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Blockade of JAK2 by Tyrphostin AG-490 Inhibits Antigen-Induced Eosinophil Recruitment into the Mouse Airways

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We studied the effect of tyrphostin AG-490, a specific Janus kinase 2 (JAK2) inhibitor, on antigen-induced eosinophil recruitment into the airways of sensitized mice and on IL-5-induced chemokinesis and adhesiveness of eosinophils. The *in vivo* administration of AG-490 prevented antigen-induced eosinophil infiltration in the airways of sensitized mice in a dose-dependent manner. However, the administration of AG-490 did not affect antigen-induced IL-5 production in the airways nor *in vitro* antigen-induced IL-5 production and T cell proliferation of spleen cells. Furthermore, AG-490 inhibited IL-5-induced chemokinesis and β1-integrin adhesiveness of eosinophils *in vitro*. Because antigen-induced eosinophil recruitment into the airways is mediated by IL-5, these results indicate that JAK2 activation is critical for antigen-induced, IL-5-dependent mobilization of eosinophils into the tissue.

Asthma is characterized by airway inflammation with prominent eosinophil infiltrates (1). In a murine model of asthma, we and others have provided direct evidence that $CD4^+$ T cells and interleukin-5 (IL-5) mediate antigen-induced eosinophil recruitment into the airways of sensitized mice (2, 3). IL-5 produced by $CD4^+$ T cells induces chemokinetic responses in eosinophils, by which eosinophils are mobilized from the bone marrow into the blood and subsequently accumulated in the lung (4, 5).

IL-5 binds to the receptors consisting of an α chain specific for IL-5 and a common cytokine receptor β chain (6). IL-5 activates Lyn (7, 8), Syk (8), and Janus kinase 2 (JAK2) (9–11) tyrosine kinases in eosinophils, which transduce signals to downstream molecules. It has been suggested that these tyrosine kinases play a critical role in the survival of eosinophils (7–9, 12). However, it remains unknown which tyrosine kinases mediate antigen-induced, IL-5-dependent eosinophil recruitment into the tissue.

Therefore, to elucidate this issue, because JAK2-deficient mice are embryonic lethal (13, 14), we studied the effect of tyrphostin AG-490, a specific JAK2 inhibitor (15), on antigen-induced eosinophil recruitment into the airways of sensitized mice and on IL-5-induced chemokinetic responses of eosinophils. Our results indicate that blockade of JAK2 tyrosine kinase prevents antigen-induced eosinophil recruitment into the airways and IL-5-induced chemokinesis and adhesiveness of eosinophils. It is thus suggested that the blocking of JAK2 activation would be a rational therapeutic approach to allergic inflammation in asthma.

MATERIALS AND METHODS

Mice and immunization. Female BALB/c mice (age 8 weeks) (Charles River Laboratories, Atsugi, Japan) were immunized intraperitoneally twice with 1 μg of ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) in 4 mg of aluminum hydroxide at a two-week interval. Fourteen days after the second immunization, the sensitized mice were challenged with aerosolized OVA as described below.

Antigen-induced eosinophil infiltration in mouse airways. The eosinophil infiltration into the airways was induced by the inhalation of antigen in sensitized mice, and the number of eosinophils recovered in the bronchoalveolar lavage fluids (BALF) was evaluated as described previously (16). Briefly, the sensitized mice inhaled aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, PA) for 20 min. As a control, 0.9% saline alone was administered by the nebulizer. Twenty-four hours after the inhalation, bronchoalveolar lavage was performed four times with 0.3 ml of phosphate-buffered saline (PBS). The lavage fluid was immediately centrifuged at 400 $\times$ g for 10 min at 4°C, and cell differentials were determined by counting 500 cells stained with Wright-Giemsa solution.

