

***In vivo* regulation of the allergic response by the IL-4 receptor α chain immunoreceptor tyrosine-based inhibitory motif**

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Background: Signaling by IL-4 and IL-13 through the IL-4 receptor α chain (IL-4R α) plays a critical role in the pathology of allergic diseases. The IL-4R α is endowed with an immunoreceptor tyrosine-based inhibitory motif (ITIM) centered on tyrosine 709 (Y709) in the cytoplasmic domain that binds a number of regulatory phosphatases. The function of the ITIM in the *in vivo* regulation of IL-4 receptor signaling remains unknown.

Objective: We sought to determine the *in vivo* function of the IL-4R α ITIM by using mice in which the ITIM was inactivated by mutagenesis of the tyrosine Y709 residue into phenylalanine (F709).

Methods: F709 ITIM mutant mice were derived by means of knock-in mutagenesis. Activation of intracellular signaling cascades by IL-4 and IL-13 was assessed by means of intracellular staining of phosphorylated signaling intermediates and gene expression analysis. *In vivo* responses to allergic sensitization were assessed by using models of allergic airway inflammation.

Results: The F709 mutation increased signal transducer and activator of transcription 6 phosphorylation by IL-4 and, disproportionately, by IL-13. This was associated with exaggerated T_H2 polarization, enhanced alternative macrophage activation by IL-13, augmented basal and antigen-induced IgE responses, and intensified allergen-induced eosinophilic airway inflammation and hyperreactivity.

Conclusions: These results point to a physiologic negative regulatory role for the Y709 ITIM in signaling through IL-4R α , especially by IL-13. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: IL-4 receptor, IL-4, IL-13, immunoreceptor tyrosine-based inhibitory motif, Src homology 2 domain-containing protein tyrosine phosphatase 1, IgE, allergic airway inflammation, asthma

The IL-4/IL-13 cytokine pathway plays a central role in regulating T_H2 responses, including induction of IgE synthesis and modulation of lymphocyte and antigen-presenting cell function and allergic tissue responses, such as airway inflammation, in patients with asthma.^{1,2} IL-4 and IL-13 share a common receptor component, the IL-4 receptor α chain (IL-4R α), which can pair with alternative subunits to assemble a complete receptor.¹⁻³ The IL-4R α associates with the common γ c chain (γ c) to form a type I IL-4 receptor complex that is found predominantly in hematopoietic cells and binds exclusively IL-4.⁴ The IL-4R α can also pair with the IL-13R α 1 subunit to form a type II IL-4 receptor capable of binding either IL-4 or IL-13. The type II receptor is expressed on both hematopoietic and nonhematopoietic cells, including airway epithelium, and is essential for allergen-induced airway hyperreactivity and mucus hypersecretion.⁵⁻⁷ Binding of IL-4 and IL-13 to these receptors activates receptor-associated Janus kinases (JAKs), which initiate a number of intracellular signaling cascades by phosphorylating specific tyrosine (Y) residues in the cytoplasmic domain of the IL-4R α .^{4,6} Phosphorylation of Y575, Y603, and Y633 of the human IL-4R α mobilizes the transcription factor signal transducer and activator of transcription (STAT) 6, 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 (MAP) kinase pathways.^{8,9} Once recruited to the IL-4R α , both insulin receptor substrate (IRS) 2 and STAT6 are subject to Jak-mediated phosphorylation, which enables their effector functions.

The sequence around Y709 at the carboxyl-terminus of the murine IL-4R α (IVYSSL) is concordant with the canonical immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence (consensus I/V/LxYxxL/V).¹⁰⁻¹² The IL-4 receptor (IL-4R) ITIM binds a number of regulatory phosphatases, including Src homology 2 domain-containing protein tyrosine phosphatase 1/2 (SHP-1/2) and Src homology 2 domain-containing inositol 5-phosphatase 1 (SHIP-1), suggesting a role for this tyrosine residue in the recruitment of these regulators to the IL-4R α .¹³

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Abbreviations used

AHR:	Airway hyperresponsiveness
alum:	Aluminum potassium phosphate
BMDM:	Bone marrow–derived macrophage
ERK:	Extracellular signal-regulated kinase
EpoR:	Erythropoietin receptor
IL-4R:	IL-4 receptor
IL-4R α :	IL-4 receptor α chain
IRS:	Insulin receptor substrate
ITIM:	Immunoreceptor tyrosine-based inhibitory motif
JAK:	Janus kinase
MAP:	Mitogen-activated protein
OVA:	Ovalbumin
PAS:	Periodic acid–Schiff
PI3:	Phosphatidylinositol 3
SHIP-1:	Src homology 2 domain–containing inositol 5-phosphatase 1
SHIP-1/2:	Src homology 2 domain–containing protein tyrosine phosphatase 1/2
STAT:	Signal transducer and activator of transcription
WT:	Wild-type

Global SHP-1 and SHIP-1 deficiency is associated with enhanced signaling through the IL-4R α and enhanced allergic airway inflammation.^{14–16} However, SHP-1, SHP-2, and SHIP-1 associate with multiple other receptors relevant to the allergic response, including Fc and mast cell inhibitory receptors, thus rendering the specific function of the ITIM in modulation of IL-4R α signaling difficult to discern. In this report we aimed to specifically examine the role of the ITIM in IL-4R α signaling in mice whose IL-4R α ITIM has been inactivated by targeted knock-in mutagenesis.

