Pathogenicity of a disease-associated human IL-4 receptor allele in experimental asthma

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Asthma is an obstructive airway disease that affects an unusually large segment of the population and results in significant morbidity, and at times mortality. It is well appreciated that predisposition to asthma is genetically determined, whereas its clinical expression is controlled by environmental factors (Gern et al., 1999). Genes of the IL-4–IL-13–IL-4R cytokine pathway have been identified as leading candidates in conferring susceptibility to asthma (Ober and Hoffjan, 2006). These functionally overlapping cytokines are key effectors of Th2-dependent responses, including stimulation of IgE synthesis, modulation of lymphocyte and antigen-presenting cell function, and induction of allergic inflammation. In the allergen-exposed airway, this inflammatory response involves recruitment of eosinophils, mucin hypersecretion, generation of airway hyperresponsiveness (AHR), and, eventually, airway remodeling, characterized by subepithelial fibrosis, neovascularization, and other permanent alterations in airway microanatomy (Elas et al., 1999).

IL-4 and IL-13 share a common receptor component, the IL-4Rα chain, that pairs with distinct subunits (Nelms et al., 1999; Hershey, 2003; Chatila, 2004). IL-4Rα pairs with the common γc chain to form a type I IL-4R complex that is found predominantly in hematopoietic cells and is exclusive for IL-4. IL-4Rα also pairs with the IL-13Rα1 subunit to form a type II IL-4R that binds both IL-4 and IL-13. The type II receptor is expressed on both hematopoietic and nonhematopoietic cells such as airway epithelium. IL-4 and IL-13 activate receptor-associated Janus kinases, which initiate several intracellular signaling cascades by phosphorylating specific tyrosine (Y) residues in the cytoplasmic domain of IL-4Rα (Nelms et al., 1999; Hershey, 2003; Chatila, 2004). Phosphorylation of Y575, Y603, and Y633 of human IL-4Rα mobilizes the transcription factor STAT6, which induces IL-4– and IL-13–responsive genes. Additional cell growth and regulatory functions are served by Y497, which ...
activates phosphatidylinositol 3 (PI3)–kinase and mitogen-activated protein kinase (MAPK) pathways, and by an immunoreceptor tyrosine-based inhibitory motif (ITIM) at Y713 that activates phosphotyrosine and inositol phosphates.

An essential role for IL-4Rα signaling in asthma pathogenesis has been established (Chatila, 2004). Increased expression of IL-13 and, to a lesser extent, IL-4 in mouse airways reproduces many of the pathophysiological changes that are typical of asthma (Elia et al., 1999). Blockade of IL-13 or deletion of IL-4Rα or STAT6 genes renders mice resistant to the induction of experimental allergic asthma (Grüning et al., 1998; Wills-Karp et al., 1998). IL-4Rα chain expression in resident airway tissues is required for development of allergic airway inflammation upon antigen exposure. Induction of AHR, goblet cell metaplasia, and mucin overproduction have been found to be dependent on IL-4Rα signaling in airway epithelial cells (Kuperman et al., 2002). Furthermore, codifying genetic polymorphisms in the human IL-4Rα chain gene have been implicated in susceptibility to both atopy and asthma (Hershey et al., 1997; Ober et al., 2000; Howard et al., 2002). Of particular interest is the Q576R polymorphism that is associated with asthma susceptibility in outbred populations, especially severe asthma (Hershey et al., 1997; Rosa-Rosa et al., 1999; Ober et al., 2000; Sandford et al., 2000; Wenzel et al., 2007). The Q576R polymorphism has also been linked to severe respiratory syncytial virus–induced bronchiolitis (Hoebee et al., 2003), rapid decline in lung function in smokers (He et al., 2003), and heightened allergen sensitization in the context of maternal smoking (Liu et al., 2004). Of interest, this allele is overrepresented in the African-American population (70% allele frequency in African Americans vs. 20% in Caucasians, giving rise to 50 and 4% homozygosity, respectively; Caggana et al., 1999; Ober et al., 2000; Wu et al., 2001; Akinbami and Schoendorf, 2002; Manno et al., 2002). In concert with increased frequency of the Q576R polymorphism, African Americans suffer from heightened asthma prevalence and severity. To address the role of the Q576R polymorphism in allergic airway inflammation, we have developed a mouse model in which the Q576R allele, which is conserved in mice, is changed to R576.

RESULTS
Generation of IL-4RαR576 mutant mice by targeted knock–in mutagenesis
To elucidate the impact of the human Q576R polymorphism on IL-4Rα function, we adopted a genetic approach that took advantage of the conservation of the Q576 motif (peptide sequence 574–GYQEQFG–579) in mouse and human to substitute the equivalent glutamine residue of the mouse receptor (also Q576) with arginine. A targeting construct was designed to replace exon 12 of Il4ra of embryonic stem (ES) cells with another bearing AG→GA substitutions at the second and third positions of the Q576 codon that changed the codon specificity to R576 (Fig. S1 A; Wrighton et al., 1992). Successfully targeted ES clones were used to derive mouse male chimera that transmitted the R576 mutation into the germline (Fig. S1 B). Matings of IL-4Rα heterozygotes (Hets) resulted in generation of R576 homozygous mutant mice in numbers consistent with Mendelian inheritance. Homozygous mutant mice of both sexes were phenotypically indistinguishable from their WT and Het littermates. The transmission and integrity of the mutant allele was verified by several methods, including Southern blot analysis, allele-specific PCR amplification, and direct sequencing of genomic DNA (Fig. S1, B–D; and not depicted). Expression of mutant receptor protein was verified by flow cytometry to be closely matched to WT controls (Fig. S1 E). Analysis revealed the T and B lymphocytes of the IL-4Rα R576 mutant mice to be normal in number and phenotype (unpublished data).

