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Fgr Deficiency Results in Defective Eosinophil Recruitment to the Lung During Allergic Airway Inflammation

Lucia Vicentini,*† Paola Mazzi,* Elena Caveggion,* Silvia Continolo,* Laura Fumagalli,* José A. Lapinet-Vera,* Clifford A. Lowell,‡ and Giorgio Berton ²*

Using a mouse model of allergic lung inflammation, we found that mice deficient of Fgr, a Src family tyrosine kinase highly expressed in myelomonocytic cells, fail to develop lung eosinophilia in response to repeated challenge with aerosolized OVA. Both tissue and airway eosinophilia were markedly reduced in fgr−/− mice, whereas mice with the sole deficiency of Hck, another Src family member, responded normally. Release of allergic mediators, such as histamine, IL-4, RANTES/CCL5, and eotaxin/CCL11, in the airways of OVA-treated animals was equal in wild-type and fgr−/− mice. However, lung eosinophilia in Fgr-deficient mice correlated with a defective accumulation of GM-CSF and IL-5 in the airways, whereas secretion of these cytokines by spleen cells in response to OVA was normal. Examination of mRNA expression in whole lung tissue allowed us to detect comparable expression of transcripts for eotaxin/CCL11, macrophage-inflammatory protein-1α/CCL3, macrophage-inflammatory protein-1β/CCL4, monocyte chemoattractant protein-1/CCL2, TCA-3/CCL1, IL-4, IL-10, IL-2, IL-3, IL-9, IL-15, and IFN-γ in OVA-sensitized wild-type and fgr−/− mice. In contrast, the increase in IL-5 and IL-13 mRNA expression was lower in fgr−/− compared with wild-type mice. These findings suggest that deficiency of Fgr results in a marked reduction of lung eosinophilia and the establishment of a positive feedback loop based on autocrine secretion of eosinophil-active cytokines. These results identify Fgr as a novel pharmacological target to control allergic inflammation.

Eosinophils are thought to play an important role in the pathobiology of asthma due to their capability to release molecules inducing tissue damage and amplifying the allergic inflammatory reaction (1–4). Mechanisms responsible for eosinophil transmigration into the lung have been well characterized in terms of adhesion molecules implicated in eosinophil-endothelium interaction, chemotactic stimuli implicated in selective eosinophil recruitment, and factors regulating eosinophil differentiation and priming (3–6). However, the intracellular signal transduction pathways triggered by adhesion receptors, receptors for lipid-derived chemoattractants and chemokines, and receptors for eosinophil-active cytokines are less well understood.

Despite the complexity in signal transduction implicated in leukocyte transmigration, both adhesion and chemoattractant receptor signaling have been reported to be blocked by inhibition, or deficiency, of tyrosine kinase activities, and tyrosine kinase inhibitors can block inflammatory responses in vivo (Refs. 4 and 7–10, and references reported therein). In our own studies with myelomonocytic cells, we found that Src family kinases play a critical role in the control of a series of adhesion-dependent neutrophil and monocyte responses likely implicated in transmigration to the inflamed tissue (11–14). Indeed, neutrophil accumulation into the liver parenchyma in the course of LPS-induced septic shock is defective in mice deficient of the Src family kinases Hck and Fgr (15).

Because Hck and Fgr have been recently implicated in signal transduction by CCR3 (16), the receptor for eotaxin/CCL11, and because Src family kinase expression by eosinophils is comparable to that of neutrophils (Refs. 17 and 18 and our unpublished observation), we addressed whether deficiency of these kinases affects pulmonary eosinophilia in a mouse model of allergic lung inflammation. In this work we show that eosinophil accumulation in the lung of both hck−/−fgr−/− and fgr−/− mice following exposure to aerosolized Ag is markedly reduced when compared with wild-type animals. However, the presence in the airway lumen of inflammatory mediators that are typically produced in allergic responses, such as histamine, IL-4, eotaxin/CCL11, and RANTES/CCL5, as well as mRNA levels for a panel of different chemokines/cytokines in lung extracts, was not different in Src family-deficient compared with wild-type animals. We conclude that Fgr deficiency results in reduced accumulation of eosinophils in the lung independently of the production of mediators of allergy. In addition, the evidence that in OVA-treated, Fgr-deficient mice the release of GM-CSF and IL-5 in the airway lumen is defective, and IL-5 and IL-13 mRNA expression in lung extracts is reduced, allows us to conclude that airway eosinophils participate in a positive feedback loop by locally generating cytokines, regulating their recruitment into inflamed tissues. These findings identify Fgr as an important target for the pharmacological control of allergic inflammation.