METHODS**Derivation of mutant mice**

The derivation by means of targeted knock-in mutagenesis of mutant mice on a BALB/c background carrying a homozygous phenylalanine (F) substitution at position Y709 of the IL-4R α (C.129I14ra^{F709/F709}) and similarly manipulated control mice that retain the native Y709 residue (C.129.I14ra^{Y709/Y709}) is detailed in the Methods section of this article's Online Repository at www.jacionline.org. PCR genotyping of wild-type (WT) and mutant mice was carried out as detailed in the Methods section of this article's Online Repository. All protocols were in accordance with National Institutes of Health guidelines and approved by the Animal Care and Use Committees at the University of California at Los Angeles and the Children's Hospital, Boston.

Flow cytometry and intracellular staining

Single-cell suspensions were stained with the indicated antibodies and analyzed on a FACSCalibur cytometer (Becton Dickinson, Mountain View, Calif). Fluorescein isothiocyanate- or phycoerythrin-conjugated mAbs used were obtained from PharMingen (San Diego, Calif) and eBioscience (San Diego, Calif). Intracellular staining with anti-phospho(p)-Y641-STAT6 (pSTAT6), anti-p-threonine (T) 308-AKT (pAKT), and anti-pT180/pY182 p38 MAP kinase (pp38) antibodies (BD Biosciences) was carried out as previously described.¹⁷ The cells were then stained with conjugated pSTAT6, pAKT, or pp38 (Alexa Fluor 647; BD Biosciences) and analyzed by means of flow cytometry.

Serum total and ovalbumin (OVA)–specific IgE antibodies, *in vitro* IgE production, and T_H cell differentiation

These assays were carried out as previously described.⁸

Cell stimulation and immunoblotting

Purified cell populations were serum starved for 4 hours at 37°C before stimulation with IL-4 or IL-13 (100 ng/mL) for the indicated time periods. Whole cell lysates were normalized for their protein content, resolved by means of SDS-PAGE, and then transferred to nitrocellulose filters and immunoblotted with the indicated antibody. Antibodies against the following phosphoproteins and proteins were used in immunoblotting: pY641-STAT6, STAT6, and pY980-JAK3 (Santa Cruz Biotechnology, Santa Cruz, Calif); anti-pY1020-SHIP-1, pY580-SHP-2, p-serine (S) 473-AKT, AKT, pT202, pY204-extracellular signal-regulated kinase (ERK) 1/2, ERK1/2, pT180/182-38, p38, pY1054/1055-TYK2, and tubulin (Cell Signaling Technology, Danvers, Mass); anti-pY1021/1022-JAK1 (Sigma-Aldrich, St Louis, Mo); and anti-pY536-SHP-1 (ECM Biosciences, Versailles, Ky). The blots were developed with horseradish peroxidase-conjugated secondary antibodies and enzyme-linked chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom).

Macrophage and lung fibroblast cultures and arginase assay

Bone marrow–derived macrophages (BMDMs) were derived by culturing bone marrow aspirates for 7 days with macrophage colony-stimulating factor, cultured in 24-well tissue culture plates, and then stimulated with IL-4 or IL-13 at the indicated concentrations and times. The arginase assay was carried out as previously described.⁵ Primary lung fibroblast cultures were prepared as previously described.¹⁸

OVA-induced allergic airway inflammation

Mice were sensitized and then boosted on days 0 and 14, respectively, with intraperitoneal injection of 100 μ g of OVA mixed in aluminum potassium phosphate (alum) and then challenged intranasally with 50 μ g of OVA daily starting on day 28 for 3 consecutive days. Control mice were sensitized and boosted with PBS mixed with alum and then challenged with OVA. Bronchoalveolar lavage was performed as previously described.⁸ Paraffin-embedded lungs sections were stained with hematoxylin and eosin and periodic acid–Schiff (PAS) as previously described.⁸ Peribronchiolar inflammation was graded with a score as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2 to 4 cell layers deep; and 4, a ring of inflammatory cells more than 4 cell layers deep.¹⁹ The abundance of PAS-positive goblet cells in the airways was scored as follows: 0, less than 5% goblet cells; 1, 5% to 25% goblet cells; 2, 25% to 50% goblet cells; 3, 50% to 75% goblet cells; and 4, greater than 75% goblet cells.¹⁹

