Syk Activation in Dendritic Cells Is Essential for Airway Hyperresponsiveness and Inflammation

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We evaluated the role of Syk, using an inhibitor, on allergen-induced airway hyperresponsiveness (AHR) and airway inflammation in a system shown to be B cell– and mast cell–independent. Sensitization of BALB/c mice with ovalbumin (OVA) and alum after three consecutive OVA challenges resulted in AHR to inhaled methacholine and airway inflammation. The Syk inhibitor R406 (30 mg/kg, administered orally, twice daily) prevented the development of AHR, increases in eosinophils and lymphocytes and IL-13 levels in bronchoalveolar lavage (BAL) fluid, and goblet cell metaplasia when administered after sensitization and before challenge with OVA. Levels of IL-4, IL-5, and IFN-γ in BAL fluid and allergen-specific antibody levels in serum were not affected by treatment. Because many of these responses may be influenced by dendritic cell function, we investigated the effect of R406 on bone marrow–derived dendritic cell (BMDC) function. Co-culture of BMDC with immune complexes of OVA and IgG anti-OVA together with OVA-sensitized spleen mononuclear cells resulted in increases in IL-13 production. IL-13 production was inhibited if the BMDCs were pretreated with the Syk inhibitor. Intratracheal transfer of immune complex-pulsed BMDCs (but not nonpulsed BMDCs) to naive mice before airway allergen challenge induced the development of AHR and increases in BAL eosinophils and lymphocytes. All of these responses were inhibited if the transferred BMDCs were pretreated with R406. These results demonstrate that Syk inhibition prevents allergen-induced AHR and airway inflammation after systemic sensitization and challenge, at least in part through alteration of DC function.

Keywords: AHR; dendritic cells; eosinophils; mice; Syk

Bronchial asthma is a complex disease of the lung characterized by reversible airway obstruction, chronic airway inflammation, and airway hyperresponsiveness (AHR). A number of cell types, including Th2 cells, eosinophils, and mast cells, are recruited to the lung and activated to release cytokines and chemokines, contributing to further airway inflammation. CD4+ T cells, especially Th2-type cells, which produce IL-4, IL-5, and IL-13, are considered pivotal in the development of AHR and eosinophilic inflammation (1). IL-13–producing effector CD8+ T cells may also play an important role in the development of AHR and eosinophilic inflammation (2). In atopic asthma, increased expression of Th2-type cytokines in lymphocytes in bronchoalveolar lavage fluid (BALF) has been demonstrated (3), emphasizing that activation of T cells is likely critical in the pathogenesis of asthma.

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Recently, the role of antigen-presenting cells (APCs), including dendritic cells (DCs), in the pathogenesis of asthma has been defined. When allergens are encountered in the airways, DCs in the airway epithelium capture allergens and migrate to the draining lymph nodes, where they reside in a mature, antigen-priming mode (4). There, antigen-specific T cells are induced to differentiate into Th effector cells or regulatory cells by these DCs. Thus, DCs are important in the initiation of T-cell differentiation and activation and indirectly contribute to the development of airway inflammation. Depletion of CD11c+ DCs during allergen challenge abrogates AHR and airway inflammation in an animal model (5).

Spleen tyrosine kinase (Syk) is a pivotal intracellular signaling molecule following ligation of B-cell or Fc receptors (6, 7). In mast cells, Syk plays a critical role in FcεR-mediated mast-cell activation, including degranulation and cytokine production. Syk is also expressed in DCs, which have Fc receptors (8, 9). Sedlik and colleagues have demonstrated that FcγR-mediated DC activation is decreased when Syk is deficient (9). Accordingly, Syk inhibition during allergen challenge could be a potential therapeutic target for preventing allergen-induced AHR and airway inflammation through the modulation of T-cell activation by DCs.

In this study, we examined the efficacy of a Syk inhibitor in the prevention of the development of AHR and airway inflammation after sensitization and challenge to allergen, an approach that has been previously shown to be mast-cell– and B-cell independent (10, 11). Specifically, we determined whether the inhibitory activity of this compound could result in the inhibition of DC function.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 8–10 wk of age (Jackson Laboratories, Bar Harbor, ME) were maintained on an ovalbumin (OVA)-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

R406

R406 was synthesized and provided by Rigel Pharmaceuticals (South San Francisco, CA). The small molecule R406 was identified as a potent inhibitor of FcεRI-dependent mast-cell activation (EC_{50} = 43 nM) using cell-based structure-activity relationships with primary human mast cells. The primary target for R406 was found to be Syk kinase. R406 is an ATP competitive inhibitor of biochemical Syk kinase activity (Ki = 30 nM). In mast cells activated by FcεRI crosslinking, R406 inhibited the phosphorylation of linker of activation of T cells tyrosine residue Y191 (a Syk kinase substrate) over 50-fold more potently than the phosphorylation of Syk itself, which is phosphorylated by Lyn kinase. Additionally, R406 was selective as assessed using a panel of cell-based assays representing specific and general signaling pathways (12, and E. S. Masuda, unpublished data).

Allergen Sensitization and Challenge

Mice were sensitized as previously described (10). Briefly, mice (8–12 wk of age) received an intraperitoneal injection of 20 μg OVA (Grade V; Sigma Chemical Co., St. Louis, MO) emulsified in 2.25 mg aluminum hydroxide (AlumImuject; Pierce, Rockford, IL) in a total volume of 100 μl on Days 0 and 14 and then challenged via the airways, using nebulized OVA (1% in 0.9% saline), with an ultrasonic nebulizer (Omron, Kyoto, Japan) for 20 min daily on Days 28, 29, and 30. On Day 32, AHR was assessed, and animals were killed for the collection of BALF, blood, and lung tissue. Oral administration of R406 (30 mg/kg, twice daily) was started 1 d before the first OVA challenge (Day 27) and was continued to Day 31. This dose of R406 was determined in preliminary *in vivo* experiments to be the minimum but most effective dose.

