvironment in determining the results of pharmacologic intervention. The roles of eotaxin and RANTES are less understood in asthma but are believed in part to mediate attraction of eosinophils and lymphocytes, respectively, to the lung following allergen exposure. This effect is consistent with the observed decrease in eosinophils and lymphocytes in mice that inhaled CD86 ASO (Fig. 4 and data not shown). Caron et al. (Caron et al., 2001) suggest that resident DC may participate in local re-stimulation of memory T-cells. This was supported by the fact that histamine-activated DC produce chemokines involved in memory T cell recruitment. We postulate that inhibiting CD86 reduces the ability of resident lung DCs to stimulate T-cell production of chemokines.

Our results differ from those of van Rijt and colleagues showing that OVA-pulsed CD80/CD86 $-/-$ mouse DCs produced Th2 pulmonary immune reactivation responses that were similar to those induced by wild type DCs upon intratracheal injection (van Rijt et al., 2004). Partial downregulation of CD86 on DCs following antisense treatment may favor a different series of cell-cell interactions than germline deletion. In addition, the presence of intact CD80 expression in our model could additionally alter the B7 family interaction dynamics. Our results may also be explained by participation of other non-DC APCs, such as alveolar macrophages and eosinophils, in the reactivation response. Recently, the generation of eosinophil-deficient mice has demonstrated that these cells are required for some of the observed allergen-induced experimental pulmonary pathology (Lee et al., 2004). Previous work had shown that release of the major eosinophil proteins, MBP and EPO, was not sufficient to produce the asthmatic phenotype in mice (Denzler et al., 2000), suggesting that the antigen presenting capacity of eosinophils (Shi et al., 2000) may play an important role in the development of pulmonary inflammation. We therefore speculate that the down-regulation of CD86 expression on infiltrating eosinophils, in addition to alveolar macrophages and dendritic cells, following the initial nebulized OVA challenge may result in reduced pulmonary inflammation by blockade of APC activity and/or interference with eosinophil activation and effector function.

The oligonucleotides used in this study did not contain any of the reported murine immunostimulatory sequence motifs (Krieg et al., 1995), including the well-documented CpG dinucleotide motif that has been shown to activate Th1 immune responses *in vivo*

and prevent allergen-induced inflammation (Krieg et al., 1995). Oligonucleotide treatment was well-tolerated in OVA-challenged mice. In ASO treated animals, no increases in airway granulocytes or lymphocytes above and beyond that induced by allergen exposure were observed, and no increases in Th1 cytokines (IFN- γ or IL-12) were detected in BAL fluid. ASO treatment produced no change in the baseline airway response to methacholine in allergen-sensitized mice and no structural changes in lung tissue, or alterations in liver or spleen weights or serum transaminase levels (unpublished observations).

There remains a significant need for improved control of symptoms and disease exacerbations in moderate and severe asthma. Inhaled ASOs have the potential to regulate transcription of individual molecular targets in lung tissue in a highly specific manner (Nyce and Metzger, 1997) and to target molecules that cannot be effectively inhibited by conventional small molecule therapies. We have shown that pulmonary CD86 expression is sufficient to support the secondary immune response to allergen provocation in a mouse model of asthma. The contribution of B7 co-stimulation to activation of mucosal T cell responses in human asthma however, remains unclear. Elucidation of the relative role of CD86 in regulating chronic pulmonary inflammation and AHR will require studies in a chronic mouse model of asthma. Our results demonstrate that inhalation of second-generation chemically-modified antisense oligonucleotides can effectively diminish expression of targeted proteins in cells with antigen presenting function in the lung while largely avoiding systemic exposure and thus highlight the potential of this approach in the clinical setting.