Induction of airway hyperresponsiveness and measurement of airway responsiveness

Mice were sensitized with 50 μ g of OVA (Sigma-Aldrich) in 2 mg of alum administered intraperitoneally. A week later, the mice were challenged with intranasal antigen (50 μ g of OVA/d) administered on 3 consecutive days. Control mice received intraperitoneal injections of alum and intranasal administrations of OVA. Twenty-four hours after the final dose, airway hyperresponsiveness (AHR) was assessed as previously described.²⁰ Briefly, mice were anesthetized with 50 mg/kg pentobarbital and instrumented for the measurement of pulmonary mechanics (BUXCO Electronics, Wilmington, NC). Mice were tracheostomized, intubated, and mechanically ventilated as previously described.²⁰ Baseline lung resistance and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to increasing doses (0.32 to 40 mg/mL) of aerosolized acetyl- β -methylcholine chloride (methacholine; Sigma-Aldrich). The 3 highest values of lung resistance obtained after each dose of methacholine were averaged to obtain the final values for each dose.

Statistical analysis

The Student *t* test, 2-way ANOVA, repeated-measures ANOVA with Bonferroni post-test analysis of groups, and the Kruskal-Wallis test with Dunn

post-test analysis of groups were used to compare test groups. A *P* value of less than .05 was considered statistically significant.

RESULTS

Characterization of the IL-4R α ITIM

To identify putative ITIMs in the IL-4R α and elucidate their function, we first analyzed the capacity of individual tyrosine motifs present in the human IL-4R α chain cytoplasmic domain to serve as ITIMs. These studies identified the tyrosine motif at position Y713 to uniquely serve as an ITIM (see Fig E1 in this article's Online Repository at www.jacionline.org). A peptide spanning phospho-Y713, but not other phospho-Y residues, bound the carboxyl-terminal SH2 domain of SHP-1 and activated SHP-1 phosphatase activity, which is consistent with its function as an ITIM. In contrast, unphosphorylated Y713 peptide failed to bind to SHP-1 (see Fig E1).

The functional consequences of ITIM inactivation were analyzed in a cellular model by using a chimeric receptor composed of the extracellular and transmembrane domains of the erythropoietin receptor (EpoR) and the cytoplasmic domain of the IL-4R α (EpoR/IL-4R α). The chimeric receptor was expressed in the human Burkitt lymphoma cell line BJAB, which does not express EpoR (data not shown). Treatment with erythropoietin induces dimerization of EpoR/IL-4R α and receptor activation independent of contribution of the γ c chain or IL-13R α 1. When stimulated with erythropoietin, BJAB cells expressing the chimeric receptor mediated activation of JAK1 and STAT6 as evidenced by their phosphotyrosine phosphorylation (see Fig E2 in this article's Online Repository at www.jacionline.org). Inactivation of the ITIM by mutagenesis of Y713 into F713 resulted in enhanced activation of JAK1 and STAT6, which is consistent with a negative regulatory function of the ITIM in receptor signaling. By using an overexpression system in HeLa cells in which EpoR/IL-4R α and SHP-1 were coexpressed, it could be demonstrated that SHP-1 associated with EpoR/IL-4R α and that mutagenesis of the ITIM Y713 to F713 abrogated this association (see Fig E3 in this article's Online Repository at www.jacionline.org).

Generation of IL-4R α F709 mutant mice by means of targeted knock-in mutagenesis

In view of the above results and to elucidate the function of the IL-4R α ITIM in an *in vivo* murine system, we exploited the virtual identity of the extended human and murine ITIMs (GIVpY⁷¹³SALTCHL and GIVpY⁷⁰⁹SSLTCHL, respectively) to mutagenize the critical Y709 residue of the murine IL-4R α ITIM to F709 by means of targeted knock-in mutagenesis of *Il4ra*. This approach, which mirrored an earlier study that analyzed the IRS-2 docking site at the Y500 residue, disabled the ITIM by preventing its phosphorylation and its association with SHP-1 (see Figs E1 and E3 in this article's Online Repository at www.jacionline.org).²¹ Homozygous mutant mice of both sexes were phenotypically indistinguishable from their WT and heterozygous littermates. Expression levels of the mutant receptor protein were verified by means of flow cytometry to be closely matched to WT IL-4R α (see Fig E4 in this article's Online Repository at www.jacionline.org). Analysis revealed the T and B lymphocytes of the IL-4R α F709 mutant mice to be normal in number and phenotype (data not shown).