Effect of tyrphostin AG-490, a specific JAK2 inhibitor. To determine whether JAK2 activation mediates antigen-induced eosinophil recruitment into the airways, we examined the effect of tyrphostin AG-490, a specific JAK2 inhibitor (15), on antigen-induced eosinophil infiltration in the airways of sensitized mice. OVA-sensitized mice were injected intraperitoneally with AG-490 (9–450 μg) (Mitsubishi Chemical Corp., Tokyo, Japan) dissolved in 0.05% DMSO in PBS at

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16 h and 3 h before the inhaled OVA challenge and at 6 h and 12 h after the challenge. As a control, OVA-sensitized mice were injected intraperitoneally with 0.05% DMSO in PBS. The eosinophil infiltration in the BALF was then evaluated at 24 h after OVA or saline inhalation.

To determine whether the *in vivo* administration of AG-490 affects cytokine production in the airways, we examined IL-2, IL-4, and IL-5 levels in the BALF of AG-490-treated mice (9–450 μg/mouse) at 24 h after OVA or saline inhalation as described below.

To determine whether the *in vivo* administration of AG-490 affects antigen-specific T cell function and thereby inhibits antigen-induced eosinophil recruitment, we examined the *in vitro* production of IL-2, IL-4, and IL-5 in spleen cells of AG-490-treated mice (9–450 μg/mouse). Twenty-four hours after OVA inhalation, *in vitro* OVA-induced production of IL-2, IL-4, and IL-5 in spleen cells of AG-490-treated or control mice was evaluated as described below.

Cytokine levels in BALF. Bronchoalveolar lavage was performed with 1.2 ml of PBS at 24 h after saline or OVA inhalation in AG-490-treated or control (PBS-treated) mice. The BALF were centrifuged at 400 $\times$ g for 10 min at 4°C, and the amount of IL-2, IL-4, and IL-5 in the supernatant was measured by the enzyme immunoassays using murine IL-2, IL-4, and IL-5 ELISA kits (Endogen Inc., Boston, MA). The assays were performed in duplicate according to the manufacturers' recommendations. The minimum significant values of these assays were 15 pg/ml of IL-2, IL-4, and IL-5.

In vitro antigen-induced cytokine production and proliferation in spleen cells. The spleen was removed from AG-490-treated or untreated mice at 24 h after OVA inhalation, and a single cell suspension of spleen cells was prepared and suspended in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Cells (2×10^5) were then cultured in triplicate in the absence or presence of OVA (100 μg/ml) in a 96-well microtiter plate at 37°C for 48 h in a humidified 5% CO_2 and 95% air. After the culture, the culture supernatant was collected and centrifuged at 400 $\times$ g at 4°C. The amount of IL-2, IL-4, and IL-5 in the supernatant was measured by the enzyme immunoassays as described above. For proliferation assays, spleen cells were cultured in the same conditions as described above, with 1 μCi of [^{3}H] thymidine added for the final 16 h.

Effect of AG-490 on JAK and Lyn phosphorylation in mouse spleen cells. Mouse spleen cells (2×10^7) were preincubated with or without AG-490 (10–50 μM) at 37°C for 10 min and then stimulated with rIL-2 (100 U/ml) and rIFN-γ (3000 U/ml) at 37°C for 15 min. For Lyn activation, cells were preincubated with or without AG-490 and then stimulated with goat anti-mouse IgM antibody (50 μg/ml) at 37°C for 15 min. The tyrosine phosphorylation of JAK kinases and Lyn kinase was detected by immunoprecipitating cell lysates with anti-mouse JAK1, JAK2, and JAK3 antibodies (Upstate Biotechnology, Lake Placid, NY) or anti-Lyn antibody (Transduction Laboratories, Lexington, KY) and then blotting with anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology).

Chemokinesis assay. Human eosinophils were purified from 100 ml of heparinized venous blood from normal individuals by Percoll density gradient centrifugation and magnetic cell sorting using MACS anti-CD16 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described by Hansel *et al.* (17). Eosinophils (purity >99%) were suspended in Hanks' balanced salt solution (HBSS).