Materials and Methods

Animals and cell and fluid sample preparation

Male and female C57BL/6J, 6- to 8-wk-old mice were used as wild-type control animals. Generation of hck−/−fgr−/− double knockout and hck−/− and fgr−/− single knockout mice has been previously described (19). Mice were treated according to a standardized procedure consisting of a first
phase of sensitization and a second phase of induction of the allergic response (20). Animals were first injected i.p. with OVA (100 μg/mouse; Sigma-Aldrich, St. Louis, MO) on day 1, then exposed to aerosolized OVA (2% in PBS) for 5 min on day 8, and finally exposed to aerosolized Ag (1% in PBS) for 20 min daily on days 15–21. Control animals were injected with PBS alone and then exposed to aerosolized PBS. Within 1 h after the last Ag challenge on the day indicated in Results, animals were anesthetized and a blood sample was taken from the abdominal aorta to prepare smears for differential cell count and serum samples. Subsequently, mice were killed and the tracheas were cannulated. Airways were washed four times with 0.5 ml of ice-cold PBS and, after centrifugation, bronchoalveolar lavage (BAL) supernatants were frozen immediately at −80°C. Cell pellets were resuspended in PBS and total cells were counted. Cytocentrifuge preparations were stored at −20°C before staining for differential cells counts. Bone marrow cells were obtained from mouse femurs and tibias, as described (21), and cytocentrifuge preparations were stored as described above for BAL cells. Lungs were excised, rolled in Tissue Tek OCT (Raymond Lamb, London, U.K.), frozen in liquid nitrogen, and stored at −80°C. For in vitro cytokine production assays, spleens were removed and kept in ice-cold PBS before cell preparation (see below).

Assessment of sensitization

OVA-specific serum IgE were determined by ELISA. Wells were incubated overnight at 4°C with 100 μl OVA (100 μg/ml) in carbonate/bicarbonate buffer (pH 9.6), washed three times with PBS-Tween 0.05%, and finally incubated at room temperature with 200 μl 0.05% PBS-Tween and 1% BSA (dilution buffer). After further washing, wells were filled with 100 μl of different serum dilutions in dilution buffer. After 2 h of incubation at room temperature, wells were washed and biotin-conjugated rat anti-mouse IgE mAb (1 μg/ml; BD Pharmingen, San Diego, CA) followed by streptavidin-HRP (1/1000; Sigma-Aldrich), both in dilution buffer, were used to detect the presence of anti-OVA IgE.

Lung histochemistry and differential cell counts

Lung cryostat sections (7 μm thickness), BAL and bone marrow cell cytocentrifuge preparations, and blood smears were stained for phenylhydrazine-resistin peroxidase, which stains specifically eosinophil granulocytes (22, 23). Myeloperoxidase was first inhibited with 0.1% phenylhydrazine in PBS for 30 min, before peroxidase staining with 3′-3′-diaminobenzidine. Nuclei were counterstained with hematoxylin. Pulmonary eosinophilia in lung sections was semiquantitatively assessed by grading eosinophil infiltration severity as follows: grade 1, absence of positive cells (or rarely detectable in the parenchyma); grade 2, few scattered groups of positive cells, mostly parenchymal or perivascular; grade 3, moderate perivascular and peribronchial infiltration of eosinophils in most fields; grade 4, diffuse, heavy eosinophil infiltration. At least 200 cells were counted in BAL cell preparations and the different cell types were expressed as a percentage of total cells. The number of eosinophils in bone marrow preparations and in blood smears was expressed as a percentage of total or of white blood cells, respectively. At least 200 cells were counted on each slide.