The F709 mutation augments STAT6 phosphorylation on IL-4R signaling

To elucidate signaling mechanisms by which the F709 substitution mediates its proallergic inflammatory effects, we examined IL-4R α signaling in hematopoietic and airway epithelial cells of C.129.*Il4ra*^{F709/F709} and control C.129.*Il4ra*^{Y709/Y709} mice. Treatment with IL-4 resulted in enhanced phosphorylation of STAT6 in C.129.*Il4ra*^{F709/F709} B lymphocytes compared with that seen in the C.129.*Il4ra*^{Y709/Y709} control lymphocytes, as detected by means of immunoblotting with an anti-pSTAT6 antibody (Fig 1, A). Although the F709 mutation augmented STAT6 phosphorylation by IL-4, it did not influence the course of STAT6 dephosphorylation after signal termination (Fig 1, B). Enhanced STAT6 phosphorylation in IL-4-treated C.129.*Il4ra*^{F709/F709} B and T cells was also documented by intracellular staining with anti-pSTAT6 antibodies, with T cells exhibiting more pronounced augmentation of pSTAT6 formation compared with B cells (Fig 1, C and D). The increase in the mean fluorescence intensity of pSTAT6 staining in the mutant cells reflected an absolute increase in STAT6 phosphorylation because the WT and mutant cells exhibited similar basal pSTAT6 mean fluorescence intensity. Collectively, these data established that the ITIM mutagenesis augmented the induction phase of STAT6 phosphorylation in response to IL-4R signaling.

The F709 mutation promotes T_H2 polarization and IgE responses

To determine whether the F709 substitution influences T_H cell differentiation as a contributing factor in its promotion of allergic airway inflammation, we compared cytokine production of T cells of C.129.*Il4ra*^{F709/F709} and control C.129.*Il4ra*^{Y709/Y709} mice that were induced to differentiate *in vitro* into T_H1 or T_H2 effector lymphocytes. C.129.*Il4ra*^{F709/F709} T_H2 cells exhibited a significant increase in IL-4 production compared with that seen in C.129.*Il4ra*^{Y709/Y709} T_H2 cells. In contrast, there was no significant difference in IFN- γ production between T_H1 cells of the respective genotype (Fig 2, A and B). These results indicated that the F709 mutation promotes IL-4 production by T_H2 cells.

The effect of the F709 mutation on humoral immunity was examined by measuring the total and antigen-specific antibody responses after immunization with OVA mixed with alum adjuvant to promote T_H2-type responses²² or with saline/alum as an immunization control. Fig 2, C, shows that the C.129.*Il4ra*^{F709/F709} mice exhibited enhanced total serum concentrations of IgE after immunization with OVA/alum compared with those seen in the C.129.*Il4ra*^{Y709/Y709} control mice. Significantly, OVA-specific antibody responses were also increased in the F709 mutant mice compared with those seen in control mice (Fig 2, D). The heightened IgE production in the F709 mutant mice was not the result of an intrinsic alteration in the capacity of the C.129.*Il4ra*^{F709/F709} B cells to produce IgE because purified mutant and control B cells produced similar levels of IgE when stimulated *in vitro* with anti-CD40 mAb plus increasing concentrations of IL-4 (Fig 2, E). Also, IL-4 stimulation did not superinduce the expression in B cells of CD23 and I-A^d, which proceeds in a STAT6-dependent mechanism. These results suggested that the increased IgE production in C.129.*Il4ra*^{F709/F709} mice was extrinsic to the B cells, possibly involving increased IL-4 production by the C.129.*Il4ra*^{F709/F709} T cells.