The R576 allele promotes antigen–induced allergic airway inflammation and remodeling
The functional consequences of the R576 mutation were analyzed in antigen-driven mouse models of allergic airway inflammation. In an acute model of allergic airway inflammation, C129.IIl4raR576/R576 and C129.IIl4raQ576/Q576 mice were i.p. sensitized and boosted with OVA in alum, and challenged at 4 wk after sensitization with inhaled OVA for three consecutive days. Control mice were immunized and boosted with PBS/alum and challenged with inhaled OVA. Examination of lung tissue of WT and mutant mice stained with hemotoxylin and eosin (H&E) revealed patchy peribronchial inflammatory infiltrates, composed primarily of eosinophils and lymphocytes, that were substantially more prominent in OVA–sensitized and –challenged C129.IIl4raR576/R576 mice as compared with C129.IIl4raQ576/Q576 controls (Fig. 1 A). In contrast, sham–immunized mice exposed to aerosolized OVA showed normal lung histology (unpublished data). Significantly, OVA–challenged C129.IIl4raR576/R576 mutants exhibited markedly increased peribronchial and perivascular inflammation and increased numbers of goblet cells compared with C129.IIl4raQ576/Q576 controls (Fig. 1, B and C; Grüning et al., 1998; Wills-Karp et al., 1998; Dabbagh et al., 1999; Kuperman et al., 2002). Analysis of bronchoalveolar lavage (BAL) fluid of OVA–sensitized and –challenged mice revealed that C129.IIl4raR576/R576 mice exhibited increased accumulation of eosinophils in the airways as compared with similarly treated controls (Fig. 1 D). These results indicated that the R576 substitution caused an increase in the airway inflammatory response to allergen.

We next examined the impact of the R576 mutation on airway inflammation and remodeling in chronic OVA–driven allergic airway inflammation (Wegmann et al., 2005). This model better reflects some of the physiological changes associated with chronic asthma, including subepithelial fibrosis. Accordingly, C129.IIl4raR576/R576 and control C129.IIl4raQ576/Q576 mice were sensitized and boosted with OVA in alum, and starting at week 4 after sensitization they were challenged with OVA by inhalation twice daily for an additional 8 wk (Wegmann et al., 2005). Histological analysis revealed that the C129.IIl4raR576/R576 mice exhibited increased perivascular inflammation and tissue eosinophilia
as compared with control mice (Fig. 2, A–E, arrows in B and D). Sirius red staining revealed markedly increased subepithelial collagen deposition in the mutant mice as compared with controls (Fig. 2, F–J). These findings indicated that the R576 mutation exacerbated chronic sequelae of airway inflammation, such as airway remodeling, that are key determinants of disease morbidity in asthma.

The R576 allele promotes IL-13–induced allergic airway inflammation

Many aspects of antigen–induced allergic airway inflammation, including AHR, eosinophilic infiltration, goblet cell metaplasia, and tissue remodeling, can be induced by the direct action of IL-13 on resident airway tissue independent of the adaptive immune response. To determine whether

Figure 1. The Q576R substitution promotes acute antigen–induced allergic airway inflammation. OVA-sensitized C.129.Ii4ra<sup>Q576Q576</sup> (Q576) and C.129.Ii4ra<sup>R576R576</sup> (R576) mice (n = 13 and 11, respectively) were challenged intranasally with OVA on three consecutive days and analyzed. (A) Lung histology (PAS staining). (B) Semiquantitative analysis of the severity of peribronchial inflammation represented by the mean of the inflammation scores, as described in Materials and methods. (C) Enumeration of PAS-positive goblet cells in the airways. (D) BAL fluid analysis for inflammatory cells. Values represent means ± SEM derived from two independent experiments. *, P < 0.05; **, P < 0.01. Bar, 200 µm.

Figure 2. The Q576R substitution promotes airway inflammation and remodeling after chronic allergen challenge. (A–E) Sham– (PBS) and OVA-sensitized C.129.Ii4ra<sup>Q576Q576</sup> and C.129.Ii4ra<sup>R576R576</sup> mice (n = 15–20 per group) were challenged intranasally with PBS and OVA, respectively, twice weekly for 12 wk. Airway inflammation was analyzed by histology (H&E staining; A–D), and inflammatory cells in the lung parenchyma were enumerated (E). Arrows in B and D represent areas of perivascular infiltration. (F–J) Detection (F–I) and quantitation (J) of subepithelial collagen deposition by Sirius red staining. Values represent means ± SEM of results from 8–10 mice derived from two independent experiments. HPF, high power field. *, P < 0.05; **, P < 0.01. Bars, 100 µm.
the R576 mutation could augment IL-13–induced airway inflammation, C.129.Il4ra<sup>R576/R576</sup> and WT BALB/c control mice were subjected to daily instillations of recombinant IL-13 or vehicle alone (BSA) for three consecutive days and compared for the magnitude of their responses. For both genotypes, the IL-13–challenged mice developed an allergic inflammatory response characterized by AHR to methacholine, eosinophilic infiltration, and goblet cell hyperplasia. (Fig. 3, A–D). However, for each of these parameters the C.129.Il4ra<sup>R576/R576</sup> mice exhibited an enhanced response as compared with WT BALB/c control mice, indicating that the R576 substitution augmented the IL-13–induced airway inflammation.

These results were validated in a second IL-13–dependent allergic airway inflammatory model that used a doxycycline-responsive IL-13 transgene (tg) driven by the Clara cell–specific promoter CC10 and maintained on a C57BL/6 background. Double-transgenic B6.129Il4ra<sup>R576/R576</sup>/IL-13tg mice were derived and compared with single-transgenic B6.129Il4ra<sup>Q576/Q576</sup>/IL-13tg mice for allergic airway inflammation both in the absence of and upon treatment with doxycycline for a total of 7 d. Both sets of mice exhibited basal airway eosinophilia and goblet cell metaplasia as compared with their respective IL-13tg-negative controls, a reflection of the leaky nature of the IL-13tg (Fig. 4 A; Zheng et al., 2000). Importantly however, the B6.129Il4ra<sup>R576/R576</sup>/IL-13tg double-transgenic mice exhibited increased basal airway eosinophilia as compared with the B6.129Il4ra<sup>Q576/Q576</sup>/IL-13tg single-transgenic mice, and this difference was further amplified upon doxycycline induction of IL-13 (Fig. 4 A). Although the single- and double-transgenic mice exhibited equivalent percentages of goblet cells at baseline, the B6.129Il4ra<sup>R576/R576</sup>/IL-13tg double-transgenic mice were more responsive to doxycycline treatment. While the percentage of goblet cells doubled upon doxycycline treatment of IL-13tg single-transgenic mice, it nearly quadrupled in the similarly treated B6.129Il4ra<sup>R576/R576</sup>/IL-13tg double-transgenic mice (Fig. 4 B). The enhanced airway eosinophilia in doxycycline-treated B6.129Il4ra<sup>R576/R576</sup>/IL-13tg mice was associated with increased detection by real-time PCR analysis of mRNA encoding the eosinophil chemotactic factor Ccl11 (eotaxin; Fig. 4 C). In contrast, transcripts of other chemokines such as Ccl2 were not significantly affected (Fig. 4 D). The capacity of the R576 mutation to up-regulate IL-13–induced Ccl11 expression in the airways was mirrored in studies on primary cell cultures of several lineages involved in allergic airway inflammation. Both IL-4– and IL-13–induced Ccl11 expression was increased in bone marrow–derived macrophages (BMDMs) and lung fibroblasts derived from R576 mutant mice as compared with WT controls (Fig. 4 E). Collectively, these results established that the R576 substitution heightened IL-13–induced allergic airway inflammation and tissue eosinophilia irrespective of the genetic background of the mice tested.