Determination of Airway Resistance

Airway resistance was determined as a change in airway function after aerosolized methacholine (MCh; Sigma) challenge. Mice were anesthetized with sodium pentobarbital (90 mg/kg, intraperitoneally), tracheostomized, and mechanically ventilated at a rate of 160 breaths/min with a constant tidal volume of air (0.2 ml). Lung function was assessed as previously described (10). Aerosolized MCh was administered for eight breaths at a rate of 60 breath/min, V_T of 500 μl by a second ventilator (Model 683; Harvard Apparatus, South Natick, MA) in increasing concentrations (1.56, 3.125, 6.25, and 12.5 mg/ml). After each MCh challenge, the data were continuously collected for 1–5 min, and maximum values of airway resistance were taken to express changes in this functional parameter.

Determination of Cell Numbers and Cytokine Levels in BALF

Immediately after the assessment of AHR, lungs were lavaged via the tracheal cannula with Hanks, balanced salt solution (1 ml/mouse). Total leukocyte numbers were measured with a Coulter Counter (Coulter Corporation, Hialeah, FL). Differential cell counts were made from cytocentrifuged preparations using a Cytospin 2 (Shandon Ltd., Runcorn, Cheshire, UK) and after staining with Leukostat (Fisher Diagnostics, Pittsburgh, PA). At least 200 cells were counted under ×400 magnification.

BAL supernatants were collected and kept frozen at −80°C until assayed. The levels of cytokine secreted into the supernatants of BALF samples were determined by ELISA. IL-4, IL-5, IFN-γ (all from BD Pharmingen, San Diego, CA), and IL-13 (R&D Systems, Minneapolis, MN) were measured following the manufacturers' directions. The limits of detection were 4 pg/ml for IL-4 and IL-5, 1.5 pg/ml for IL-13, and 10 pg/ml for IFN-γ.

Measurement of Total and OVA-Specific Antibodies

Serum levels of total IgE and OVA-specific IgE, IgG_1, IgG_{2a}, and IgG_{2b} were measured by ELISA as described (10). The OVA-specific antibody titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per milliliter. Total IgE levels were calculated by comparison with known mouse IgE standards (BD Pharmingen). The limit of detection was 100 pg/ml for total IgE.

Preparation of Bone Marrow–Derived Dendritic Cells

Bone marrow-derived dendritic cells (BMDCs) were differentiated from bone marrow cells according to the procedure described by Inaba and colleagues (13, 14), with some modification. In brief, bone marrow cells obtained from femurs and tibias of mice were placed in T-75 flasks for 2 h at 37°C in RPMI-1640 containing 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO, Carlsbad, CA), 10 ng/ml recombinant mouse GM-CSF, and 10 ng/ml recombinant mouse IL-4 (R&D Systems). Nonadherent cells were collected by aspirating the medium and transferred into fresh flasks. On Day 8, nonadherent cells were collected, centrifuged, and resuspended in fresh medium. The purity of the DCs was demonstrated to be more than 95% by CD11c staining.

Co-Culture of BMDCs and Spleen Cells

Immune complexes of OVA (10 μg/ml) and anti-OVA IgG (50 μg/ml; Sigma) were incubated with BMDCs (1×10^6 cells/ml) overnight at 37°C. R406 (0.3–3 μM) or 0.1% DMSO (vehicle) were added to BMDCs 1 h before the addition of the immune complexes. BMDCs were thoroughly washed 1 h after the addition of the immune complex mixture. At the concentrations used, there were no effects on cell viability based on trypan blue dye exclusion.

Mice were sensitized twice (on Days 0 and 14) with OVA plus alum, and spleens were isolated 14 d after the last sensitization (on Day 28). Cells were harvested by mincing the tissues and subsequently passing them through a stainless steel sieve. Cells were washed and suspended in culture medium. Mononuclear cells were isolated by HISTOPAQUE-1083 (Sigma) gradient centrifugation at 2,000 rpm for 20 min. The mononuclear cells (2×10^5 cells/sample) were added to suspension of BMDCs (6.7×10^3 cells/sample), and the mixture of cells was incubated at 37°C for 6 d. After centrifugation at 1,500 rpm for 5 min, the supernatants were collected, and levels of IL-13 were assayed by ELISA (R&D Systems).

Development of AHR and Airway Inflammation in Mice Receiving BMDCs

Nonpulsed or immune-complex–pulsed BMDCs were transferred intratracheally into naive mice (5×10^5 cells/mouse). Two days after the transfer of BMDCs, mice were challenged via the airways to OVA (1% in saline solution) for 20 min on three consecutive days. AHR was assessed 48 h after the last OVA challenge, and BALF and lung cells were collected.

Antigen Internalization

To examine OVA uptake, immune complexes consisting of FITC-conjugated OVA (10 μg/ml; Molecular Probes, Eugene, OR) and anti-OVA IgG (50 μg/ml; Sigma) were incubated with BMDCs (1×10^6 cells/ml) for 1 h at 37°C. R406 (3 μM) or 0.1% DMSO (vehicle) was added to BMDCs 1 h before the addition of immune complexes. BMDCs were thoroughly washed to separate surface-bound OVA and stained with PE-conjugated anti-CD11c. Double-positive cells for PE and FITC were enumerated, and mean fluorescent intensity (MFI) was calculated by flow cytometric analysis.

Expression of Co-Stimulatory Molecules

Immune complexes of OVA and anti-OVA IgG were incubated with BMDCs (1×10^6 cells/ml) overnight at 37°C. R406 (3 μM) or 0.1% DMSO (vehicle) was added to BMDCs 1 h before addition of the immune complexes. BMDCs were thoroughly washed by centrifugation. Cells were analyzed after immunostaining of BMDC surface molecules. The mononuclear antibodies used were: FITC-, PE-, PerCP-, or APC-conjugated anti-CD11c; anti-CD45; anti-MHC II; anti-CD80; anti-CD86; anti-CD40; anti-CCR7; anti-B7H1; anti-ICAM1; and anti-mouse IgG (all from BD PharMingen). Cell-surface staining was performed according to standard techniques, and flow cytometric analysis was performed with a FACSCalibur using CellQuest Pro software (BD Labware, Mountain View, CA). MFI values for MHC II, CD80, CD86, CD40, B7H1, CCR7, or ICAM1 were calculated.