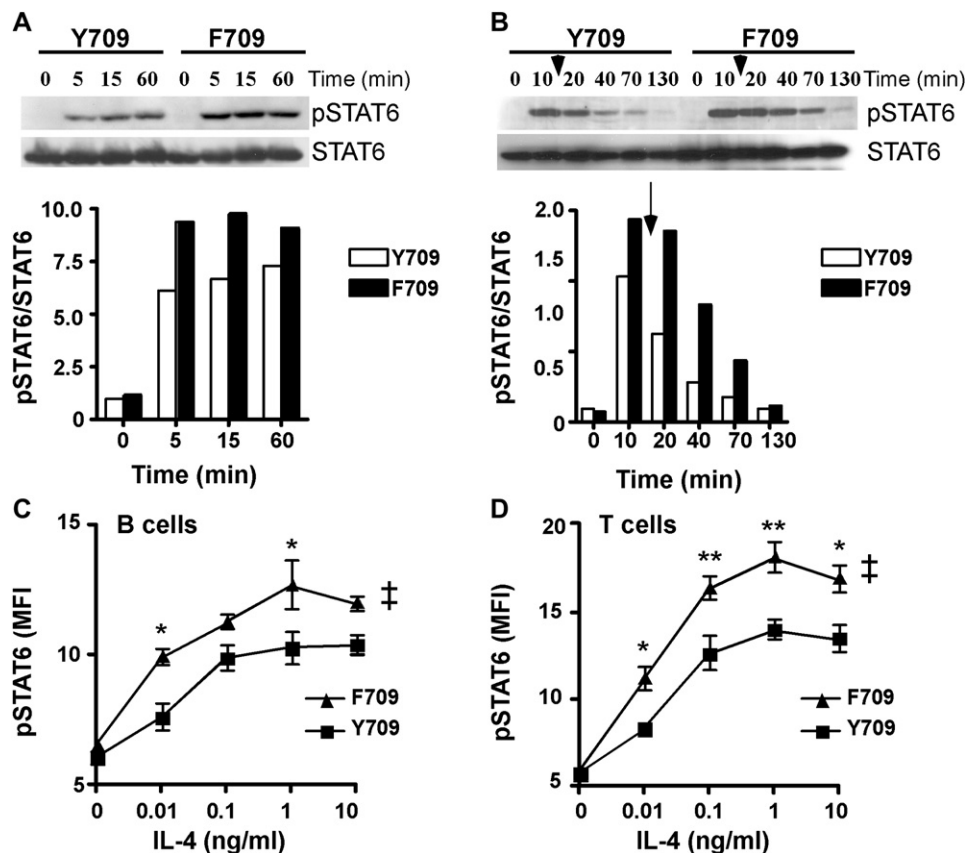


FIG 1. Enhanced STAT6 activation in the C.129.*Il4ra*^{F709/F709} mutant mice. **A**, upper and middle panels, Western blot analysis of pSTAT6 induction in B cells at different time points after treatment with IL-4 (100 ng/mL). Lower panel, Densitometric measurement of pSTAT6 normalized for STAT6 content. **B**, upper panel, Decay of pSTAT6 signal in B cells on cessation of IL-4 treatment. Cells were treated for 10 minutes with IL-4 (100 ng/mL) and then washed (arrow) and analyzed at the indicated time points for pSTAT6 content. Lower panel, Densitometric measurement of pSTAT6 normalized for STAT6 content. **C** and **D**, Flow cytometric analysis of pSTAT6 induction in WT (Y709) and mutant (F709) B cells (**Fig 1, C**) or T cells (**Fig 1, D**) after treatment with graded concentrations of IL-4 for 15 minutes. Results for **Fig 1, A** and **B**, are representative of 2 independent experiments each, and those for **Fig 1, C** and **D**, are the average of 3 mice per group per experiment, with similar results observed in 3 independent experiments. ‡*P* < .0001 for genotype effect (F709 vs Y709; repeated-measures 2-way ANOVA); **P* < .05 and ***P* < .01 (2-way ANOVA with Bonferroni post-test analysis of groups).

The F709 mutation promotes alternative macrophage activation

Alternative macrophage activation is regulated by IL-4R α /STAT6 signaling and is more effectively achieved by signaling through the type I IL-4R.^{5,6,23} To elucidate the effect of the F709 mutation on alternative macrophage activation, we examined the induction by IL-4 and IL-13 of STAT6 activation, the expression of the STAT6-regulated genes *Arg1* and *Chi3l3*, and arginase enzymatic activity in BMDMs of C.129.*Il4ra*^{F709/F709} mutant and C.129.*Il4ra*^{Y709/Y709} WT control mice. BMDMs are normally more sensitive to IL-4 treatment compared with IL-13 treatment.^{5,6} Both IL-4 and, to a lesser extent, IL-13 induced increased pSTAT6 formation in Y709 BMDMs, which reached a plateau at 10 ng/mL and 100 ng/mL, respectively (**Fig 3, A**). Although the F709 mutation upregulated pSTAT6 formation by both cytokines, it disproportionately augmented pSTAT6 formation by IL-13 compared with that seen in similarly treated WT BMDMs (**Fig 3, A** and **B**). In contrast, the F709 mutation did not alter the activation by IL-4 and IL-13 of the protein kinase AKT, a downstream

target of the IL-4R-coupled IRS2/PI3-kinase pathway (see **Fig E5** in this article's Online Repository at www.jacionline.org). Similarly, it did not affect the induction by IL-4 and, more modestly, IL-13 of p38 MAP kinase activation, whereas activation of p42/p44 ERK kinases was not induced in either WT or mutant BMDMs at greater than baseline levels (see **Fig E5** and data not shown). Overall, these results indicated a measure of specificity in the effect of the F709 mutation on IL-4R-coupled signaling pathways.