Eosinophils (1×10^6/ml, 200 μl) were placed in the upper chamber of Transwell filters (5-μm pore diameter) (Costar Corp., Cambridge, MA) that were placed in individual wells of a 24-well cell culture plate containing 300 μl of HBSS. To evaluate chemokinesis of eosinophils, recombinant human IL-5 (0.1 and 1 nM) (Wako Pure Chemical, Tokyo) was placed in the upper and lower chambers in a checkerboard pattern. To determine whether JAK2 mediates IL-5-induced chemokinesis in eosinophils, eosinophils were incubated with AG-490 (10 and 20 μM) for 30 min at 37°C before being placed in the upper chamber. Chambers were incubated for 3 h at 37°C. Cells that migrated into the lower chamber were then counted by a flow cytometry. In our previous studies, greater than 20 μM of AG-490 prevented IL-5-induced JAK2 phosphorylation in human eosinophils (18, 19).

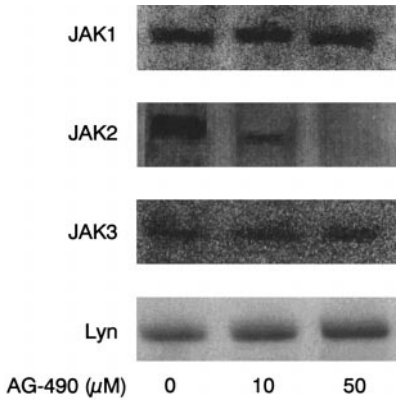

FIG. 1. Effect of AG-490 on JAK and Lyn phosphorylation in mouse spleen cells. Mouse spleen cells were preincubated with or without AG-490 (10–50 μM) at 37°C for 10 min and then stimulated with rIL-2 (100 U/ml) and rIFN-γ (3000 U/ml) at 37°C for 15 min. For Lyn activation, cells were preincubated with or without AG-490 and then stimulated with goat anti-mouse IgM antibody (50 μg/ml) at 37°C for 15 min. Cell lysates were immunoprecipitated with anti-JAK1, JAK2, and JAK3 antibodies or anti-Lyn antibody and then blotting with anti-phosphotyrosine antibody (4G10). The positions of phosphorylated JAK kinases and Lyn kinase are at 130 kDa and at 53 kDa, respectively. The data are representative of three experiments.

Binding assay of VLA-4 to fibronectin. ^{51}Cr-labeled eosinophils were preincubated with or without AG-490 (10–50 μM) for 30 min at 37°C and then stimulated with rIL-5 (10^{-10} M) for 10 min at 37°C. The binding assay of ^{51}Cr-labeled eosinophils (5×10^5) to the fibronectin-coated well of tissue culture plates was performed in triplicate for 10 min at 37°C in the presence or absence of anti-$\alpha 4\beta 1$ integrin (VLA-4) antibody (HP2/1; 10 μg/ml) (Cosmo Bio Co., Tokyo, Japan) as described previously (20).

Data analysis. Data are summarized as mean $\pm$ SD. The statistical analysis of the results was performed by the analysis of variance using Fisher's least significant difference test for multiple comparisons. p values <0.05 were considered significant.

RESULTS

Tyrphostin AG-490 Inhibits Antigen-Induced Eosinophil Infiltration into the Mouse Airways

It has been shown that tyrphostin AG-490 specifically inhibits JAK2 tyrosine kinase but does not block other tyrosine kinases including Lyn, Syk, Src, Lck, and Btk (15). We confirmed that AG-490 dose-dependently prevented the activation of JAK2 kinase but not of JAK1, JAK3, or Lyn kinases in mouse spleen cells (Fig. 1). The *in vivo* administration of JAK2 inhibitor AG-490 prevented antigen-induced eosinophil infiltration in the airways of sensitized mice. The intraperitoneal preinjection with AG-490 (450 μg/mouse) at 16 h and 3 h before the inhaled OVA challenge and