Cytokine Levels in BMDC Cultures

Immune complexes of OVA and anti-OVA IgG were incubated with BMDCs (1×10^6 cells/ml) overnight at 37°C. R406 (3 μM) or 0.1% DMSO (vehicle) was added to BMDCs 1 h before addition of the immune complexes. Cytokine levels in culture supernates were assayed as described previoulsy.

Data Analysis

Data were compared using Student's *t* test. A P value of < 0.05 was considered statistically significant. Values for all measurements were expressed as the mean ± SEM.

RESULTS

Effect of R406 on OVA-Induced AHR and Airway Inflammation

Airway responsiveness to MCh was significantly increased in sensitized mice after three airway challenges to OVA (Figure 1).

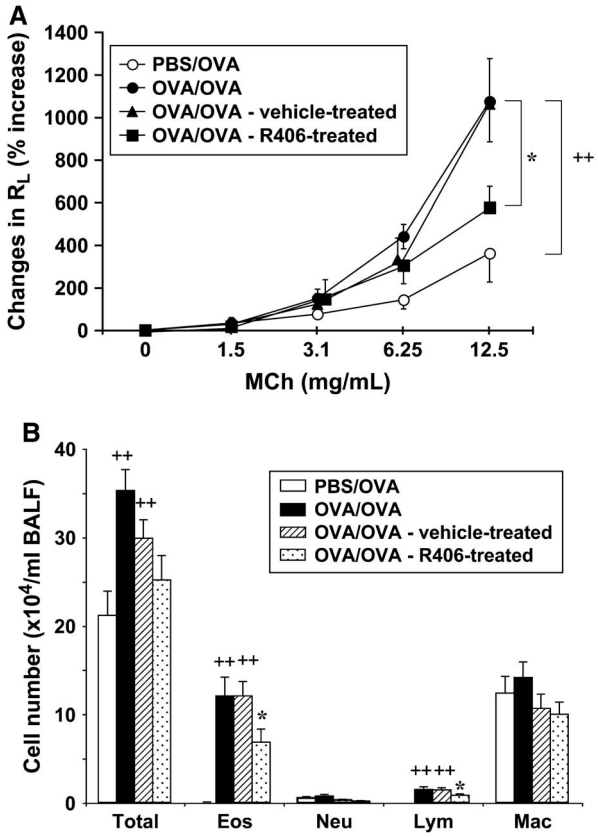

Figure 1. Effects of R406 on development of AHR and airway inflammation. (*A*) R406 suppresses antigen-induced AHR. Sensitized mice received oral R406 (30 mg/kg,twice daily) or vehicle, administered from 1 d before the first OVA challenge to the day before measurement of AHR. Results are expressed as the percentage increase in airway resistance after MCh inhalation. (*B*) R406 suppresses allergen-induced inflammatory cell infiltration in the airways. PBS/OVA: nonsensitized and challenged; OVA/OVA: sensitized and challenged. Results represent mean ± SEM from three separate experiments ($n = 12$). $^{++}P < 0.01$ comparing sensitized and challenged to challenged alone. $^{*}P < 0.05$ comparing vehicle-treated with R406-treated sensitized and challenged mice.

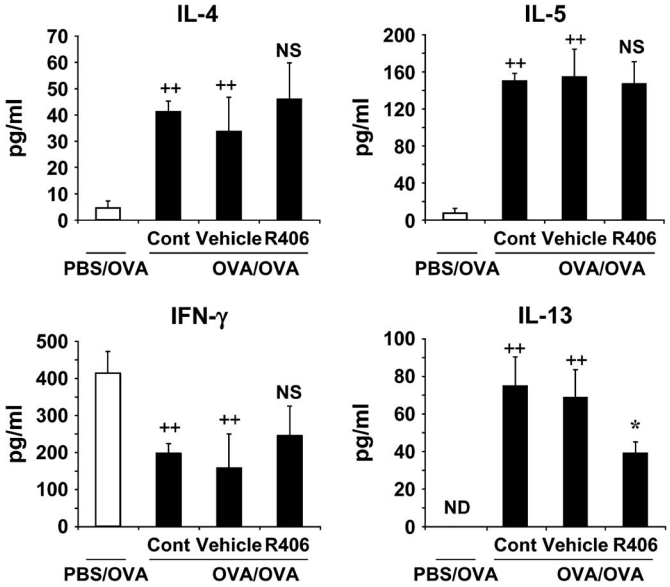

Figure 2. Cytokine levels in the BALF. Mice were the same as in the legend to Figure 1. $^{++}P < 0.01$ comparing PBS/OVA with OVA/OVA; $^{*}P < 0.05$ comparing vehicle-treated and R406-treated groups. NS: nonsignificant comparing vehicle-treated with R406-treated group. ND: none detected.

The effect of R406 on lung function was assessed when administered after sensitization but before allergen challenge. Orally administered R406 (30 mg/kg) significantly inhibited the development of AHR (Figure 1A). In contrast, vehicle treatment did not alter AHR.

R406 also altered the composition of BAL inflammatory cells. Sensitized and challenged mice developed significant increases in total cell numbers, eosinophils, and lymphocytes (Figure 1B). Vehicle treatment showed comparable levels of inflammatory cells. Treatment with R406 significantly ($P < 0.05$) reduced the numbers of eosinophils and lymphocytes in the BALF.