In Y709 BMDMs IL-4 induced markedly higher levels of *Arg1*, *Chi3l3*, and *Ccl11* transcripts and arginase enzymatic activity with a left-shifted dose-response curve compared with IL-13 (**Fig 3, C-F**). The F709 mutation markedly augmented the induction by IL-13 of *Arg1*, *Chi3l3*, and *Ccl11* transcripts and of arginase enzymatic activity, whereas it more modestly upregulated the IL-4 responses or left them unaffected (**Fig 3, C-F**). Furthermore, examination of responses to IL-4 and IL-13 in primary lung fibroblast cultures of F709 mice revealed a similar pattern of differential upregulation of *Ccl11* transcription by IL-13 but

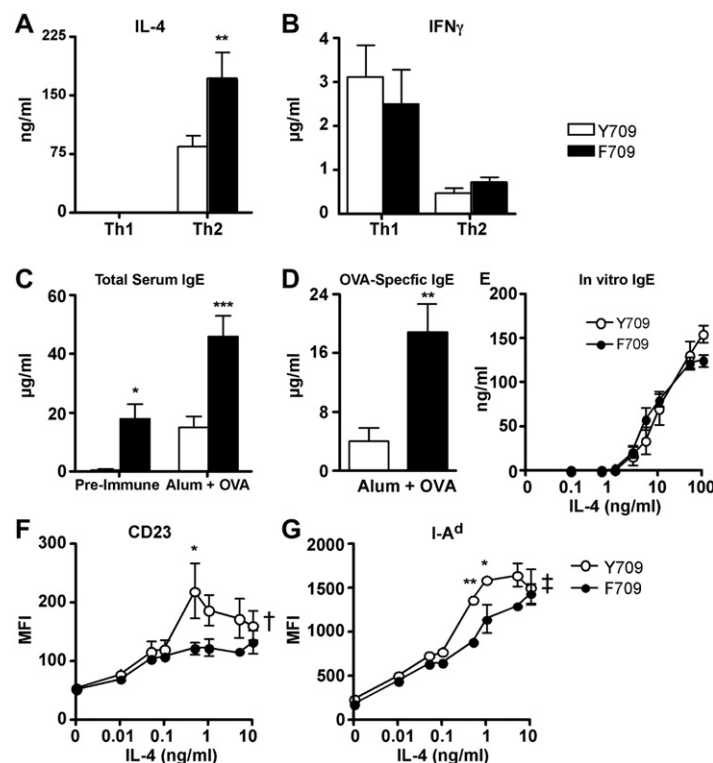


FIG 2. The F709 mutation enhances IL-4 production by T_H2 cells and IgE responses *in vivo*. **A** and **B**, Cytokine production by T_H cells. Naive WT (Y709) and F709 splenic T cells ($n = 3-7$ mice per group) were differentiated into T_H1 (**Fig 2, A**) or T_H2 (**Fig 2, B**) cells and then stimulated with anti-T-cell receptor β mAb for 48 hours. Culture supernatants were analyzed for IL-4 and IFN- γ production by means of ELISA. **C** and **D**, Total and OVA-specific serum IgE levels in F709 and WT control mice ($n = 6-8$ per group) collected before (*Pre-Immune*) or after immunization with OVA/alum. **E**, *In vitro* IgE production by purified splenic B cells of WT and F709 mice ($n = 5$ per group) after stimulation with anti-CD40 mAb plus IL-4. There was no significant difference between the 2 groups (repeat-measures 2-way ANOVA). **F** and **G**, Upregulation of CD23 and I-A^d expression in Y709 and F709 B cells treated with increasing concentrations of IL-4 ($n = 3$ per group). * $P < .05$, ** $P < .01$, *** $P < .001$, † $P < .01$, and ‡ $P < .0001$ for genotype effect (Student *t* test for **Fig 2, D**, and 2-way ANOVA with post-test analysis for **Fig 2, A, C, F**, and **G**). *MFI*, Mean fluorescence intensity.

not IL-4 (**Fig 3, G**, and data not shown). These results are consistent with differential enhancement by the F709 mutation of IL-13-induced alternative macrophage activation through the type II IL-4R.

To elucidate mechanisms by which the F709 substitution augmented IL-4 and IL-13 signaling, we examined the phosphorylation of the ITIM substrates SHP-1, SHP-2, and SHIP-1 in response to IL-4 and IL-13 treatment of BMDMs derived from C.129.*Il4ra*^{F709/F709} and control C.129.*Il4ra*^{Y709/Y709} mice. Phosphorylated proteins were detected by means of immunoblotting with specific anti-phospho antibodies. SHIP-1 was found to be constitutively phosphorylated in BMDMs. Both IL-4 and IL-13 induced partial dephosphorylation of SHIP-1 in WT cells, whereas this dephosphorylation was abrogated in C.129.*Il4ra*^{F709/F709} mutant BMDMs (**Fig 4**). IL-4 treatment induced an early increase in SHP-2 phosphorylation that was, in general, of similar magnitude in WT and mutant BMDMs. IL-13 induced delayed phosphorylation of SHP-2 that was observed only at high cytokine concentrations and that was also similar between WT and mutant BMDMs (**Fig 4**). IL-4 also induced SHP-1 phosphorylation in WT BMDMs, the magnitude of which was attenuated in F709 BMDMs. The attenuation by the F709 mutation of SHP-1 phosphorylation was more