Assays in cell-free BAL supernatants

IL-4 and IL-5 content in the BAL was measured by ELISA kits from Endogen (Cambridge, MA). GM-CSF, RANTES/CCL5, IL-1α, and eotaxin/CCL11 levels were determined by ELISA kits from R&D Systems (Minneapolis, MN). Assays were performed in duplicate, according to the protocols provided by the manufacturers. Histamine content in BAL supernatant was determined by an enzyme immunoassay kit (ImmunoTech, Marseille, France). The albumin content in the BAL was assayed by ELISA using rabbit anti-murine albumin as described (24) with reagents kindly provided by Dr. A. Mocsai (Semmelweis University of Medicine, Budapest, Hungary).

In vitro Ag-induced cytokine production by spleen cells

Spleen cells, obtained at day 18 of OVA challenge, were suspended at 4 × 10^6/ml in RPMI 1640 medium supplemented with 10% FCS, 10 mM glucose, 100 μM penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Cells (6 × 10^6/well) were cultured in the presence or absence of OVA (100 μg/ml) at 37°C, in a humidified 5% CO2 atmosphere, for 48 h. The amount of IL-1α, GM-CSF, and IL-5 in the supernatant was measured by ELISA, as described above.

RNA isolation and RPA

Total RNA was extracted from ~100 mg of fresh lung tissue by the guanidinium isothiocyanate method and then analyzed by RNase protection assay (RPA) (25). The RiboQuant MC-K-1b, MC-K-2b, and MC-K-5 Multi-Probe Template sets were used according to the manufacturer’s instructions (BD Pharmingen). The extent of hybridization was quantitatively analyzed in an Instamager (Packard Instrument, Meriden, CT) and plotted after actin normalization.

Statistical analysis

Data are expressed as mean values ± SD. Statistical significance between different groups of mice was calculated by unpaired Student’s t test.

Results

Defective OVA-induced eosinophil accumulation in the lung of hck−/−fgr−/− double knockout and fgr−/− single knockout mice

To address the role of the Src family tyrosine kinases Hck and Fgr in eosinophil migration to inflammatory sites, we exploited a widely used model of OVA-induced lung allergic inflammation (20). Mice were sensitized by i.p. injection of OVA on day 1, followed by repeated exposures to aerosolized Ag on day 8 and then on days 15–21 (see Materials and Methods). Within a few days after exposure to aerosolized OVA, eosinophils begin to accumulate in the lung of OVA-treated animals and can be easily detected in both the interstitium and the bronchoalveolar space. Staining frozen sections of lung for eosinophil-specific peroxidase (see Materials and Methods) allowed us to detect a marked eosinophil infiltrate in the lung parenchyma of OVA-treated wild-type mice on day 18 (Fig. 1b) or 21 (data not shown) of treatment. In marked contrast, eosinophils were rarely seen in the lung parenchyma of hck−/−fgr−/− mice (Fig. 1c). Pulmonary eosinophilia was semiquantitatively assessed by examining lung sections stained for eosinophil-specific peroxidase, then grading the size of pulmonary infiltrates from 1 (absence of any infiltrating eosinophil) to 4 (heavy perivascular and peribronchiolar infiltration) (see Materials and Methods) (Fig. 1, d and e). On day 18, no eosinophil infiltrate was detected in the lung of OVA-treated hck−/−fgr−/− mice (Fig. 1d, filled bars), and the slight eosinophil infiltration observed in the lung of these animals on day 21 was significantly lower than that observed in OVA-treated wild-type mice (Fig. 1e).

To determine whether deficiency of both Hck and Fgr, or loss of only single kinase, was responsible for the impaired eosinophil recruitment seen in hck−/−fgr−/− animals, we examined the responses of hck−/− and fgr−/− single mutant mice. As shown in Fig. 1, d and e, hck−/− mice responded to OVA challenge equivalently to wild-type animals on days 18 and 21. However, in fgr−/− mice pulmonary eosinophilia in response to OVA was reduced to the levels found in hck−/−fgr−/− animals, demonstrating that Fgr alone plays the predominant role in regulating eosinophil transmigration into the lung.