Effect of R406 on OVA-Induced Cytokine Production in BALF

IL-4, IL-5, and IL-13 levels were significantly increased in the BALF of sensitized mice after OVA challenge (Figure 2). R406 at a dose of 30 mg/kg significantly inhibited the increases in levels of IL-13 but did not alter the levels of IL-4 or IL-5, which were increased after sensitization and challenge or the levels of IFN-γ, which were decreased after sensitization and challenge.

Goblet Cell Metaplasia

Goblet cell metaplasia is a marker of airway inflammation and an indicator of the activity of IL-13 (15) and can be quantitated in lung tissue sections stained with PAS (Figures 3A and 3B). After OVA challenge, challenged-only mice showed no increases in PAS-positive cells, whereas sensitized and challenged mice with or without vehicle treatment showed increases in the number of PAS-positive cells (87.3 ± 2.3 and 84.6 ± 4.2 PAS-positive cells/mm BM, respectively). Sensitized and challenged mice treated with R406 at a dose of 30 mg/kg showed decreased numbers of PAS-positive cells (46.4 ± 5.2 PAS-positive cells/mm BM).

Allergen-Specific Antibodies in Serum

R406 treatment did not show any effects on the levels of total IgE or OVA-specific IgE, IgG_1, IgG_{2a}, and IgG_{2b} (data not shown).

Effect of R406-Treated BMDCs on IL-13 Production in OVA-Sensitized Spleen Cells *In Vitro*

IL-13 levels in culture supernates were assayed after co-culture of OVA-sensitized spleen cells and immune complex–pulsed or nonpulsed BMDCs. Co-culture of spleen cells and nonpulsed BMDCs did not result in any detectable levels of IL-13 in the culture supernates (Figure 4). After culture with pulsed BMDCs, IL-13 levels were significantly increased; pre-treatment of the BMDCs with R406 at a concentration of 3 μM inhibited the production of IL-13 by more than 65%. IL-4 levels were also reduced in the presence of R406, whereas levels of IL-5 and IFN-γ were not significantly different from vehicle controls.

Effect of R406 on Immune Complex-Activated BMDCs Induction of Airway Responsiveness and Airway Inflammation

We examined the effects of R406 on BMDC function *in vivo*. We determined if adoptive transfer of immune complex-pulsed BMDCs into naive mice via the trachea triggered the development of AHR and airway inflammation after allergen challenge. After three consecutive days of OVA challenge, mice receiving immune complex–pulsed BMDCs developed significant increases

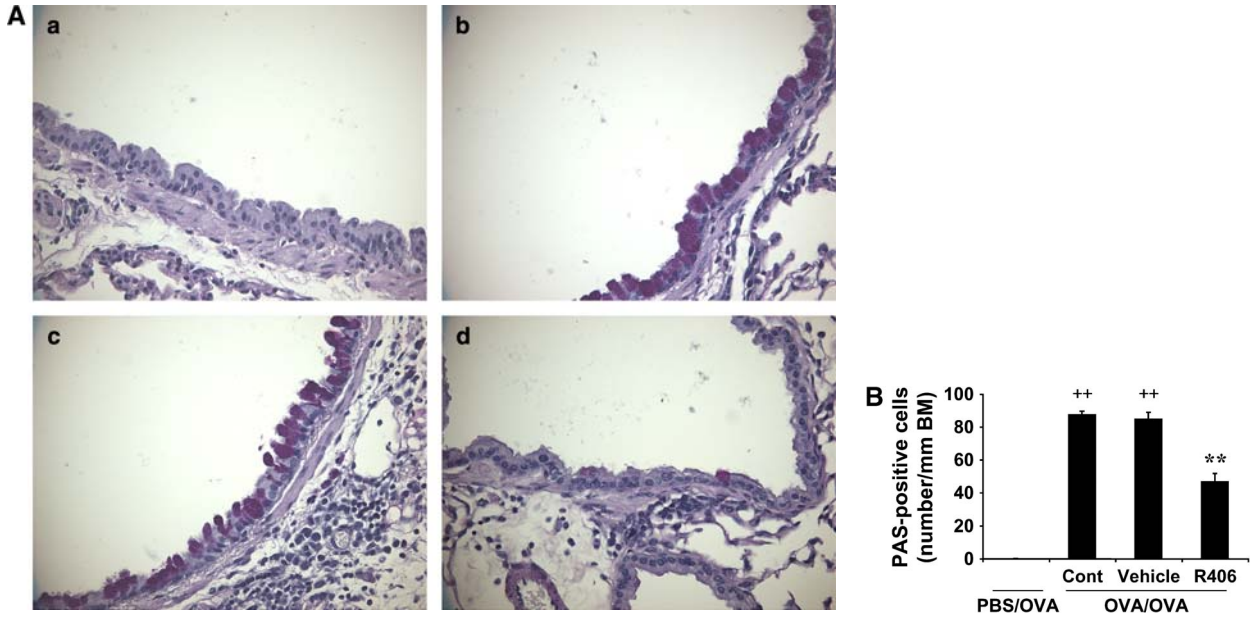

Figure 3. R406 suppresses goblet cell metaplasia. Goblet cell metaplasia was detected by PAS staining 48 h after the last OVA challenge. (*A*) Airway epithelium stained with PAS. (*a*) PBS/OVA; (*b*) OVA/OVA; (*c*) OVA/OVA after vehicle treatment; (*d*) OVA/OVA after treatment with 30 mg/kg R406. (*B*) Quantification of goblet cell metaplasia. Goblet cell numbers were determined in lung tissue stained with PAS. Results are expressed as the number of PAS-positive cells per millimeter of basement membrane (BM). Each column represents the mean ± SEM of three separate experiments ($n = 12$). $^{++}P < 0.01$ comparing PBS/OVA with OVA/OVA; $^{**}P < 0.01$ comparing vehicle-treated with R406-treated mice.

in airway resistance to inhaled MCh (Figure 5A). Transfer of nonpulsed BMDCs failed to induce increases in lung resistance. Intra-tracheal administration of pulsed BMDCs that were treated with R406 failed to increase airway responsiveness, and levels of airway resistance were similar to mice receiving nonpulsed BMDCs, throughout the MCh dose-response curve.