Figure 3. The Q576R substitution promotes airway hyperreactivity, airway eosinophilia, and goblet cell metaplasia after IL-13 administration. (A) WT BALB/c mice (Q576; genotype Il4ra<sup>Q576/Q576</sup>) and C.129.Il4ra<sup>R576/R576</sup> (R576) mutant mice (n = 14–15 mice per group) were tested for their enhanced pause (Penh) response, a proxy measure of AHR, to aerosolized methacholine at 96 h after three daily intranasal instillations of IL-13 or BSA (5 µg each). Group responses were analyzed by repeated measures ANOVA (P < 0.001, R576/IL-13 vs. Q576/IL-13 groups). (B) Eosinophil count in BAL fluid. (C) PAS staining of IL-13–treated lung tissue. Arrowheads represent PAS+ goblet cells. (D) Enumeration of PAS-positive goblet cells. Values represent means ± SEM derived from three to four independent experiments. **, P < 0.01; ***, P < 0.001. Bar, 100 µm.
Expression profiling revealed that the induction by doxycycline of IL-13tg expression resulted in the up-regulation of related sets of canonical IL-13–responsive genes in the B6.129Il4raQ576/Q576/IL–13tg and B6.129Il4raR576/R576/IL–13tg mice as compared with their respective control groups (Tables S1 and S2). Comparison of the gene sets up-regulated by the IL–13tg revealed several asthma-related genes that met a rather restrictive criterion for selection (a twofold change cutoff in expression) on the R576 but not the Q576 background (Tables S3 and S4). A more direct comparison of the magnitude of gene expression in doxycycline–treated B6.129Il4raR576/R576/IL–13tg versus B6.129Il4raQ576/Q576/IL–13tg mice, done at a more inclusive 1.2-fold change cutoff, continued to reveal enhanced expression of several IL–13–responsive genes in the homozygous R576 allele group as compared with their Q576 counterparts (Fig. 5 A and Table S5). These included genes involved in airway inflammation and remodeling such as Retnlb (resistin-like β) and Alox15 (15 lipoxygenase; Chu et al., 2002; Mishra et al., 2007), goblet cell metaplasia (Clca3 and Clca1) (Nakanishi et al., 2001), mucin and mucin granule components (Muc5b and Gp2), regulators...
of ion transport (Fxld3 and Fxld4) (Geering, 2006), protease and protease inhibitors (Adam8 and Timp4) (King et al., 2004), and epithelial barrier formation (Spr2a; Zimmermann et al., 2005; Segre, 2006). When normalized for gene expression in the respective control groups, several IL-13–responsive genes persisted in being overexpressed in doxycycline-treated B6.129Il4raR576/R576/IL-13tg as compared with similarly treated B6.129Il4raQ576/Q576/IL-13tg mice, including Adha2a, Alox15, Clev3, Fxld4, Gp2, Muc5b, and Spr2a (Fig. 5 B). The results of the gene array studies were further validated by real-time PCR analysis, which confirmed the up-regulation by the R576 substitution of several IL-13–induced genes, including Alox15, Retnlb, and Spr2a (Fig. 5 C). These results established that the R576 substitution differentially up-regulated the transcription of a subset of IL-13–responsive genes, especially ones involved in mucus secretion and airway inflammation and remodeling.

The R576 mutation promotes IL-4 production by Th2 cells and IgE responses

To determine whether the R576 substitution influenced Th cell differentiation as a contributing factor in its promotion of allergic airway inflammation, we compared cytokine production of T cells of C.129. Il4raR576/R576 and control C.129. Il4raQ576/Q576 mice that were induced to differentiate in vitro into Th1 or Th2 effector lymphocytes. C.129. Il4raR576/R576 Th2 cells exhibited a significant increase in IL-4 production as compared with C.129. Il4raQ576/Q576 Th2 cells. In contrast, production of the Th2 cytokines IL-5 and IL-13 was not significantly different between the two populations (Fig. 6 A). Similarly, there was no significant difference in IFN-γ production between Th1 cells of the respective genotype. These results indicated that the R576 substitution selectively up-regulated IL-4 production by Th2 cells.

The impact of the Q576R polymorphism on humoral immunity was examined by measuring the total and antigen–specific antibody responses after immunization with OVA mixed with alum adjuvant to promote Th2-type responses (Brewer et al., 1999), or with saline/alum as immunization control. Fig. 6 B revealed that the C.129. Il4raR576/R576 mice exhibited increased total serum concentrations of IgE after immunization with OVA/alum as compared with C.129. Il4raQ576/Q576 control mice. In contrast, IgG1 levels were unchanged (unpublished data). Significantly, OVA-specific antibody responses were also increased in the Q576R mutant mice as compared with controls (Fig. 6 B). The heightened IgE production in the Q576R mutant mice was associated with an increased capacity of the C.129. Il4raR576/R576 B cells to produce IgE but not IgG1 when stimulated in vitro with anti-CD40 mAb plus IL-4 (Fig. 6 C). Also, the R576