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Footnotes

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Legends for Figures

FIGURE 1. Specific and hybridization-dependent reduction of CD86 expression and T cell co-stimulatory activity in MH-S alveolar macrophage cells following transfection with a murine CD86 ASO. (A) Evaluation of sequence-specific CD86 target RNA reduction in MH-S cells by quantitative RT-PCR 24 hours following transfection with CD86 ASO or 1, 3, 5, or 7 base pair mismatch (MM) control oligonucleotides. (B) Analysis of CD80 and CD86 cell surface protein levels in MH-S cells transfected with, CD86 ASO or a 3 base mismatch control oligonucleotide, as assayed 24 hours later by flow cytometry. (C) Inhibition of the T cell co-stimulatory activity of MH-S cells following transfection with a murine CD86 ASO or treatment with a CD86-specific monoclonal antibody. IL-2 was quantified by ELISA using supernatants recovered after 16 hours of co-culture of MH-S cells with anti-CD3 activated primary CD4⁺ T cells. The data is presented as the mean $\pm$ SD and is representative of duplicate (A and B) or triplicate (C) experiments with four replicate values for each data point. *, statistically significant ($p < 0.05$) compared with control (Dunnett's test).

FIGURE 2. Reduction of CD86 protein levels in lung antigen presenting cells from OVA-challenged mice following inhalation of a CD86 ASO but not a 7 base mismatch (MM) control oligonucleotide. Lungs were harvested 6 hours following a second local OVA challenge and cells were recovered after collagenase treatment of the tissue and analyzed by flow cytometry. The level of CD86 expression was quantitated by percent CD86 positive cells (A) and by mean fluorescence intensity (MFI) (B) either on total

cells, lung granulocytes (CD11b-positive, GR-1 high), macrophages and eosinophils (CD11b-positive, GR-1 negative or low), and dendritic cells (CD11c-positive, MHC class II-positive). Cells were isolated from vehicle-treated naïve mice or OVA-challenged mice treated with vehicle, an inhaled CD86 ASO or a 7 base mismatch control oligonucleotide. (C, D), Lack of inhibition of CD80 protein levels on total cells, lung granulocytes, macrophages, eosinophils and dendritic cells following inhalation of CD86 ASO. Data is expressed as the group mean percent CD86-positive cells or MFI +/- SE, N=4/group. * $p < 0.05$ for groups compared to vehicle using Dunnett's-test.

FIGURE 3. CD86 is required for allergen-mediated AHR in previously sensitized and challenged mice. OVA sensitized and challenged mice were rested for one month before treatment with aerosolized CD86 ASO or a 7-base mismatch (MM) control oligonucleotide (Day 56, 59, 61, 63 and 66) and subsequent re-challenge with nebulized OVA (day 66 and 67). (A), AHR data is collected on day 68 and presented as Penh values [percent vehicle (saline) control] measured in treated mice exposed to methacholine (100 mg/mL; similar effects were observed at lower concentrations of methacholine; data not shown). (B, C) Measures of airway resistance (RL) and lung compliance (CL) in naïve or allergen challenged mice treated with CD86 ASO (100 mcg/kg), control oligonucleotide (100 mcg/kg), or saline (vehicle) and evaluated following anaesthesia, tracheostomy, intubation, and mechanical ventilation, using a Flexivent apparatus. Data is presented as group means +/- SE, N=10/group except RL and CL, where N=4-6/group. * $p < 0.05$ for groups compared to vehicle using 2-way

ANOVA and Dunnett's test. For B and C, comparisons were made using area under the curve analysis.

FIGURE 4. Allergen-induced airway recruitment of eosinophils is inhibited by treatment with an inhaled CD86 ASO but not a mismatched control oligonucleotide. Airway cells were recovered by BAL on Day 68 of the re-challenge protocol and evaluated by cytospin and differential cell count analysis. Kinetic studies demonstrated peak eosinophil recruitment to the airways at this respective time point in the re-challenge model (data not shown). Data is presented as group mean % cell type $\pm$ SE, N=16/group. * $p < 0.05$ for groups compared to vehicle using Dunnett's-test.