In the experiments reported in Fig. 1, lung eosinophilia was examined within 1 h after the last exposure to OVA (see Materials and Methods). When eosinophil accumulation in the lung of hck−/−fgr−/− mice was examined at later times (3 and 24 h) after the last OVA challenge on day 18, we found an almost total absence of eosinophil infiltration (Fig. 1f). Together with the data reported in Fig. 1, d and e, this finding suggests that reduction of lung eosinophil infiltration in Fgr-deficient mice reflects more an absolute impairment of, rather than just a delay in, eosinophil transmigration to the tissue.

Defective eosinophil accumulation in the airway lumen of hck−/−fgr−/− double knockout and fgr−/− single knockout mice

To know whether Fgr deficiency also affected eosinophil infiltration into the airway lumen, differential cell counts were performed

3 Abbreviations used in this paper: BAL, bronchoalveolar lavage; RPA, RNase protection assay; MMP, macrophage-inflammatory protein.
in BAL from PBS- or OVA-treated mice (Fig. 2). Cells present in the BAL of PBS-treated mice were mainly macrophages, but after OVA treatment eosinophils represented the major BAL cell population in wild-type mice (Fig. 2a). In marked contrast, in double knockout hck\(^{-/-}\)fgr\(^{-/-}\) mice OVA treatment induced a much lower eosinophil accumulation into the airway lumen (Fig. 2b). In concert with the parenchymal eosinophil accumulation (Fig. 1, d and e), eosinophil number in the BAL of OVA-treated hck\(^{-/-}\) mice was quite similar (Fig. 2c). However, single mutant fgr\(^{-/-}\) mice manifested markedly reduced eosinophil infiltration in the airway lumen (Fig. 2d). Defective eosinophil accumulation in the airways of fgr\(^{-/-}\) mice was selective, as indicated by the finding that macrophage number increased in the BAL of OVA-sensitized fgr\(^{-/-}\) mice. Lymphocyte and neutrophil counts were variable and accounted for <10% of the total BAL cells in all strains of mice, and no significant differences in cell numbers were observed in wild-type vs knockout mice.

As previously reported (20), we also found that eosinophil accumulation in the BAL increases with time after exposure to aerosolized OVA on day 15. In wild-type mice, eosinophils constituted 45.7 ± 10.3% (n = 3), 62.3 ± 28.9% (n = 4), and 74.7 ± 5% (n = 4) of the total BAL cells on days 16, 18, and 21 of OVA treatment, respectively. In hck\(^{-/-}\)fgr\(^{-/-}\) mice, the percentages of eosinophils at the same time points were 2.8 ± 1.7% (n = 4, day 16, p < 0.01), 7.1 ± 5.8% (n = 5, day 18, p < 0.05), and 11.5 ± 9.9% (n = 4, day 21, p < 0.01). These data show that eosinophil infiltration in the airway lumen is markedly defective in hck\(^{-/-}\)fgr\(^{-/-}\) mice independently of the time of exposure to the Ag.

Normal induction of inflammatory cytokines/chemokines in the airways of hck\(^{-/-}\)fgr\(^{-/-}\) double knockout and fgr\(^{-/-}\) single knockout mice

We asked whether defective eosinophil accumulation into the lung of knockout animals could be due to a defect in the induction of