Figure 5. The R576 substitution heightens the expression of a subset of IL-13–induced genes in the airways. (A) Differential expression of a subset of IL-13–induced genes in doxycycline-treated (Dox) B6.129Il4raR576/R576/IL-13tg double-transgenic versus B6.129Il4raQ576/Q576/IL-13tg single-transgenic mice (n = 11 and 8, respectively). Results depict mean fold change values scoring P < 0.05 by the Student’s unpaired two-tailed t test. (B) Selected results from A were normalized for gene expression levels in doxycycline-treated, IL-13tg–negative B6.129Il4raR576/R576, and B6.129Il4raQ576/Q576 littermate controls, respectively (n = 5 for each control group). (C) Real-time PCR analysis of Alox15, Retnlb, and Spr2a mRNA levels in total lung extracts of doxycycline-treated and IL-13tg–negative and –positive Q576 and R576 mice (n = 4–11 per group). Values represent means ± SEM derived from two to four independent experiments. *, P < 0.05; and **, P < 0.01 for doxycycline-treated R576/IL-13tg versus Q576/IL-13tg groups.
We also examined the impact of the R576 substitution on the induction in BMDMs of the STAT6–dependent genes arginase 1 (Arg1) and chitinase (Chi3l3) and of arginase enzymatic activity (Gordon, 2003; Munitz et al., 2008; Ramalingam et al., 2008). Although the R576 substitution augmented Ccl11 gene expression in BMDMs (Fig. 4), it did not influence the induction of Arg1 and Chi3l3 transcripts nor the expression of arginase enzymatic activity in BMDM cells treated with IL-4 or IL-13 (Fig. S2). Collectively, these results established that the R576 substitution selectively augmented some IL-4R–dependent responses in T and B cells and in BMDMs but not others.

The R576 substitution does not augment proximal STAT6 activation
To elucidate signaling mechanisms by which the R576 substitution mediates its proallergic inflammatory effects, we examined the capacity of the R576 mutation to augment the activation of STAT6 in different cell types in response to IL-4 and IL-13. Results revealed that the R576 substitution did not augment the increase in STAT6 phosphorylation in response to IL-4 in T or B cells, nor did it alter the time course of STAT6 dephosphorylation in both cell types upon the cessation of IL-4 treatment (Fig. 7, A–C). Because T and B cells express the type I but not type II IL-4R and, as such, do not respond to IL-13, we examined STAT6 activation in response to IL-4 and IL-13 in BMDMs, which express both the type I and II IL-4Rs. There were no differences in the induction of STAT6 phosphorylation, or in its dephosphorylation upon signal termination, between WT and R576 mutant BMDMs treated with IL-4 or IL-13 (Fig. 7, D–F and G–I, respectively). These results established that the R576 substitution did not directly affect STAT6 activation in T and B lymphocytes or BMDMs.

We also examined the activation of several pathways downstream of IL-4R that have been previously implicated in receptor signaling. The R576 substitution was not associated with enhanced Shc phosphorylation, nor was it associated with enhanced activation of the PI3-kinase–Akt pathway, suggesting that the association at the I4R motif and the subsequent activation of the adaptor IRS2, which lies upstream of PI3-kinase–Akt, was similarly unperturbed (Fig. S3).

Figure 6. The R576 substitution enhances IL-4 production by Th2 cells and IgE responses in vivo. (A) Cytokine production by Th cells. Naive WT and R576 splenic T cells (n = 12 mice per group) were differentiated into Th1 or Th2 cells, and stimulated with anti-TCRβ mAb for 48 h. Culture supernatants were examined for IL-4 and IFN-γ production by ELISA. (B) Total and OVA-specific serum IgE levels in Q576R and WT control mice (n = 13 per group) after i.p. immunization with OVA/alum. Horizontal bars represent means. (C) IgE and IgG1 production by purified splenic B cells of Q576R and WT control mice (n = 6 per group) stimulated in vitro for 1 wk with anti-CD40 mAb increasing concentrations of IL-4. (D) Up-regulation of CD23 and I-A^d expression in WT and R576 B cells treated with increasing concentrations of IL-4 (n = 3 per group). Values represent means ± SEM derived from two to four independent experiments. MFI, mean fluorescence intensity. **, P < 0.01; †, P < 0.05 by repeat measures two-way ANOVA.
back to background levels in Q576 control mice. In contrast, the STAT6-deficient R576 mutant mice continued to exhibit significantly increased eosinophil infiltration, reaching up to 50% of that observed in STAT6-sufficient R576 mutant mice (Fig. 8, C and D). The residual, STAT6-independent tissue eosinophilia in the triple-transgenic B6.IL4raR576/R576/STAT6−−/IL-13tg mice involved a Ccl11- and Alox15-independent mechanism, as the increased Ccl11 and Alox15 expression induced by the IL-13tg was abolished in both WT and mutant mice by concurrent STAT6 deficiency (Fig. 8, E and F). Hence, although the R576 mutation augmented several STAT6-dependent responses, the promotion by R576 allele of tissue eosinophilia involved both STAT6-dependent and -independent mechanisms.

Activation of alternative signaling pathways by the R576 substitution

To determine potential alternative mechanisms by which the R576 substitution promoted allergic airway inflammation, we examined the activation of other pathways downstream of IL-4R that have been previously implicated in receptor signaling. Induction of IL-13tg expression by doxycycline treatment was associated with enhanced MAPK phosphorylation in lung extracts of B6.129Il4raR576/R576/IL-13tg mice as compared with similarly treated B6.129.Il4raR576/R576/IL-13tg controls (Fig. S3 A). Similarly, treatment of primary cultures of tracheal airway epithelial cells (TECs) revealed that IL-4 treatment of C.129.IL4raR576/R576 cells was associated with enhanced Erk1/2 kinase activation (Fig. S3 B). IL-4 (and IL-13) treatment of C.129.IL4raR576/R576 TEC cultures was not associated with enhanced Shc phosphorylation, suggesting that the enhancement of MAPK activation proceeded by a distinct mechanism (Fig. S3 B and not depicted). Treatment of TEC cultures with IL-4 resulted in increased expression of Ccl11 transcripts, with a twofold superinduction noted in mutant C.129.IL4raR576/R576 TECs as compared with C.129.IL4raQ576/Q576 controls. This differential induction of Ccl11 transcripts in mutant TEC cells was sensitive to inhibition by the MAPK inhibitor PD98059 (Fig. S3 C). Activation of other downstream kinases, including AKT and p38 back to background levels in Q576 control mice. In contrast, the STAT6-deficient R576 mutant mice continued to exhibit significantly increased eosinophil infiltration, reaching up to 50% of that observed in STAT6-sufficient R576 mutant mice (Fig. 8, C and D). The residual, STAT6-independent tissue eosinophilia in the triple-transgenic B6.IL4raR576/R576/STAT6−−/IL-13tg mice involved a Ccl11- and Alox15-independent mechanism, as the increased Ccl11 and Alox15 expression induced by the IL-13tg was abolished in both WT and mutant mice by concurrent STAT6 deficiency (Fig. 8, E and F). Hence, although the R576 mutation augmented several STAT6-dependent responses, the promotion by R576 allele of tissue eosinophilia involved both STAT6-dependent and -independent mechanisms.