In parallel, transfer of immune complex-pulsed BMDCs followed by OVA challenge significantly increased the numbers of BAL eosinophils and lymphocytes when compared with mice receiving nonpulsed BMDCs (Figure 5B). Transfer of pulsed and R406-treated BMDCs failed to result in increased numbers of eosinophils and lymphocytes.

In the BALF, IL-13 levels were significantly increased in mice receiving pulsed BMDC before airway challenge (Figure 5C). However, no increases in BAL IL-13 levels were detected in recipients of nonpulsed BMDCs or pulsed and R406-treated BMDCs. Levels of IL-4, IL-5, and IFN-γ showed little change.

Effect of R406 on Antigen Internalization

Uptake of FITC-OVA MFI was significantly ($P < 0.01$) increased after addition of immune complexes consisting of FITC-OVA and OVA-IgG (Figure 6). R406 significantly ($P < 0.05$) reduced MFI, but overall the effects were small. MFIs were lower when FITC-OVA was incubated with BMDC not as an immune complex, and R406 did not show any effects on these increases in FITC-OVA uptake, suggesting that R406 may have been more effective on Fc-dependent, but not Fc-independent, antigen uptake.

Effect of R406 on Expression of Co-Stimulatory Molecules on BMDCs

After pulsing with OVA-IgG immune complexes, surface expression of MHC class II, CD86, CD40, ICAM-1, and B7H1 were increased compared with nonpulsed BMDCs (Table 1). Treatment with R406 significantly reduced the expression of MHC II ($P < 0.05$), CD80 ($P < 0.05$), CD86 ($P < 0.05$), CD40 ($P < 0.05$), and ICAM1 ($P < 0.01$) on BMDCs. The compound did not significantly inhibit the expression of CCR7 and did not influence the expression of B7H1, an inhibitory surface molecule on APCs.

Effect of R406 on BMDC Cytokine Production

After culture of BMDCs with OVA-IgG complexes, levels of IL-1β, IL-10, IL-12, and IL-13 were significantly increased (Figure 7). Incorporation of the Syk inhibitor significantly reduced the levels of IL-10, IL-12, and IL-13 to virtually undetectable levels; levels of IL-1β were unaffected by R406.

DISCUSSION

There is increasing interest in targeting signaling molecules that are essential to cells that have been implicated in the pathogenesis of asthma. Because the number of cells that may play a role in the development and maintenance of asthma are numerous, those signaling molecules that are important in a number of cell types may provide greater therapeutic benefit. Syk is one such potential target because it is expressed in many cell types putatively involved in asthma, including B cells, eosinophils, mast cells, and other cell types expressing Fc receptors (6, 7, 16). T lymphocytes do not express Syk but express ZAP-70, a kinase in the same family as Syk (17). As a result, a number of studies demonstrated that inhibition of Syk may be associated with suppression of allergic responses and airway inflammation. In many of these previous studies of Syk inhibition and the effects on allergic responses (18–21), it was not clear which cells were targeted at the doses used.

To define which cells and which pathways play essential roles in the development of AHR and airway inflammation, we developed several approaches in an attempt to focus or isolate critical components of the response to sensitization and challenge. In

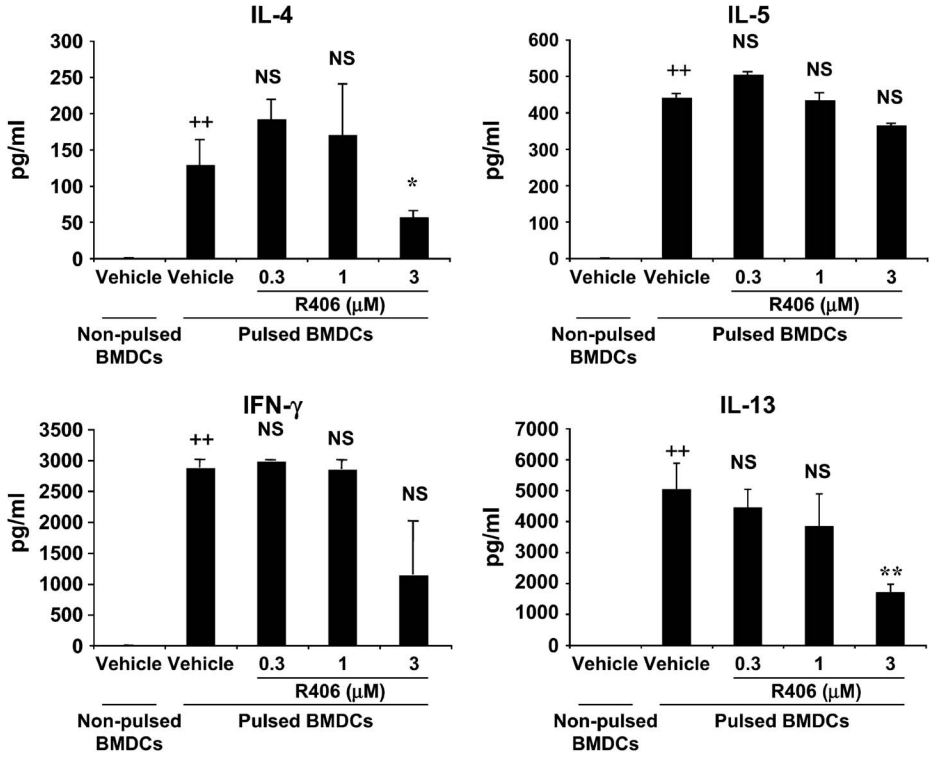

Figure 4. Decreased IL-13 release from OVA-sensitized spleen cells cultured with Syk inhibitor-treated BMDCs. BMDCs were pre-incubated with 0.3–3 μM of R406 for 1 h. The cells were pulsed with anti-OVA IgG/OVA for 1 h, thoroughly washed, and co-cultured with sensitized spleen mononuclear cells for 6 d. Cytokine levels in supernates were assayed by ELISA. Each column represents mean ± SEM from two experiments ($n = 8$). $^{++}P < 0.01$ comparing pulsed and non-pulsed BMDCs; $^{**}P < 0.01$ and $^{*}P < 0.05$ comparing vehicle-treated and R406 (3 μM)-treated and pulsed BMDCs. NS: nonsignificant comparing vehicle-treated and R406-treated groups.