FIGURE 5. CD86 ASO treatment reduces mucus production in the airways of OVA-challenged mice. Lungs were inflated with formalin, and removed on day 68 of the protocol after the mice had been re-challenged with OVA on days 66 and 67. Mucus expression was visualized by staining of formalin-fixed, paraffin-embedded lung sections with periodic acid-Schiff's (PAS) stain. Representative sections from vehicle, naïve, CD86 ASO or MM control oligonucleotide treated mice are shown (A). Mucus production in all groups was quantitated by digital image analysis and shown in (B). Mucus production analyses were carried out in separate groups of mice from those used for other endpoints. * $p < 0.05$ for groups compared to vehicle using Dunnett's-test.

Table I. **Antisense and control oligonucleotide sequences.**

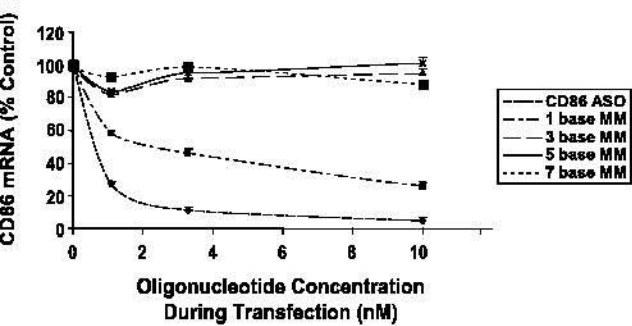
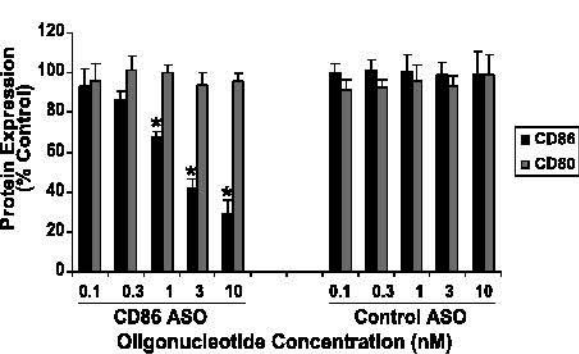
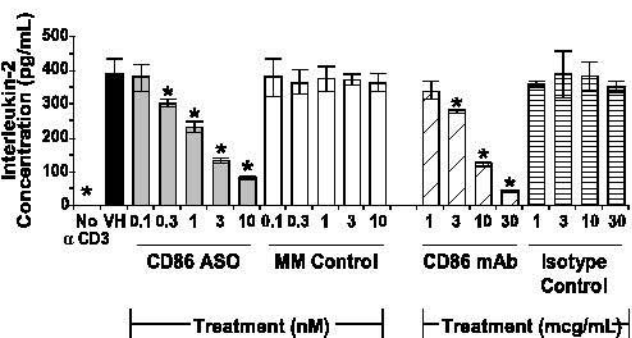
<u>Name</u>	<u>Antisense Sequence</u>	<u>Target</u>
121874	<u>TCAAG</u> TTTCTCTGTG <u>CCCAA</u>	CD86
131903	<u>TCAAG</u> TTTCTC <u>CG</u> TG <u>CCCAA</u>	1 base mismatch to CD86
131904	<u>TCAAG</u> T <u>C</u> TCTC <u>CGCG</u> <u>CCCAA</u>	3 base mismatch to CD86
131905	<u>TCAAG</u> T <u>C</u> <u>T</u> <u>T</u> C <u>CACG</u> <u>CCCAA</u>	5 base mismatch to CD86
131906	<u>TCAAG</u> T <u>CCT</u> T <u>CACA</u> <u>CCCAA</u>	7 base mismatch to CD86