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Figure 7. The R576 substitution does not affect STAT6 activation by IL-4 or IL-13. (A) Intracellular staining of pSTAT6 in WT (Q576) and mutant R576 B cells stimulated for 15 min at 37°C with 10 ng/ml IL-4. (B and C) Flow cytometric analysis of pSTAT6 induction in WT and mutant R576 B cells (B) or T cells (C) treated with increasing concentrations of IL-4. (D–I) Flow cytometric analysis of pSTAT6 induction in BMDMs. (D and G) Intracellular staining of pSTAT6 in BMDMs treated with IL-4 (D) or IL-13 (G). (E and H) pSTAT6 induction at increasing concentrations of IL-4 (E) or IL-13 (H). (F and I) Decay of pSTAT6 signal upon cessation of IL-4 and IL-13 treatment. Cells were treated for 10 min with 10 ng/ml IL-4 or 100 ng/ml IL-13, and washed (arrow) and analyzed at different time points thereafter for pSTAT6 content. Results represent means ± SEM of lymphocytes, and BMDM cultures from three to five mice per group from two to three independent experiments. MFI, mean fluorescence intensity.
MAPK, appeared unaffected by the R576 substitution (unpublished data). Collectively, these findings identified enhanced MAPK activation as one putative effector mechanism of the IL-4Rα R576 substitution.

DISCUSSION

Several factors restrict the interpretation of association studies examining the role of genetic polymorphisms in the pathogenesis of allergic diseases, including asthma. These include the genetic heterogeneity of outbred human populations, which results in confounding genetic influences from other disease susceptibility and modifier loci. In addition, the polymorphisms under study might be but markers in linkage disequilibrium with other true disease susceptibility loci. Finally, any particular polymorphism may influence only selective aspects of a complex phenotype such as asthma. In the present study, we have addressed some of these restrictions as they apply to the IL-4Rα Q576R polymorphism by studying the functional impact of this polymorphism in the context of the mouse receptor, in which the Q576 residue and the motif within which it is embedded are conserved. By comparing the functional attributes of the WT and the R576 mutant

![Figure 8. STAT6-dependent and -independent effects of the R576 substitution.](image-url)

(A) IL-13tg+ B6.II4raQ576/Q576 and B6.II4raR576/R576 mice, and tg-negative controls, that were either STAT6 sufficient or deficient were examined by immunofluorescence staining for expression of MUC5AC after doxycycline (Dox) treatment (red-staining cells). (B) MUC5AC+ cells (red-staining cells; A) were counted and expressed as the percentage of total airway epithelial cells (DAPI [blue]-staining nuclei) in the respective airways. (C and D) Lung tissue eosinophilia. (C) Mouse groups as in A were treated with doxycycline, and their lungs were stained with anti-eosinophil MBP1 antibodies (red-staining cells). (D) MBP1+ cells were counted and expressed as mean cell numbers per 20× objective field (n = 10 fields per group). (E and F) Real-time PCR analysis of Ccl11 and Alox15 expression in the different mouse groups treated with doxycycline. Values represent means ± SEM of 4–15 mice per group derived from three to five independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bars, 100 μm.
alleles on a homogenous genetic background, we have identified a role for this polymorphism in promoting atopic responses and allergic airway inflammation and remodeling.

The R576 allele potentiated allergic airway inflammation in an antigen-dependent (OVA sensitization) and –independent manner (IL-13 inhalation and transgenic expression). In both cases, the inflammation was associated with increased airway eosinophilia, which persisted in the chronic antigen-driven airway inflammation model. Given that airway eosinophilia plays a critical role in unfolding the acute and chronic changes associated with asthma, including AHR, mucous production, and subepithelial fibrosis, the increased airway eosinophil load would provide one mechanism by which the R576 allele promotes allergic airway inflammation (Humbles et al., 2004; Lee et al., 2004). Gene expression profiling identified additional mechanisms by which the R576 allele may exert proinflammatory functions. A subset of IL-13–regulated genes associated with airway inflammation, remodeling, and mucus production, including Alox15, Retnllb, Clca3, and others were superinduced by the R576 allele. Although some metabolites of 15-lipoxygenase, such as the lipoxins, may serve an antiinflammatory function, others such as 15(S)-hydroxyeicosatetraenoic acid may promote airway remodeling (Chu et al., 2002; Kühn and O’Donnell, 2006). In addition, expression of Clca3 is sufficient for induction of goblet cell metaplasia in mice (Patel et al., 2006). Collectively with the recent demonstration of the induction of subepithelial fibrosis by resistin-like β, these findings point to potential targets of intervention to interrupt the evolution of chronic airway changes promoted by the R576 allele (Mishra et al., 2007).

The R576 allele enhanced total and antigen-specific IgE responses, a likely reflection of both increased IL-4 production by Th2 cells and IL-4–driven isotype switching to heavy chain in B cells. An increased atopic response and a heightened resident airway tissue response to IL-4 and IL-13 may both contribute to the aggressive allergic airway inflammatory phenotype of the R576 allele. However, the capacity of the R576 allele to potentiate IL-13–induced allergic airway inflammation, which proceeds in the absence of an IgE–associated adaptive immune response, indicates that the airway inflammatory phenotype can be uncoupled from the atopic response. Nevertheless, it is likely that the two mechanisms interact synergistically in vivo in the context of an antigen-driven allergic airway inflammation.