FIGURE 1. Eosinophil accumulation in the lung parenchyma of OVA-sensitized animals is defective in mice deficient of Fgr. Frozen sections of lungs from PBS-treated wild-type (a), OVA-sensitized wild-type (b), and OVA-sensitized hck\(^{-/-}\)fgr\(^{-/-}\) (c) mice were stained for phenylhydrazine-resistant peroxidase and counterstained with hematoxylin as described in Materials and Methods. Pulmonary eosinophilia was assessed by grading the size of pulmonary infiltrates (see Materials and Methods) on day 18 (d) or 21 (e) of OVA treatment or after different hours from OVA challenge at day 18 (f). Error bars indicate SD; *, p < 0.05 and **, p < 0.01 compared with wild-type mice. Mean results of four to six (day 18) and three (day 21) experiments performed with PBS-treated mice are reported. Mean results of eight (wild type) and six (knockouts) experiments on day 18 of OVA treatment, and of 10 (wild type) and six (knockouts) on day 21, are reported. In f, mean results of six (wild type) and five (knockouts) experiments after 3 or 24 h from OVA challenge are reported.
Eosinophil accumulation in the airway lumen of OVA-sensitized animals is defective in mice deficient of Fgr. Cytocentrifuge preparations from BAL of PBS- or OVA-treated animals were stained for phenylhydrazine-resistant peroxidase and counterstained with hematoxylin as described in Materials and Methods to calculate the percentage of eosinophils (Eos), macrophages (Macr), neutrophils (PMN), and lymphocytes (Lymph). Cell number for each cell type was calculated from the number of total cells counted in the BAL. Error bars indicate SD. Significant differences in BAL cell number between knockout and wild-type mice are indicated: *, p < 0.05; **, p < 0.01; ***, p < 0.001. Results are means of 8 (PBS) and 16 (OVA) experiments with wild-type, 8 (PBS) and 13 (OVA) with hck\(^{-/-}\)fgr\(^{-/-}\), 5 (PBS) and 14 (OVA) with fgr\(^{-/-}\), and 4 (PBS) and 6 (OVA) with hck\(^{-/-}\) mice. Results obtained at days 18 and 21 of treatment were pooled, because values were comparable.

inflammation by examining accumulation in the BAL fluid of typical markers of the allergic response. On day 18 after Ag challenge, histamine (Fig. 3a), a marker of mast cell degranulation, and IL-4 (Fig. 3b), a cytokine released by Th2 lymphocytes and mast cells, increased to a comparable extent in the BAL fluid of wild-type, double knockout hck\(^{-/-}\)fgr\(^{-/-}\) and single knockout fgr\(^{-/-}\) animals. In addition, a proinflammatory cytokine such as IL-1\(\alpha\) (Fig. 3c) and two chemokines known to act as chemoattractants for eosinophils such as RANTES/CCL5 (Fig. 3d) and eotaxin/CCL11 (Fig. 3e) increased in the BAL fluid of knockout mice equally as well as in the BAL fluid of wild-type animals. IL-4, RANTES/CCL5, eotaxin/CCL11, and IL-1\(\alpha\) are produced by different cells implicated in allergic inflammation, such as Th2 lymphocytes, mast cells, macrophages, and tissue epithelial/endothelial cells, and have been shown to play an essential role in regulating pulmonary eosinophilia (2–6). We conclude that deficiency of Fgr does not result in a defective activation of different cell types participating in the development of the inflammatory response but causes a selective defect in the recruitment of eosinophils to the lung. That the development of an allergic type of response to the sensitization protocol used occurred normally in knockout animals was confirmed by experiments showing that the increase in anti-OVA-specific IgE was equal in the serum of wild-type and knockout animals (data not shown).

Despite the fact that mutant mice released normal levels of histamine and proinflammatory cytokines following OVA challenge, the markedly reduced infiltration of eosinophils into the lung resulted in decreased damage to the endothelial and bronchoepithelial barrier as revealed by detection of albumin in the BAL fluid (Fig. 4).

Defective eosinophil accumulation in the lung of hck\(^{-/-}\)fgr\(^{-/-}\) double knockout and fgr\(^{-/-}\) single knockout mice is accompanied by decreased accumulation of the eosinophil-active cytokines IL-5 and GM-CSF in the airway lumen

Eosinophil accumulation into tissues in allergic inflammation is thought to depend not only on release of chemokines selectively recruiting this cell type but also on cell priming by cytokines such as IL-5 and GM-CSF (1, 2, 4). IL-5 and GM-CSF potentiate the eosinophil response to specific chemoattractants and also induce eosinophil differentiation and release into the blood stream. Correlated with the reduced eosinophil recruitment in the lung of hck\(^{-/-}\)fgr\(^{-/-}\) and fgr\(^{-/-}\) mice, we found that levels of IL-5 and GM-CSF in the BAL fluid from these animals did not increase upon induction of the allergic response (Fig. 5, a and b). In contrast, these cytokines were dramatically increased in the airway lumen of wild-type mice following OVA sensitization. Defective IL-5 and GM-CSF production in OVA-treated knockout animals correlated with a significant reduction of blood eosinophilia as well (Fig. 5c). Although the level of blood eosinophils did increase in hck\(^{-/-}\)fgr\(^{-/-}\) and fgr\(^{-/-}\) mice following OVA sensitization (see Fig. 5), the change was significantly less than in wild-type animals. Additionally, bone marrow eosinophilia was reduced in knockout mice and correlated with lower eosinophil-active cytokine production (Fig. 5d).