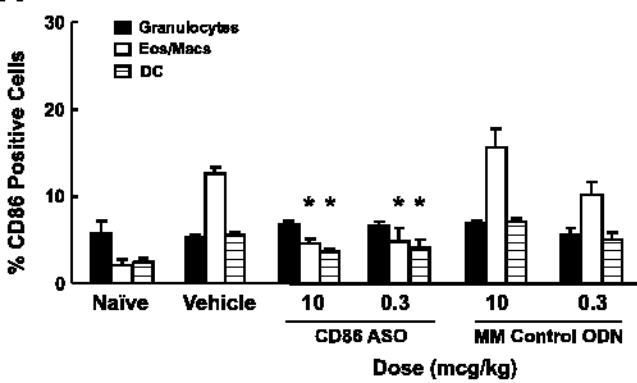
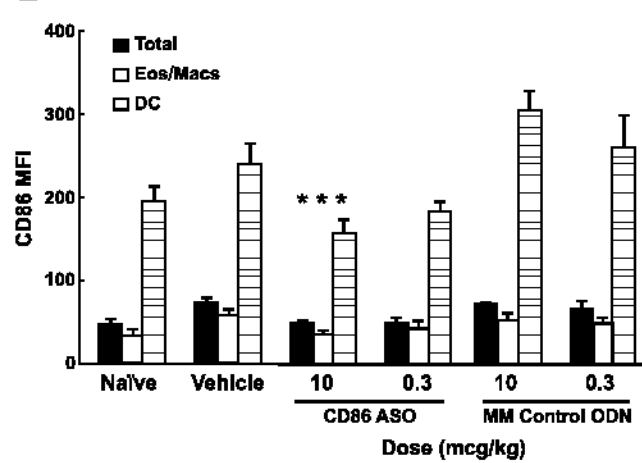
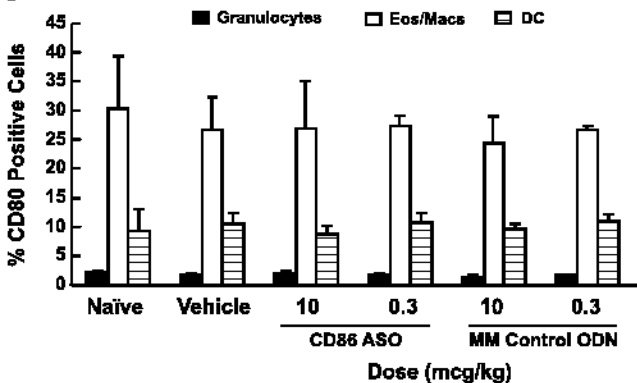
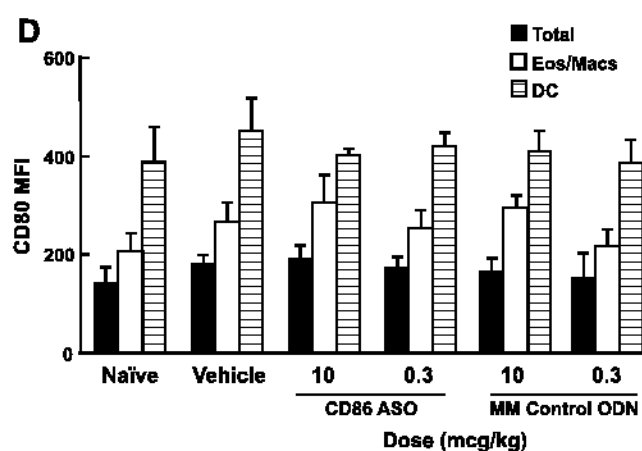
2'-MOE modifications are underlined; base mismatches are emboldened and underlined.