The proallergic inflammatory phenotype of the R576 allele was associated with the selective enhancement of some STAT6–dependent responses in a tissue- and gene–specific manner but in the absence of increased STAT6 phosphorylation on the regulatory tyrosine residue or the activation of pathways such as PI3–kinase–Akt, Shc, or p38 MAPK. These findings are in agreement with an earlier report that failed to document evidence of enhanced STAT6 activation in mouse cell lines transfected with a human IL–4R allele carrying the R576 substitution (Wang et al., 1999).

The capacity of the R576 substitution to selectively augment some STAT6–dependent responses in the absence of increased proximal STAT6 activation may be best explained by the activation by the R576 substitution of an alternative signaling mechanism that acts in synergy with STAT6 in a gene- and tissue–specific manner. This conclusion is supported by the finding that the enhanced eosinophil accumulation in lung tissues of Il4ra<sup>R576/R576</sup>/IL-13<sup>tg</sup> mice proceeds in part by STAT6–independent mechanisms (Fig. 8). Increased MAPK activation was observed in lung tissues of Il4ra<sup>R576/R576</sup>/IL-13<sup>tg+</sup> mice and in cultured Il4ra<sup>R576/R576</sup> primary TECs, indicating that this may represent one such mechanism (Duan et al., 2005; Lee et al., 2006). However, the precise mechanisms by which the R576 alter signaling via the IL–4R, and the role of MAPK and other signaling pathways in mediating the effects of the R576 substitution, remains to be fully established.

Earlier studies have linked the R576 substitution to altered binding at Y575 with increased association with SHP-1 and SHP-2 (Hershey et al., 1997; Kruse et al., 2002). More detailed analysis revealed that recombinant SHP-1 protein selectively binds to the ITIM at positions Y713 and Y709 in the human and mouse receptors, respectively. Mutagenesis of the ITIM in vivo was associated with enhanced STAT6 phosphorylation, grossly elevated baseline serum IgE levels, and exaggerated total and antigen–specific IgE responses upon antigen sensitization (unpublished data). These findings indicated that the effects of the R576 substitution are distinct from those associated with disruption of a bona fide ITIM.

Epidemiological surveys have linked different IL-4Rα polymorphisms to distinct phenotypes of atopy and asthma. In particular, the R576 allele has been linked to severe asthma and low lung function in asthmatics. Other alleles such as the cysteine 431 arginine (C431R) have been linked to atopy, whereas the E400A has been linked to both severe asthma and atopy (Ober et al., 2000; Wenzel et al., 2007). Furthermore, the linkage to both asthma and atopy appears to be strengthened when analysis involves combinatorial haplotypes of different IL–4Rα polymorphisms, indicative of interaction among the alleles (Ober et al., 2000; Risma et al., 2002; Hytönen et al., 2004). These findings are consistent with some of the IL–4Rα polymorphisms acting by distinct though possibly overlapping mechanisms to promote atopy and asthma. It would be important to decipher the molecular mechanisms underlying these interactions, as they are likely to reflect on the different clinical phenotypes associated with asthma, including disease progression and severity.

Previous analysis has indicated that the association of the R576 polymorphism with severe asthma is independent of the racial group analyzed (Wenzel et al., 2007). However, the R576 polymorphism exists at a much higher prevalence among the racial group analyzed (Wenzel et al., 2007). However, the precise mechanisms by which the R576 alter signaling via the IL–4R, and the role of MAPK and other signaling pathways in mediating the effects of the R576 substitution, remains to be fully established.
interactions between IL-13 and IL-4Rα alleles have been demonstrated for African Americans with asthma (Battle et al., 2007). The specific contribution of the R576 allele to asthma incidence and severity among different ethnic groups, especially African Americans, requires further investigation. Its association with a distinct mechanism of cellular activation via the IL-4Rα suggests, however, that novel therapeutic interventions may be beneficial to asthma treatment in this and other ethnic groups.

MATERIALS AND METHODS

Construction of targeting vector and generation of mutant mice. Genomic DNA used to construct the targeting vector were isolated from a bacterial artificial chromosome clone derived from the 129X1Sv/Ej mouse strain that contained the mouse IL-4Rα gene (Genesis Systems; Wrighton et al., 1992). The replacement-type targeting vector (pKO; Lexicon) contained 13.5 kb of IL-4Rα sequences that extended from a XhoI site proximal to exon 9 to a KpnI site distal to exon 12 of the gene. An AG→GA substitution was introduced by site-directed mutagenesis (QuickChange; Agilent Technologies) into the second and third positions of codon Q576 (corresponding to bp 1964–1965 of the mouse IL-4Rα cDNA; Mosley et al., 1989), changing it to R576 (CGA). The targeting construct included a floxed neomycin resistance gene (neo) and a diphtheria toxin gene for positive/negative selection. Targeting plasmids were transfected into RW4 ES cells and subjected to G418 selection. Successfully targeted clones were transiently transfected with a Cre recombinase to remove the inserted neomycin gene.

Heterozygous ES cells were injected into C57BL/6 blastocysts, and the resultant male chimeras were mated with BALB/c females. Offspring were screened for Hets by Southern blotting and PCR analysis. Heterozygous animals were further bred for 10 generations on BALB/c (Taconic) and C57BL/6 (Charles River Laboratories) backgrounds to generate the congenic strains C.129OlaIl4raQ576/Q576 and B6.129Il4raQ576/R576. A targeted clone in which the floxed neo cassette was incorporated (later removed by Cre recombinase action) but which retained the WT Q576 codon was used to generate the control C.129OlaIl4raQ576/Q576 congenic mouse strain. WT BALB/c and IL-4Rα KO mice (BALB/c-Illtra tm1Sz) were obtained from Taconic and the Jackson Laboratory, respectively. All protocols were in accordance with National Institutes of Health (NIH) guidelines and approved by the Animal Care and Use Committee at the University of California, Los Angeles. The C.129OlaIl4raQ576/Q576 strain has been deposited at the Jackson Laboratory (strain no. 007746).