earlier studies, we examined the effect of the Syk inhibitor R406 in mice exposed exclusively to allergen via the airways in the absence of systemic sensitization and adjuvant (12). In this approach, which was shown to be dependent on IgE synthesis and mast cell activation (22), R406 prevented the development of AHR but did not alter the synthesis of IgE. *In vitro*, R406 showed effects on mast cell activation, degranulation, and IL-13 cytokine production (12). *In vivo* treatment with the inhibitor reduced AHR, airway inflammation (eosinophilia), and BAL IL-13 levels in allergen-exposed mice. These results suggest that at the dose used, R406 (30 mg/kg) selectively inhibited some but not all responses after exposure to allergen. These results were confirmed in mice that were passively sensitized with allergen-specific IgE (thereby bypassing the need for B-cell activation and IgE synthesis) and exposed to limited airway challenge, further confirming the activity of R406 on mast cell function *in vivo* (12).

To further examine the effect of Syk inhibition on AHR and allergic inflammation *in vivo*, we examined R406 in a model of systemic sensitization (together with adjuvant) and airway challenge, an approach shown to be independent of the need for B cells, IgE, or mast cells (10, 11, 22). The results demonstrated that R406 was a potent inhibitor of the development of altered airway function (lung resistance) to inhaled MCh, airway eosinophilia, and goblet cell metaplasia. As shown previously (12), R406 did not prevent the increases in serum allergen-specific antibody levels or alter the levels of IL-4, IL-5, or IFN-γ, further confirming the selectivity of the effects of Syk inhibition, at least at the dose used *in vivo*. However, R406 did significantly reduce the levels of IL-13 in the BALF of sensitized and challenged mice. The mechanism underlying the selectivity of R406 in this and other approaches is unclear. IL-13 is an important effector cytokine: Administration of IL-13 can induce AHR, lung eosinophilic inflammation, and goblet cell metaplasia in naive mice or recombination activating gene-deficient mice (14). IL-13 effects on AHR are mediated independently of IL-5 and exotoxin and may directly affect airway smooth muscle function (23). Further, IL-13 can increase the expression of numerous chemokines involved in the regulation of eosinophil chemotaxis (24–27). Perhaps most critically, IL-13 is the major inducer of goblet cell metaplasia and mucus production, triggering differentiation of mature goblet cells through induction of the MUC5AC gene (28). Targeting IL-13 alone results in the inhibition of AHR and airway inflammation in a number of *in vivo* approaches, both mast cell-dependent and -independent (22). Thus, the major activity of R406 may be to prevent IL-13 production after allergen sensitization and challenge.

There are several potential sources of IL-13 in these models, but a major source is thought to be activated T cells (22). CD4+ and CD8+ T cells have been described to be essential to the development of AHR and airway inflammation (1), and both subtypes are important sources of IL-13. Because mature T cells do not express Syk and in the absence of a requirement for B cells or mast cells in this model of systemic sensitization, we explored the possibility that R406 targeted APC function and DC in particular.

Immature DCs capture antigens by macropinocytosis or receptor-mediated internalization (endocytosis and phagocytosis) (29). FcγRs are well characterized components engaged in phagocytosis by APCs (30–32), and these receptors mediate efficient antigen uptake and strongly enhance the efficiency of antigen presentation to T cells (9, 33). Syk signaling seems to be essential for phagocytosis through FcγRs (34–36). After antigen uptake, DCs present processed antigenic peptides to MHC class II-restricted CD4+ T cells and to class I-restricted CD8+ T cells. Although peptides presented on MHC class I molecules are generally derived from cytosolic antigens, DCs also cross-present peptides from exogenous antigens to MHC class-I restricted CD8+ T cells (29).

In the present study, we focused on the functional activity of Syk-inhibited DCs. The functional activation of antigen-pulsed BMDCs was first evaluated *in vitro* after co-culture of immune