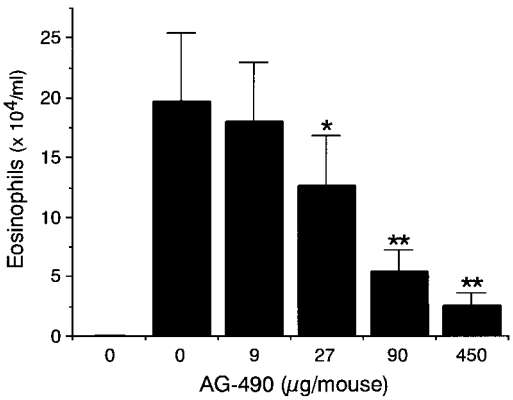

FIG. 2. Effect of AG-490 on antigen-induced eosinophil infiltration into the mouse airways. OVA-sensitized mice were injected intraperitoneally with AG-490 (9–450 μg) or 0.05% DMSO in PBS at 16 h and 3 h before the inhaled OVA challenge and at 6 h and 12 h after the challenge. The number of eosinophils recovered in the BALF was then counted at 24 h after OVA (solid columns) or saline (open column) inhalation. Data are means ± SD for 6 mice in each group. *,**Significantly different from the mean value of the control response (0.05% DMSO in PBS), *$p < 0.05$, **$p < 0.001$.

at 6 h and 12 h after the challenge significantly decreased OVA-induced eosinophil infiltration into the airways of OVA-sensitized mice at 24 h after OVA inhalation by 87% (control 19.7 ± 5.7 versus AG-490 2.6 ± 0.9 cells × 10^4/ml, mean ± SD, n = 6 mice in each group, $p < 0.001$) (Fig. 2).

The dose-dependent effect of AG-490 on antigen-induced eosinophil infiltration in the airways was then examined. The intraperitoneal preinjection with AG-490 (27 and 90 μg/mouse) from 16 h before OVA inhalation significantly decreased the OVA-induced eosinophil infiltration at 24 h by 36% and 72%, respectively (n = 6 mice in each group, $p < 0.05$) (Fig. 2). However, the administration of AG-490 (9–450 μg/mouse) did not significantly affect blood eosinophil counts at 24 h compared with those of control mice (data not shown), suggesting that the inhibition of the antigen-induced eosinophil infiltration by AG-490 was not due to the suppression of systemic eosinophilopoiesis.

Effect of AG-490 on Antigen-Induced IL-5 Production in the Airways

Because it has been shown that antigen-induced eosinophil infiltration into the airways of sensitized mice is mediated by IL-5 (2, 3), we then examined the effect of the administration of AG-490 on antigen-induced IL-5 production in the airways of sensitized mice. IL-5 levels in BALF of control mice were significantly increased at 24 h after OVA inhalation (120.3 ± 25.7 pg/ml, n = 6 mice, $p < 0.001$) as compared with those of saline inhalation (26.6 ± 3.9 pg/ml, n = 6 mice) (Fig. 3A).

However, the preinjection with AG-490 (9–450 μg/mouse) did not significantly affect IL-5 levels in BALF of sensitized mice at 24 h after OVA inhalation (120.6 ± 26.3 pg/ml, at 450 μg/mouse, n = 6 mice) as compared with those of control mice (Fig. 3A). In addition, the preinjection with AG-490 (450 μg/mouse) did not significantly affect IL-2 and IL-4 levels in BALF of sensitized mice at 24 h after OVA inhalation as compared with those of control mice (data not shown).