Control and IL-13tg strains. The mouse Il4ra exists as two distinct allelotypes, each restricted to different strains of inbred mice, that differ at multiple polymorphic residues (Schulte et al., 1997). The 129X1Sv/J and C57BL/6 strains share the same allotype targeted by mutagenesis in this study, whereas BALB/c mice carry the other. Accordingly, for studies on the BALB/c background, the C.129OlaIl4raQ576/Q576 mice were used as controls except for experiments specifically indicated in the figures in which native BALB/c mice were used. For studies on the C57BL/6 background, native C57BL/6 mice were used as controls. The doxycycline-inducible IL-13tg mice (CC10-tTA;IL-13, on a C57BL/6 background; designated in this study as B6.IlltraQ576/Q576/IL-13tg) were a gift of J. Elias (Yale University School of Medicine, New Haven, CT; Zheng et al., 2000). B6.129 IItraQ576/R576 mice were backcrossed on the IL-13tg mice to create the B6.129.IlltraQ576/R576/IL-13tg strain.

PCR analysis. Screening of WT and mutant Illtra alleles was performed by PCR amplification using genomic DNA and the following primers: common forward (F) primer, 5′-TTTGACACATCCTGCTTA-3′; WT-specific reverse (R) primer, 5′-ACTGCTCTAGACAAACACTCCT-3′; and mutant-specific R primer, 5′-ACTGCTCTAGACAAAACACTCCT-3′. The following primers were used for PCR screening of the residual LoxP-containing allele: F, 5′-GGTGTCTATTTTAGTGCC-3′, and R, 5′-TCTCCTCCTCTACTCTGTGCT-3′. For RT-PCR analysis, total RNA was extracted from splenocytes of WT and IL-4Rα Q576R mutant mice using TRIzol (Invitrogen). The RNA was treated with Dnase I to remove residual genomic DNA contamination and reverse transcribed. Il4ra transcripts were amplified by a two-step process using the following pairs of nested primers: outer pair F (exon 11), 5′-CAG-ACCAGAACCCAGGACTAAC-3′, and R, (exon 12) 5′-CCCTT-GCTTCACTGCCTGACAAAC-3′; and inner pair (both exon 12) F, 5′-GAGAGCTCTCCACACG-3′, and R, 5′-ACGGCTCTGCAAA-GACTCCT-3′. For GAPDH transcripts, the following primers were used: F, 5′-ACACAGTCCTGACATCACC-C-3′, and R, 5′-TCCACACCT-GTTGCTGTA-3′. For real-time PCR analysis, cDNA was reverse transcribed from 2 µg RNA per sample and used in real-time PCR assays that used commercially available oligonucleotides and SYBR green–based reagents (SA Biosciences). The comparative Ct (threshold cycle) method normalized to GAPDH was used to analyze relative changes in gene expression. The fold change was calculated as 2−ΔΔCt for negative ΔCt values (indicating a fold-change increase) and −2ΔΔCt for positive ΔΔCt values (indicating a fold-change decrease).

Serum total and OVA-specific IgE antibodies, in vitro IgE production, and Th cell differentiation. These assays were performed as previously described (Blaejer et al., 2003).

Cell preparation, stimulation, and immunoblotting. Purified T and B cell populations were derived by magnetic bead separation (Blaejjer et al., 2000). BMDMs, primary lung fibroblast cultures, and primary air–liquid interface cultures of mTECs were established as previously described (You et al., 2002; Sugiyura et al., 2007). Cells were serum starved for 4 h (T and B cells) or overnight (BMDMs, fibroblasts, and mTECs) at 37°C before stimulation with IL-4 or IL-13 at the concentrations and time periods indicated in the figures. Immunoblotting was performed as previously described (Blaejjer et al., 2000). The following antibodies were used: anti-pSTAT6 and -STAT6 (Santa Cruz Biotechnology, Inc.), and anti-pAKT, -pERK1/2, -ERK1/2, -p38, and -p38 (Cell Signaling Technology). The blots were developed using horseradish peroxidase-conjugated secondary antibodies and enzyme-linked chemiluminescence (ECL; GE Healthcare).

Flow cytometry and intracellular staining. Single-cell suspensions were stained with the antibodies indicated in the figures and analyzed on a cytometer (FACS caliber; BD). FITC- or PE-conjugated mAbs used were obtained from BD and ebioscience. Intracellular staining with anti–phospho-Y641–STAT6 (pSTAT6), anti–phospho–threonine 308–AKT (pAKT), and anti–pT180/pY182 p38 MAPK (pp38) antibodies (BD) was performed as previously described (Krutzik et al., 2004). Cell aliquots were stimulated with IL-4 or IL-13 (PeproTech) for the periods and concentrations indicated in the figures. The cells were then fixed with 1.6% paraformaldehyde and permeabilized for 10 min with methanol. After washing, the cells were stained with conjugated pSTAT6, pAKT, or pp38 (Alexa Fluor 647; BD) and analyzed by flow cytometry.

Immunofluorescence. Tissue section preparation was performed as previously described (Gomperts et al., 2007). Primary antibodies used were mouse anti–Muc5AC mAb 14.100 (1:100; Novemarkers) and 0.4 µg/ml of monoclonal rat anti–mouse eosinophil MBP1 mAb 14.7.4 (a gift of J. Lee; Mayo Clinic Arizona, Scottsdale, AZ). Sections were incubated with either Alexa Fluor 568 anti–mouse MUC5AC mAb or biotinylated secondary antibodies and enzyme-linked chemiluminescence (ECL; GE Healthcare).
Macrophage and lung fibroblast cultures and arginase assay. BMDMs were derived by culturing bone marrow aspirates for 7 d with M-CSF, cultured in 24-well tissue culture plates, and stimulated with IL-4 or IL-13 at the concentrations and times indicated in the figures. The arginase assay was performed as previously described (Ramaalingam et al., 2008). Primary lung fibroblast cultures were prepared as previously described (Sugura et al., 2007).

Allergic airway inflammation models. For the acute antigen–induced airway inflammation model, mice were sensitized and boosted with a mixture of OVA in alum, and challenged 4 wk later intranasally with 50 µg OVA daily for three consecutive days. Control mice were sensitized and boosted with PBS mixed with alum, and challenged with OVA. For the chronic model, mice were immunized and boosted with OVA/alum, and starting on week 4, challenged twice weekly with intranasal OVA through week 12. Control mice were immunized with PBS/alum challenged with PBS. Inhaled IL-13–induced allergic airway inflammation was accomplished by instilling 5 µg of recombinant IL-13 intranasally daily for three consecutive days. Control instillation was performed using 5 µg BSA in 50 µl PBS. The IL-13tg–induced airway inflammation model was effected by feeding IL-13tg mice and their tg-negative littermates with doxycycline-containing chow (300 ppm; Research Diets, Inc.) for 1 wk.