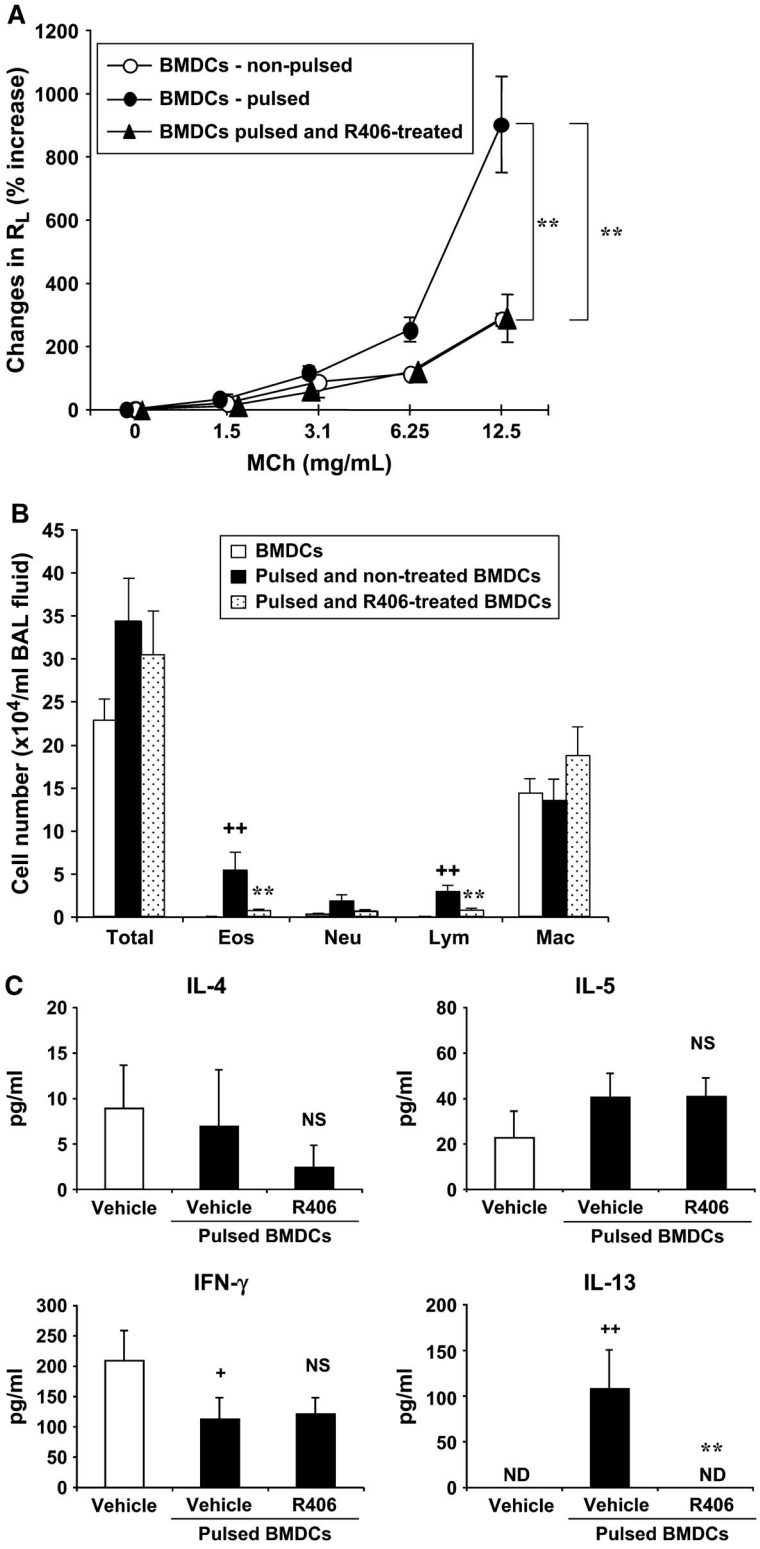

Figure 5. Effects of intratracheal transfer of Syk inhibitor-treated BMDCs. Pulsed and nonpulsed BMDCs were prepared as described, and 5×10^6 BMDCs were instilled intratracheally after sensitization but before OVA challenge. (*A*) AHR ($n = 8$ in each group). $^{**}P < 0.01$ comparing recipients of nonpulsed and pulsed BMDC. $^{**}P < 0.01$ comparing vehicle-treated and R406-treated BMDC recipients. (*B*) BAL inflammatory cell composition ($n = 8$ in each group). $^{++}P < 0.01$ comparing recipients of pulsed and nonpulsed BMDC; $^{**}P < 0.01$ comparing vehicle-treated and R406 (3 μM)-treated and pulsed BMDCs. (*C*) BAL cytokine levels ($n = 8$ in each group). $^{++}P < 0.01$ and $^{+}P < 0.05$ comparing recipients of pulsed and nonpulsed BMDC; $^{**}P < 0.01$ comparing vehicle-treated and R406 (3 μM)-treated and pulsed BMDCs. NS: nonsignificant comparing vehicle-treated and R406-treated groups.

complex-pulsed and R406-treated BMDCs and spleen mononuclear cells. IL-13 production from spleen cells was used as functional readout for BMDC activity. The results showed that R406-treated BMDCs reduced IL-13 (and IL-4) release when cultured with sensitized spleen cells *in vitro*.

Sung and colleagues (37) showed that OVA- and $OVA_{323–339}$ peptide-pulsed splenic DCs, when introduced intratracheally, are potent inducers of AHR, lung eosinophilia, and goblet cell metaplasia by recruiting lymphocytes to the lung draining lymph nodes and by stimulating Th2 responses. *In vivo*, we similarly monitored BMDC function after their intratracheal instillation into naive mice followed by limited airway allergen exposure. Mice receiving nonpulsed BMDC failed to develop any of these responses. The time course of this experimental approach bypasses any role for endogenous (recipient) DC or B-cell function, focusing on the transferred DCs and their activation by antigen-pulsing or

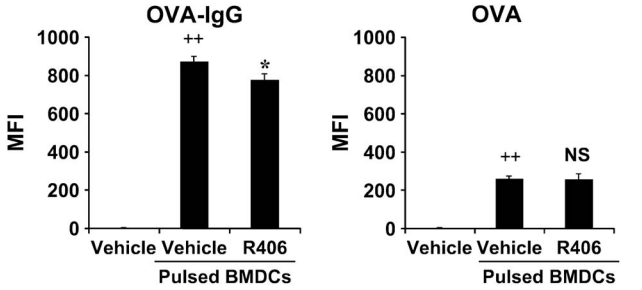

Figure 6. Effect of R406 on FITC-conjugated antigen uptake in BMDCs. BMDCs were incubated with R406 (3 μM) for 1 h before pulsing with FITC-OVA-IgG or FITC-OVA alone. Cells were washed 60 min after pulsing, and the amount of antigen uptake was measured by flow cytometry. The values were expressed as FITC-MFI calculated with Cell Quest Pro Software. Each column represents mean ± SEM from two experiments ($n = 6$). $^{**}P < 0.01$ comparing pulsed and nonpulsed BMDCs; $^{*}P < 0.05$ comparing vehicle-treated and R406-treated and pulsed BMDCs. NS: nonsignificant comparing vehicle-treated and R406-treated groups.