We further determined whether the *in vivo* administration of AG-490 affected antigen-specific T cell functions in sensitized mice. *In vitro* OVA-induced IL-5 production from spleen cells was similarly observed between AG-490-treated and untreated mice (n = 5 mice) (Fig. 3B). *In vitro* OVA-induced IL-2 and IL-4 production was not significantly different between AG-490-treated and untreated mice, either (data not

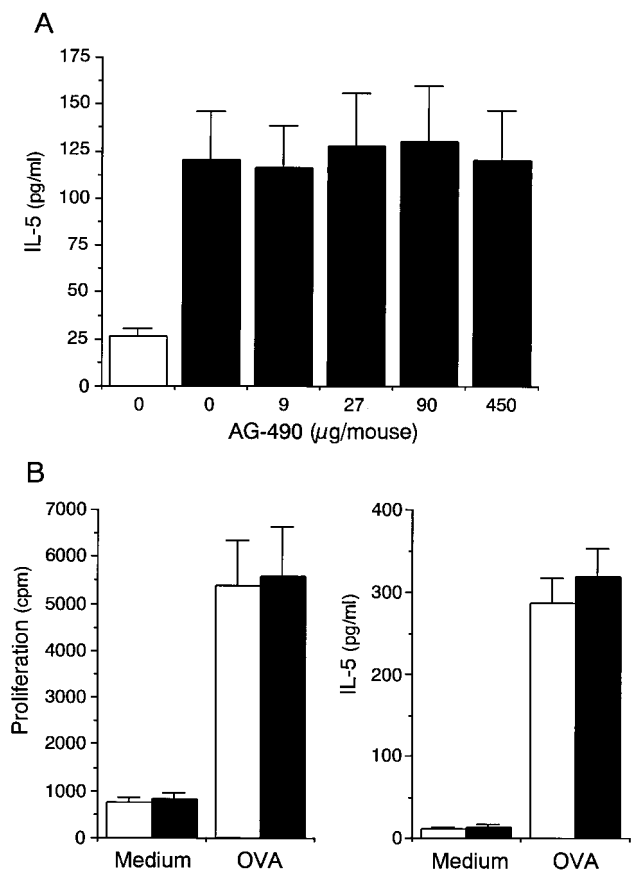

FIG. 3. Effect of AG-490 on antigen-induced IL-5 production in the mouse airways (A) and on *in vitro* antigen-induced IL-5 production and proliferation in mouse spleen cells (B). (A) OVA-sensitized mice were treated with or without AG-490 (9–450 μg/mouse) as described in Fig. 1. At 24 h after OVA (solid columns) or saline (open column) inhalation, IL-5 levels in the BALF were determined by ELISA. Data are means ± SD for 6 mice in each group. (B) At 24 h after OVA inhalation, spleen cells were prepared from OVA-sensitized, AG-490 (450 μg/mouse)-treated (solid columns) or untreated (open columns) mice, and *in vitro* OVA-induced proliferation (left) and IL-5 production (right) by spleen cells were determined by [^{3}H] thymidine uptake and ELISA, respectively. As a control, spleen cells were stimulated with medium alone. Data are means ± SD for 5 mice in each group.

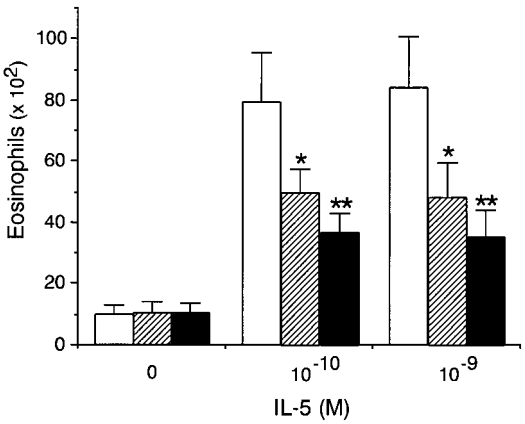

FIG. 4. Effect of AG-490 on IL-5-induced chemokinesis in human eosinophils. IL-5-induced chemokinesis of human eosinophils was evaluated by adding recombinant human IL-5 (0.1 and 1 nM) in the upper and lower chambers in a checkerboard pattern. Eosinophils were preincubated with AG-490 (10 μM; hatched columns and 20 μM; solid columns) or HBSS alone (open columns) for 30 min at 37°C before being placed in the upper chamber. Chambers were incubated for 3 h at 37°C, and cells that migrated into the lower chamber were then counted by a flow cytometry. Data are means ± SD for 3 experiments. *,**Significantly different from the mean value of the corresponding control response, *$p < 0.05$, **$p < 0.02$.

shown). *In vitro* OVA-induced T cell proliferation of spleen cells was also similarly observed between AG-490-treated and untreated mice (n = 5 mice) (Fig. 3B). These results suggest that the *in vivo* administration of AG-490 did not significantly affect antigen-induced T cell activation in sensitized mice.