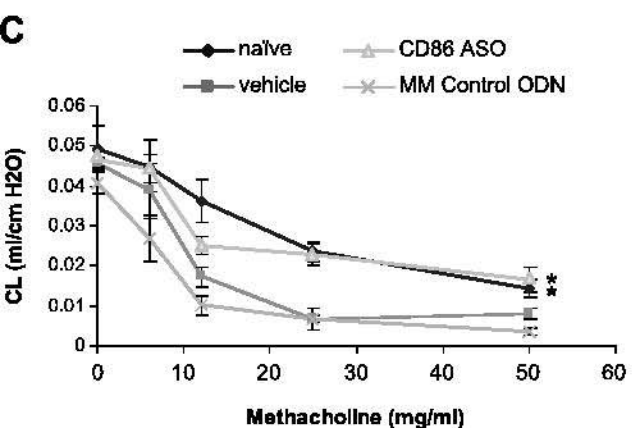
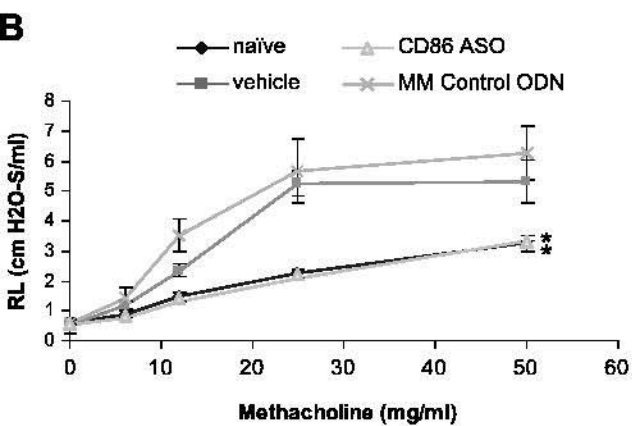
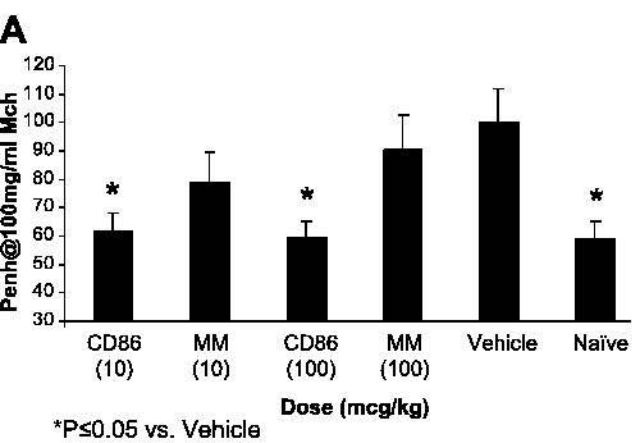
Table II. . **Inhibition of chemokines in aerosolized CD86 ASO but not MM control oligonucleotide treated allergic mice.**

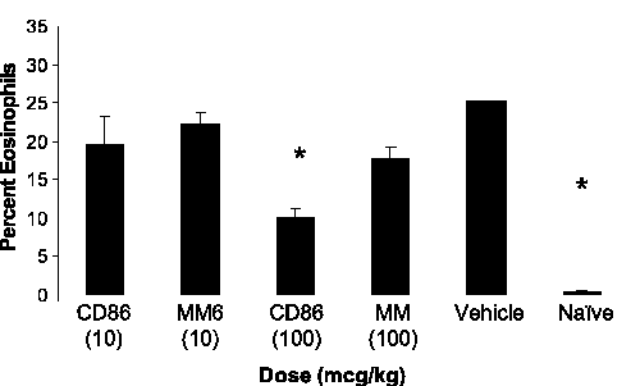
<u>Cyt/Chemokine</u>	<u>Naïve</u>	<u>Vehicle</u>	<u>CD86 ASO</u>	<u>MM ASO</u>
IL-5	6.3 +/- 1.8*#	558 +/- 104	413 +/- 134	427 +/- 97
IL-13	71 +/- 10*	102 +/- 5.8	87 +/- 12	94 +/- 9.7
Eotaxin	86 +/- 17*#	2932 +/- 639	1082 +/- 381*#	1615 +/-575
Rantes	8.7 +/- 2.1*	15.1 +/- 2.8	8.9 +/- 0.56*	15.1 +/- 2.7