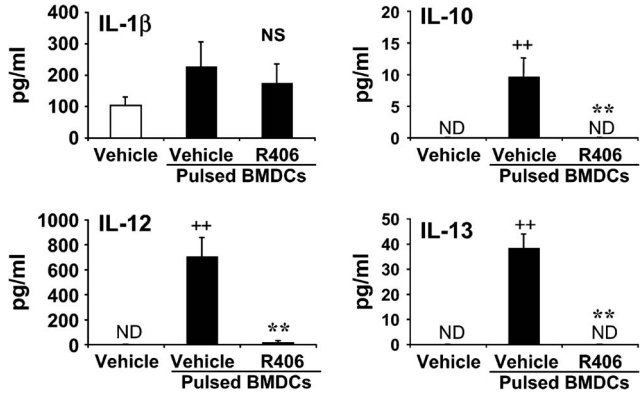

Figure 7. Effect of R406 on cytokine production in immune complex-pulsed BMDCs. BMDCs were incubated with R406 (3 μM) for 1 h before pulsing with OVA-IgG. The cells were cultured overnight, and cytokine levels in supernates were assayed by ELISA as described. Each column represents mean ± SEM from two experiments ($n = 6$). $^{++}P < 0.01$ comparing pulsed and nonpulsed BMDCs; $^{**}P < 0.01$ comparing vehicle-treated and R406-treated and pulsed BMDCs. NS: nonsignificant comparing vehicle-treated and R406-treated groups.

challenge. Naive mice receiving immune-complex–pulsed DCs developed AHR, pulmonary eosinophilia, goblet cell metaplasia, and increases in IL-13 levels in BALF after OVA challenge. These findings strongly suggest that pulsed BMDCs can prime and activate T cells to induce allergic AHR and airway inflammation after allergen challenge. Treatment of the pulsed BMDC before their transfer failed to trigger any of the responses seen after transfer of nontreated but pulsed BMDC.

Studies were initiated to begin to define how inhibition of Syk affected BMDC function *in vitro* and *in vivo*. Three areas were initially targeted: antigen uptake, co-stimulatory molecule expression: and cytokine production. R406 did inhibit immune-complexed FITC-OVA uptake. Although the decreases were significant, they were relatively small. When expression of a number of co-stimulatory molecules was examined, the levels of many of them were significantly reduced. Although it is not clear which ones may be more important than others, cumulatively, these reductions could have accounted for the reduced DC functional activity. The largest decrease was in CD86 expression (~50%), a surface molecule implicated in the development of AHR and airway inflammation (38). In keeping with their central role as initiators of allergic responses in the lung, the cytokines released by DC play an important role in directing the nature of the immune response that develops. Immune-complex–pulsed BMDC were capable of inducing AHR, airway eosinophils, and increased BAL levels of IL-13 in recipients exposed to challenge alone. Because addition of immune complexes induced the release of increased levels of IL-10, IL-12, and IL-13 from BMDC and because this was abolished when R406 was added to the cultures in parallel to the loss of their activity *in vivo*, it may be assumed that these cytokines also contributed to the BMDC activity *in vivo*. It is not certain if one or all of these cytokines are essential to the activity of BMDC in triggering allergen responses in the lung. Overall, it seems that inhibition of Syk affects a number of the responses of BMDC after immune-complexed antigen exposure, including cytokine uptake, accessory molecule expression, and cytokine production. Likely, the end result is the cumulative effects of the inhibition of all of these processes, although further study is necessary to determine if a hierarchy exists.

TABLE 1. EFFECT OF R406 ON EXPRESSION OF CO-STIMULATORY MOLECULE EXPRESSION

	Nonpulsed BMDCs (*MFI*)	OVA-IgG–Pulsed BMDCs (*MFI*)	
Co-stimulatory Molecules	Vehicle	Vehicle	R406
MHC II	461.7 ± 13.8	571.6 ± 45.8*	403.1 ± 40.4‡
CD80	106.4 ± 2.9	121.9 ± 7.9	93.8 ± 7.3‡
CD86	785.7 ± 35.8	1219.7 ± 168.4*	614.9 ± 114.1‡
CD40	134.5 ± 3.5	192.9 ± 13.0*	139.6 ± 9.5‡
CCR7	45.8 ± 1.4	44.8 ± 4.0	37.3 ± 2.1
ICAM1	459.8 ± 5.3	676.7 ± 12.2†	501.7 ± 28.8§
B7H1	766.0 ± 30.8	861.7 ± 15.2*	897.5 ± 17.6

Definition of abbreviations: BMDC, bone marrow–derived dendritic cell; IgG, immunoglobulin G; MFI, mean fluoresence intensity; OVA, ovalbumin.

Effect of R406 on BMDC expression of co-stimulatory molecules. BMDCs (1×10^6 cells/ml) were incubated with R406 (3 μM) for 1 h before pulsing with OVA-IgG. The cells were cultured overnight and levels of expression of co-stimulatory molecules were analyzed by flow cytometry as described. Levels of expression of the co-stimulatory molecules was calculated and expressed as MFI. Each value represents mean ± SEM from two experiments ($n = 6$).

* $P < 0.05$ comparing which treated pulsed and nonpulsed BMDC.

† $P < 0.01$ comparing which treated pulsed and nonpulsed BMDC.

‡ $P < 0.05$ comparing vehicle-treated and pulsed BMDCs.

§ $P < 0.01$ comparing vehicle-treated and pulsed BMDCs.

Together, our results confirm that inhibition of Syk activity is a potent inhibitor of allergen-induced AHR and inflammation after systemic sensitization and challenge, where B cells and mast cells are not essential to these responses. Moreover, at the doses used, R406 exhibits selectivity in the functions affected, sparing IL-4, IL-5, and IFN-γ production and antibody synthesis. Because T cells are not directly targeted by the Syk inhibitor, the data pointed to another cell type in the cascade initiated by allergen exposure of sensitized mice. *In vitro* and *in vivo* experiments identified that DC function is an important target of Syk inhibition, adding to the increasing body of evidence supporting the targeting of Syk in the treatment of allergic airway disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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