Normal, OVA-induced cytokine production by spleen cells of OVA-sensitized hck\(^{-/-}\)fgr\(^{-/-}\) double knockout and fgr\(^{-/-}\) single knockout mice

To determine whether reduced accumulation of eosinophil-active cytokines in the airway lumen is due to a cell-intrinsic inability in cytokine production, we examined cytokine production by spleen cells obtained from OVA-sensitized animals. As reported in Fig. 6, spleen cells from OVA-treated wild-type, hck\(^{-/-}\)fgr\(^{-/-}\), and fgr\(^{-/-}\) mice produced equally high amounts of the proinflammatory cytokine IL-1\(\alpha\) and the eosinophil-active cytokines GM-CSF and IL-5 when exposed in vitro to OVA for 48 h. These data indicate that deficiency of Hck and Fgr does not cause an intrinsic block in GM-CSF or IL-5 production.

Expression of chemokines/cytokines mRNA in the lung of OVA-sensitized wild-type and fgr\(^{-/-}\) knockout mice

To strengthen the conclusion that Fgr deficiency affects eosinophil responses selectively, we analyzed expression of a wide panel of chemokines and cytokines released by different cell types known to infiltrate the lung in allergic reactions or resident in the lung
parenchyma (Fig. 7). Using RPA, we did not detect any difference in the OVA-induced increase of transcript levels for eotaxin/CCL11, macrophage-inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, monocyte chemoattractant protein-1/CCL2 and TCA-3/CCL1 in the lung of wild-type and fgr mice. Also, the expression of mRNA for IL-4 and IL-10, two classical Th2-type cytokines, was equal in lung extracts from wild-type and fgr mice. In contrast, mRNA levels for two other Th2-type cytokines, IL-5 and IL-13, increased to a much lower extent, albeit significantly, in the lung of OVA-treated fgr mice. Normal expression of IL-4 concomitantly with reduced expression of IL-5 mRNA in the lung of fgr mice is concordant with the levels of the IL-4 and IL-5 protein detected in the airways (Figs. 3 and 5). Because IL-13 is thought to be produced by eosinophils (26, 27), we conclude that reduced IL-5 and IL-13 mRNA levels in the lung of knockout animals likely reflect a reduced eosinophil accumulation in the tissue. Conversely, normal expression of IL-4, IL-10, and TCA-3/CCL1, another Th2-specific marker (28, 29), may be taken as a strong indication that Th2 lymphocytes accumulate normally in the lung parenchyma of fgr mice, as expected by the lack of expression of Fgr in T lymphocytes (30). No difference was found between wild-type and fgr mice in lung expression of mRNA for a wide panel of cytokines reflecting Th1-mediated responses such as IL-2, IL-3, IL-9, IL-15, IFN-γ (Fig. 7), or IL-1α and IL-12 (data not shown).

Discussion
Asthma is characterized by infiltration into the lung of different leukocyte subsets. Among cells present in the airways, Th2 cells, mast cells, and eosinophils are thought to play a major role in the development of allergic inflammation (2). Macrophages and resident lung cells also contribute to changes that characterize this inflammatory response (20). The mediators produced during allergic responses have been elucidated in great detail. IL-4 produced by Th2 lymphocytes is essential to promote synthesis of IgE, as
well Th2 differentiation and recruitment (31). Upon encounter with
the Ag, IgE triggers release of different mast cell products, includ-
ing lipid-derived inflammatory mediators, histamine, and cyto-
kines that, besides inducing alteration of endothelial permeability
and edema formation, participate in the recruitment and activation
of other cell types (2). Among these, eosinophils are considered to
play a major role in further development of inflamma-
tion, tissue damage, and tissue remodeling (3).