pronounced in the case of IL-13 treatment. Examination of JAK1 revealed that it was hyperphosphorylated at baseline in F709 relative to Y709 BMDMs. It became hypophosphorylated on activation in both cell types, while maintaining increased pY content in F709 cells. Both JAK3 and Tyk2 underwent activation-induced hyperphosphorylation in F709 BMDMs relative to that seen in WT control cells. Collectively, these data established that the ITIM mutagenesis impaired SHP-1 phosphorylation and, reciprocally, exaggerated receptor-associated kinase activation and STAT6 phosphorylation, which is consistent with defective recruitment of SHP-1 as one likely mechanism by which the ITIM mutagenesis exerts its effects.

The F709 mutation results in enhanced antigen-induced allergic airway inflammation

The functional consequences of the F709 mutation were analyzed in antigen-driven murine models of allergic airway inflammation. In an acute model of antigen-induced allergic airway inflammation, C.129.*Il4ra*^{F709/F709} and C.129.*Il4ra*^{Y709/Y709} mice were sensitized on day 0 and boosted on day 14 with OVA mixed with alum and then challenged at 4 weeks after sensitization with inhaled OVA for 3 consecutive days. Control mice were

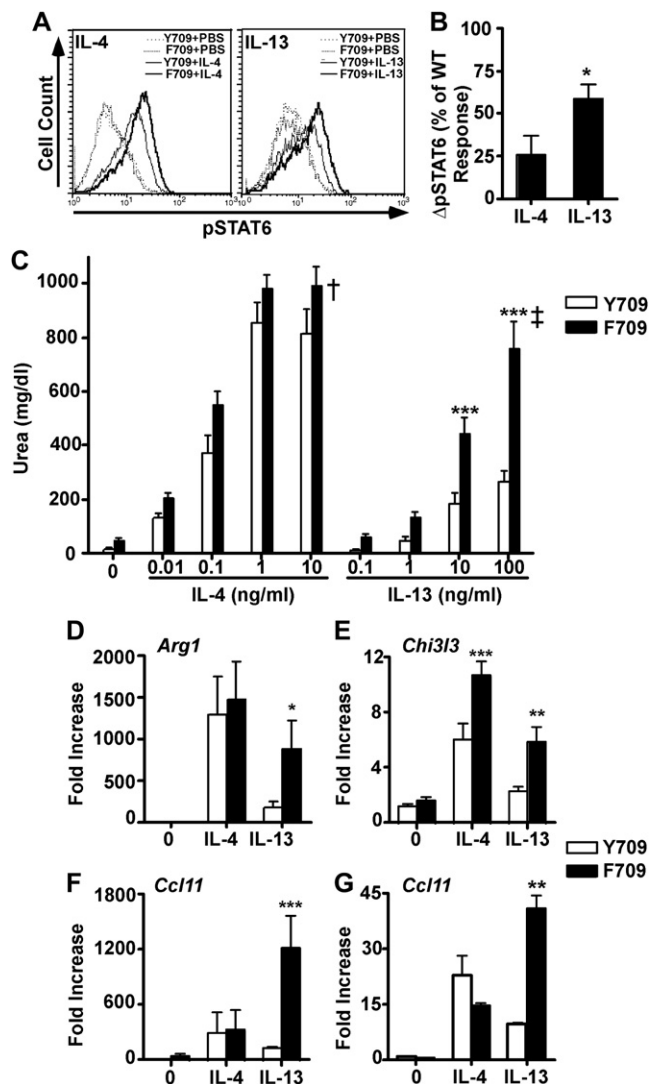


FIG 3. The F709 mutation enhances alternative macrophage activation. **A**, Intracellular staining of pSTAT6 in WT (Y709) and F709 BMDMs stimulated for 15 minutes at 37°C with IL-4 (10 ng/mL) or IL-13 (100 ng/mL). **B**, Bar graph plot of the difference in the relative increase in pSTAT6 formation (Δ pSTAT6) in IL-4- and IL-13-treated F709 BMDCs compared with similarly treated Y709 BMDCs ($n = 3$ mice per group). **C**, Arginase activity in lysates of BMDMs treated with various concentrations of IL-4 or IL-13 and analyzed after 48 hours by means of measurement of urea production ($n = 9$ –12 per group). **D–G**, Real-time PCR of genes encoding arginase 1 (*Arg1*), chitinase 3-like 3 (*Chi3l3*), and *Ccl11* in BMDMs (**Fig 3, D–F**) and *Ccl11* in primary lung fibroblast cultures (**Fig 3, G**) stimulated for 20 hours with IL-4 or IL-13 at 10 ng/mL each ($n = 7$ –12 per group). * $P < .05$, ** $P < .01$, *** $P < .001$, † $P < .01$, and ‡ $P < .0001$ for genotype effect (Student t test for **Fig 3, B**, and 2-way ANOVA with post-test analysis for **Fig 3, C–G**).