Measurement of airway tissue and functional responses. Paraformaldehyde–embdded lungs were stained with H&E and periodic acid–Schiff (PAS) staining as previously described (Blaeser et al., 2003). Peribronchiolar inflammation was graded with a score as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of greater than four cells deep (Ford et al., 2001). Subepithelial collagen deposition was detected by Sirius red staining (Wegmann et al., 2005). BAL was performed as previously described (Blaeser et al., 2003). Airway functional responses were examined in mice treated intranasally with either recombinant IL-13 or BSA (5 µg/50 µl PBS for three consecutive days), and were measured at 24 h after the last inhalation treatment using whole-body plethysmography (Buxco Research Systems; Hamelmann et al., 1997; Blaeser et al., 2003). Responses to increasing concentrations of aerosolized methacholine were determined and expressed as enhanced pause (Penh) values.

Gene expression profiling. Whole-lung extracts were used to generate total RNA, which was then amplified and used to create probes for Affymetrix 430 2.0 chips. For the analysis of gene expression measures, all Affymetrix data were normalized using the masrRNA algorithm from the Bioconductor group (Gentleman et al., 2004). Pairwise comparisons were made for all experimental conditions such that probe sets that showed at least a 1.2–2-fold change in expression, as specified for the respective group comparisons, were selected for further analysis. Listed genes had to additionally show a group mean difference above a threshold, chosen to be the 10th percentile of all group mean differences across all probe sets. Results represent mean fold change values derived from 5–11 independent arrays (one mouse per array) for the respective genotype, and scoring P < 0.05 by the Student’s unpaired two-tailed t test. Microarray data have been deposited in the Gene Expression Omnibus under accession no. 18010.

Statistical analysis. The Student’s t test, two-way analysis of variance (ANOVA), and repeat measures two-way ANOVA with the Bonferroni posttest analysis of groups were used to compare test groups, as appropriate. P < 0.05 was considered statistically significant.

Online supplemental material. Fig. S1 details the derivation of Il4raR576/R576 mutant mice. Fig. S2 shows the induction of arginase activity, and Arg1 and Chi333 transcripts in IL-4 and IL-13–treated Il4raQ576/Q576 and Il4raR576/R576 BMDMs. Fig. S3 demonstrates the induction of MAPK activation in Il4raQ576/Q576 and Il4raR576/R576 mice and TECs in response to IL-13 and IL-4. Gene expression profiles in the lungs of nontransgenic and IL-13tg Il4raQ576/Q576 mice are detailed in Tables S1–S5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091480/DC1.

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SUPPLEMENTAL MATERIAL

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Figure S1. Targeted knock-in mutagenesis of the mouse IL-4Rα. (A) Targeting strategy. WT exon 12 was replaced with one carrying an AG→GA substitution (indicated by an asterisk) in the second and third positions of codon Q576, changing it to R576. (B) Southern blot analysis of EcoRI-digested mouse-tail genomic DNA of WT Q576 (WT), heterozygous (HET), and homozygous (HOMO) R576 mutant mice. The introduction of a novel EcoRI site in intron 11 of targeted IL4A reduces the size of an EcoRI genomic fragment detected with an exon 4 probe from 12.5 kb in the WT allele to 8.5 kb in the mutant allele. (C) PCR analysis of mouse-tail DNA using Q576 and R576 allele-specific primers (top and middle, respectively), or primers spanning the residual 34 bp loxP site left in intron 11 after Cre-mediated excision of the floxed Neo cassette. (D) Sequence analysis of RT-PCR products of IL-4Rα transcripts of WT (Q576) and R576 mutant mice. (E) Flow cytometric analysis of IL-4Rα expression in IL-4Rα Q576 and R576 lymphocytes. Splenic B cells of WT (Q576/Q576), IL-4Rα-deficient (IL-4Rα−/−), and IL-4Rα homozygous R576 mutant (R576/R576) mice were stained with PE-conjugated rat anti-mouse IL-4Rα mAb (mIL4R-M1). Control staining was performed on WT splenic B cells using a PE-conjugated, isotype-matched antibody.
Figure S2. Impact of the Q576R substitution on alternative macrophage activation by IL-4 and IL-13. (A and B) Arginase activity in lysates of BMDMs treated with various concentrations of IL-4 (A) or IL-13 (B). BMDM lysates were derived at 48 h after stimulation and examined for urea production ($n = 9–12$ mice/group). C, D. Real-time PCR of gene transcripts encoding arginase 1 (Arg1) and chitinase 3-like 3 (Chi3l3) in BMDMs stimulated for 20 h with IL-4 or IL-13 at 10 ng/ml each ($n = 7–12$ mice/group). Results represent means ± SEM derived from 7–12 independent experiments.
Figure S3. The R576 substitution augments MAPK activation upon IL-4 and IL-13 treatment of lung tissues. (A) Left: Erk1/2 phosphorylation in lung extracts of untreated and doxycycline-treated (48 h) IL-13tg+ Il4ra^{Q576/Q576} and Il4ra^{R576/R576} mice. [right] Compiled results from densitometric analysis of three to five independent experiments per test group. (B) Phosphorylation of Erk1/2, STAT6, and Shc in IL-4–treated TECs derived from Il4ra^{Q576/Q576} and Il4ra^{R576/R576}. Cells were stimulated with 100 ng/ml IL-4 for the indicated time periods. The cell lysates were probed with the indicated phospho-specific antibodies followed by antibodies against the respective unmodified protein. Results represent means ± SEM of three to six independent experiments. (C) Induction of Ccl11 expression in TECs and its inhibition by the MAPK inhibitor PD98059. Cells were stimulated with IL-4 for 6 h in the absence or presence of the indicated concentrations of PD98059, and analyzed for Ccl11 transcripts by real-time PCR. Results represent means ± SEM of three independent experiments. *, P < 0.05; **, P < 0.01.