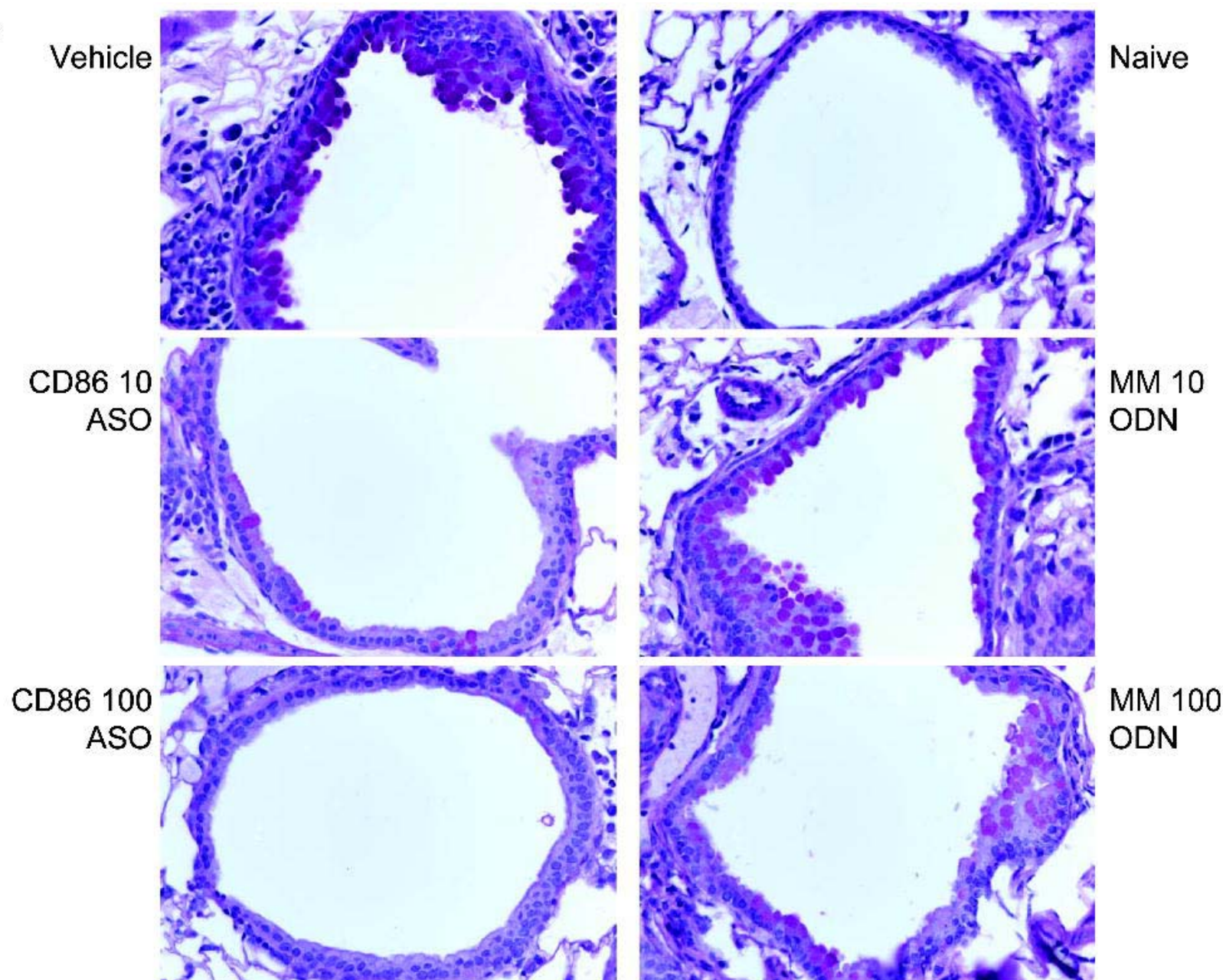
Cytokines and Chemokines were quantitated by ELISA on Day 67, 6 hours after the second nebulized OVA challenge. Data is expressed as the group mean +/- SE, N=4/group. *p < 0.05 for groups compared to vehicle using *Student's T-test and #Dunnett's-test.

A**B****C**

A**B****C****D**





A**B**