AG-490 Inhibits IL-5-Induced Chemokinesis in Human Eosinophils

To determine whether JAK2 mediates IL-5-induced chemokinesis in eosinophils, we examined the effect of AG-490 on IL-5-induced chemokinesis in human eosinophils. Chemokinesis of eosinophils was significantly induced by 10^{-10} M and 10^{-9} M recombinant human IL-5 which was placed in the upper and lower chambers in a checkerboard pattern (Fig. 4). Preincubation of eosinophils with AG-490 (10 μM and 20 μM) for 30 min at 37°C significantly inhibited IL-5 (10^{-10}–10^{-9} M)-induced chemokinesis in eosinophils by 43 to 49% (at 10 μM) and 62 to 66% (at 20 μM), respectively ($p < 0.05$ and $p < 0.02$, n = 3 experiments) (Fig. 4), indicating that JAK2 activation is essential for IL-5-induced chemokinesis in eosinophils. On the other hand, AG-490 (20 μM) did not significantly affect platelet-activating factor (10^{-8}–10^{-6} M)-induced chemotaxis in eosinophils (data not shown). Because IL-5-induced chemokinesis of eosinophils is required for antigen-induced eosinophil recruitment into the airways (5), these results suggest that the *in vivo* administration of AG-490 prevented antigen-induced eosinophil infiltration in the mouse airways by inhibiting the IL-5-induced mobilization of eosinophils into the airways.

AG-490 Inhibits IL-5-Induced Adhesive Function of $\alpha 4\beta 1$ Integrin (VLA-4) in Human Eosinophils

We also examined the effect of AG-490 on IL-5-induced adhesive function of VLA-4 on eosinophils to its ligand fibronectin. IL-5 (10^{-10} M) significantly increased eosinophil binding to fibronectin (control 13.6 ± 2.2 vs. IL-5 51.0 ± 5.5% of total eosinophils added, n = 3, $p < 0.001$) (Fig. 5). Anti-VLA–4 antibody abolished the IL-5-induced increase in eosinophil binding to fibronectin (24.3 ± 4.9% of total eosinophils added, n = 3, $p < 0.005$) (Fig. 5). Preincubation of eosinophils with AG-490 (20 μM and 50 μM) for 30 min at 37°C significantly inhibited IL-5-induced eosinophil binding to fibronectin by 55% and 63%, respectively ($p < 0.05$, n = 3) (Fig. 5). These results indicate that JAK2 activation is involved in IL-5-induced VLA-4 binding to its ligand in eosinophils.

DISCUSSION

In this study, we demonstrated that tyrphostin AG-490 prevented antigen-induced eosinophil recruitment into the airways and IL-5-induced chemokinesis and