Although mouse models of allergic lung inflammation do not
reproduce all the features of the human asthmatic reaction (32),
mechanisms regulating pulmonary eosinophilia have been ad-
dressed in great detail by exploiting the availability of mice with
the genetic deficiency of specific genes implicated in asthma. For
example, genetic deficiency of ICAM-1/2 (33, 34) and VCAM-1
(20), or knockout of the VCAM-1-inducing cytokine IL-12 (35),
have been reported to result in reduced pulmonary eosinophilia. In
addition, mice with the knockout of IL-4 (36) and IL-5 (Ref. 37
and reviewed in Refs. 2 and 38), present a marked defect in the
development of a lung allergic reaction and eosinophil accumu-
lation. Whereas defective IL-4 production likely affects different re-
sponses, such as Th2 generation and recruitment, IgE secretion,
and other cytokine production (39–41), a lack of IL-5 directly
hampers eosinophil responses. In fact, together with other eosino-
phil-active cytokines, IL-5 plays an essential role in eosinophil
differentiation and priming for an enhanced chemotactic response
(1–4). Regulation of lung eosinophilia in allergic inflammation has
been recently addressed at the intracellular level. For example,
mice deficient in STAT-6 (42–44) or the p50 subunit of NF-κB

**FIGURE 5.** Deficiency of Fgr results in defective IL-5 and GM-CSF accumulation in the airway lumen and a reduced blood and bone marrow eosinophilia. BAL was collected from PBS- or OVA-treated animals at day 18, and cytokines were assayed in the cell-free supernatants as described in Materials and Methods. The percentage of eosinophils was calculated by counting eosinophil-specific peroxidase-positive cells in blood smears or bone marrow cytograms (see Materials and Methods). Error bars indicate SD; *, p < 0.05 and **, p < 0.01 compared with wild-type animals. After OVA treatment, the increase in the percentage of blood eosinophils was statistically significant in all mouse strains, whereas the increase in the percentage of bone marrow eosinophil was significant only in wild-type mice. Results are means of 9–10 (PBS) and 9–12 (OVA) experiments.

**FIGURE 6.** Spleen cells from OVA-sensitized, Fgr-deficient mice are not defective in the release of proinflammatory cytokine IL-1α and the eosino-
phil-active cytokines GM-CSF and IL-5 in response to OVA Ag. Spleen cells were obtained from OVA-sensitized (day 18) animals and cultivated for 48 h
in the absence (control) or the presence (plus OVA) of 100 μg/ml OVA before assaying IL-1α and GM-CSF in cell-free supernatants (see Materials and
Methods). Mean results ± SD of four independent experiments are reported.
Importantly, this defective eosinophil response in Fgr-deficient mice results in a marked reduction in eosinophil accumulation into the lung in the course of an allergic response. In fact, in Fgr-deficient mice, IgE and release in the airways of inflammatory mediators, such as histamine, IL-4, and IL-12 in the lung of OVA-treated fgr−/− mice equaled that found in wild-type mice. Normal release of inflammatory mediators from mast cells, T lymphocytes, and tissue cells in Fgr-deficient mice is understandable in light of the selective expression of this Src family kinase in the myelomonocytic lineage, normal and transformed B lymphocytes, and NK cells (30, 49, 50). Like neutrophils, eosinophils express predominantly the three Src family members Lyn, Hck, and Fgr (Refs. 16–18 and our unpublished observation).

Defective eosinophil accumulation in the lung of OVA-sensitized fgr−/− mice correlated with a markedly reduced release of the eosinophil-active cytokines IL-5 and GM-CSF in the airways. In addition, expression of mRNA for IL-5 and IL-13, another cytokine regulating tissue eosinophilia (26, 27), was lower in the lung of fgr−/− mice. In accord with the well-established role of IL-5 and GM-CSF in eosinophil maturation, fgr−/− mice also developed a reduced blood and bone marrow eosinophilia upon allergen challenge. The normal level of histamine and prototypical Th2 cytokines present in the lung of fgr−/− mice suggests that the reduced levels of IL-5 and GM-CSF are probably not due to defects in mast cell or Th2 cell recruitment to the lung during the OVA-induced response. Moreover, the normal production of IL-5 and GM-CSF by spleen cells from OVA-sensitized fgr−/− mice demonstrates that the mutation does not cause a cell-intrinsic block in production of these cytokines. The simplest conclusion from our findings is that eosinophils themselves produce significant amounts of IL-5, IL-13, and GM-CSF, as has been shown in previous studies (2, 26, 27, 38), and that impaired recruitment of these cells to the lung in Fgr-deficient mice is the cause of the reduced eosinophil-active cytokine levels. Previous studies found the same correlation between defective eosinophil accumulation in the lung and reduced BAL IL-5 secretion in OVA-sensitized ICAM-1-deficient mice (33).