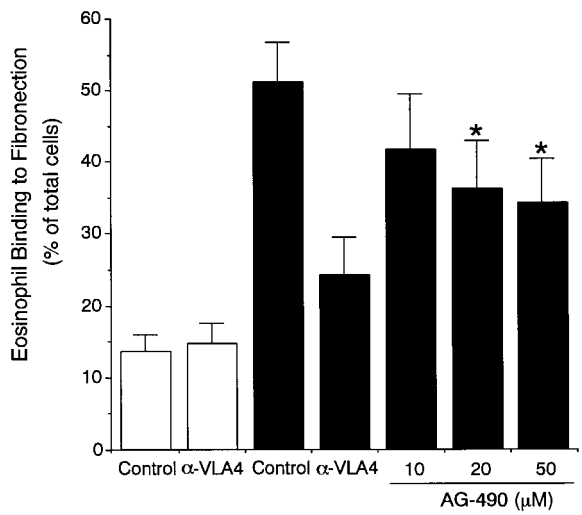

FIG. 5. Effect of AG-490 on IL-5-induced adhesive function of $\alpha 4\beta 1$ integrin (VLA-4) in human eosinophils. ^{51}Cr-labeled eosinophils were preincubated with or without AG-490 (10–50 μM) for 30 min at 37°C and then stimulated with rIL-5 (10^{-10} M) (solid columns) or HBSS alone (open columns) for 10 min at 37°C. The binding assay of ^{51}Cr-labeled eosinophils (5×10^5) to the fibronectin-coated well of tissue culture plates was performed for 10 min at 37°C in the presence or absence of anti-VLA-4 antibody (10 μg/ml). After unbound cells were washed off, bound cells were lysed by the addition of HBSS containing 1% Triton X-100 and radioactivity of the cells was counted in a gamma counter. Data are means ± SD for 3 experiments. *Significantly different from the mean value of the IL-5-induced control response, *$p < 0.05$.

adhesiveness of eosinophils (Figs. 2, 4, and 5). We also found, however, that AG-490 did not affect antigen-induced IL-5 production in the airways nor *in vitro* antigen-induced IL-5 production and T cell proliferation of spleen cells (Fig. 3). Because antigen-induced eosinophil recruitment into the airways is mediated by IL-5 (2, 3), these results indicate that JAK2 activation is critical for antigen-induced, IL-5-dependent mobilization of eosinophils into the tissue. In addition, the finding that AG-490 did not affect antigen-induced IL-5 production is consistent with the observation that JAK pathway is not involved in T cell receptor-mediated signaling leading to cytokine production (21, 22).

JAK2 associates with the common cytokine receptor β chain of IL-5 receptors (9–11). Previous studies showed that JAK2 was involved in IL-5-induced anti-apoptotic signaling in eosinophils (12, 23). In this study, we show that JAK2 is required for IL-5-induced chemokinesis of eosinophils. Because it has been shown that, in other cell types, phosphatidylinositol 3-kinase plays a central role in regulating cell migration (24, 25) and we have previously shown that IL-5 activates phosphatidylinositol 3-kinase as well as JAK2 in eosinophils (18), phosphatidylinositol 3-kinase may be a downstream target of JAK2 for IL-5-induced chemokinesis of eosinophils.

It was recently shown that *in vivo* administration of AG-490 inhibited the development of experimental autoimmune encephalomyelitis by inhibiting the infiltration of pathogenic T cells into the brain and that AG-490 decreased the binding affinity of β1-integrin on T cells to vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (26, 27). Because it has been shown that antigen-induced eosinophil recruitment into the airways is mediated by the interaction between β1-integrin on eosinophils and VCAM-1 on endothelial cells (28), it is also suggested that AG-490 may inhibit the infiltration of eosinophils into the airways by decreasing the adhesive function of β1-integrin on eosinophils (Fig. 5).

Our findings that AG-490 prevents antigen-induced eosinophil recruitment into the airways of sensitized animals have important clinical relevance to the treatment of airway inflammation in asthma. It has been shown that IL-5 plays a central role in allergic inflammation in asthma and that the IL-5-mediated allergic inflammation can be abrogated by the blockades both at the ligand level (2, 3) and at the receptor level (29). Thus, JAK2 inactivation by AG-490 is another candidate for modulation of IL-5-mediated allergic inflammation.

In summary, we have shown that tyrphostin AG-490 prevents antigen-induced eosinophil recruitment into the airways and IL-5-induced chemokinesis and adhesiveness of eosinophils. These results suggest that the blockade of JAK2 activation would be a new therapeutic approach to allergic inflammation in asthma.

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