Defective eosinophil accumulation in the lung of OVA-sensitized fgr−/− mice may depend on at least three different, albeit not mutually exclusive, alterations. Fgr-deficient eosinophils may have an intrinsic defect in their capability to emigrate from blood into tissues. Alternatively, Fgr may be implicated in a signaling pathway regulating autocrine cytokine secretion in response to integrin ligation or other stimuli. According to this last hypothesis, lack of secretion of cytokines or other mediators by Fgr-deficient eosinophils upon migration into the lung would prevent triggering of a positive feedback loop responsible for eosinophil recruitment into the tissue. Finally, Fgr deficiency may result in alterations in eosinophil differentiation and priming by eosinophil-active cytokines. In this context, it is important to note that Lyn, another Src family kinase, has been recently shown to be implicated in IL-5-stimulated eosinophil differentiation (18). Despite the fact that this study provided evidence that both Fgr and Hck were unlikely involved in IL-5 signaling in eosinophils, the hypothesis that Fgr and Hck do not play any role in eosinophil differentiation must be formally proved. Insights on the role of Fgr in regulating different eosinophil responses await appropriate protocols to isolate from knockout mice an eosinophil number high enough to make in vitro studies feasible.

It is tempting to speculate, based on the data so far available on the role of Src family kinases in regulating granulocyte responses, that the primary defect resulting from Fgr deficiency is at the level of eosinophil transmigration from the blood to the tissue. Hck and Fgr were found to associate with CCR3 following eotaxin/CCL11 binding in human eosinophils (16), and tyrosine kinase inhibitors block eosinophil migration (16, 51, 52). The participation of Src family kinases in signaling by trimeric G protein-coupled receptors...
(53, 54) suggests that Fgr may have a role in eotaxin/CCL11 signaling, which could contribute to the reduced eosinophil accumulation in the lung of fgr−/− mice. However, neutrophils derived from fgr−/− mice have no apparent defects in responses to chemokines such as MIP-1α/CCL3, MIP-2/CXCCL1, or chemotractions such as FMLP (Y. Zhang and C. A. Lowell, unpublished data). In contrast, we demonstrated that Src family kinases play a central role in signal transduction by integrins in myelomonocytic cells (11, 12, 14) and, at the same time, regulate their movement both in vitro (21) and in response to inflammatory injuries in vivo (Refs. 13 and 15 and R. Holmes, M. Zhou, G. Fann, G. Benton, and C. A. Lowell, unpublished data). It should be also noted that, in the context of granulocyte-endothelium interaction and transmigration, integrin signaling may serve to trigger rearrangement of the cell cytoskeleton, thus promoting firm adhesion, and to activate selective cell functions such as reactive oxygen intermediate generation and degranulation (12, 14). Interestingly, cross-linking of both β2 and β1 integrins triggers human eosinophil-reactive oxygen intermediate generation (55) and degranulation (L. Vicentini, P. Mazzi, and G. Benton, unpublished data). The recent finding that β2 integrin signaling triggers the release of heparin-binding protein/tepin/CAP37 from neutrophils, thus inducing formation of gaps in endothelial monolayers, add clues to envision the possible role of integrin-induced cell responses in the regulation of granulocyte recruitment into inflamed tissues (56).

In all the in vitro and in vivo studies we have done with Src family kinase knockout mice, we found that the deficiency of only Fgr or Hck was insufficient to cause any phenotypic alterations in myelomonocytic cell responses (12, 14, 15, 21). We report in this work that the sole deficiency of Fgr is sufficient to markedly reduce eosinophil accumulation in lung allergic inflammation. Because inhibition of Fgr should not affect host defenses, its role in regulating tissue eosinophilia makes it a powerful potential target to control allergic reactions.

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