immunized and boosted with PBS/alum and challenged with inhaled OVA. Microscopic examination of hematoxylin and eosin- and PAS-stained lung sections of WT and mutant mice revealed patchy peribronchial inflammatory infiltrates composed primarily of eosinophils and lymphocytes that were substantially more prominent in OVA-sensitized and challenged C.129.*Il4ra*^{F709/F709} mice compared with those in C.129.*Il4ra*^{Y709/Y709} control mice (**Fig 5, A**, and data not shown). In contrast, sham-immunized mice exposed to aerosolized OVA showed normal lung histology (data not shown). Significantly, OVA-challenged

C.129.*Il4ra*^{F709/F709} mutants exhibited markedly increased peribronchial and perivascular inflammation and increased numbers of goblet cells compared with those seen in C.129.*Il4ra*^{Y709/Y709} control mice (**Fig 5, B and C**).^{24–27} Analysis of bronchoalveolar lavage fluid of OVA-sensitized and challenged mice revealed that OVA-sensitized C.129.*Il4ra*^{F709/F709} mice exhibited increased accumulation of eosinophils in the airways compared with that seen in similarly treated control animals (**Fig 5, D**). A more limited antigenic sensitization protocol was used to better discriminate the genotype effect to determine whether the F709 mutation increased AHR after antigenic sensitization and challenge (see the Methods section). Results revealed that OVA-sensitized C.129.*Il4ra*^{F709/F709} mice exhibited augmented AHR in response to OVA challenge compared with similarly treated Y709 control BALB/c mice (**Fig 5, E**). The heightened AHR in the F709 mutants induced by the limited OVA sensitization protocol was also associated with a dramatic increase in airway eosinophilia compared with that seen in Y709 control mice (see **Fig E6, A** in this article's Online Repository at www.jacionline.org). These results indicated that the F709 substitution caused an increase in allergic airway inflammation and AHR in response to allergen sensitization and challenge.

To determine the effect of the F709 mutation on the transcription of STAT6-responsive genes induced during allergic airway inflammation, we measured the relative transcript levels of a battery of STAT6-responsive genes in OVA- and sham-sensitized C.129.*Il4ra*^{F709/F709} and C.129.*Il4ra*^{Y709/Y709} mice after challenge with OVA. Results revealed that the F709 mutation upregulated the expression of a number of STAT6-responsive gene transcripts both at baseline and after OVA challenge, including transcripts of the cysteinyl leukotriene receptor 1 and 2 genes (*Cyslr1* and *Cyslr2*, respectively), 5-lipoxygenase (*Alox5*), and resistin like β (*Retnlb*; **Fig 5, F–I**). Transcripts encoded by the T_H2 cytokine genes *Il4* and *Il9* were also significantly increased in lung tissues of allergen-challenged C.129.*Il4ra*^{F709/F709} mutant mice compared with those seen in WT control animals, whereas those encoded by *Il5* and *Il13* were increased but did not achieve significance (see **Fig E6, B–E**). These results indicated that the F709 mutation upregulated the expression of a number of STAT6-responsive genes involved in allergic airway inflammation.

DISCUSSION

Our findings reveal a significant regulatory function of the IL-4R α chain ITIM in receptor signaling *in vivo*. Inactivation of the ITIM site disrupted the normal homeostasis of allergic effector pathways, giving rise to spontaneously increased IgE levels, goblet cell metaplasia of the bronchial epithelium, and influx of eosinophils into the airways of allergen-naïve mice. Isolated T, B, and BMDM cells evidenced enhanced receptor signaling in response to IL-4 and (for BMDMs) IL-13. Furthermore, the F709 mutation resulted in amplification of the allergic response to the allergen OVA, as evidenced both by increased total and antigen-specific IgE production and by exaggerated airway inflammation, mucus metaplasia, and AHR. These results identify a negative regulatory function for the IL-4R α ITIM in controlling allergic tone both in the unchallenged basal setting and after challenge with allergens.

Induction of STAT6 phosphorylation in ITIM mutant cells was enhanced at different concentrations of IL-4 and IL-13. Signal enhancement started at the earliest time points examined, and it

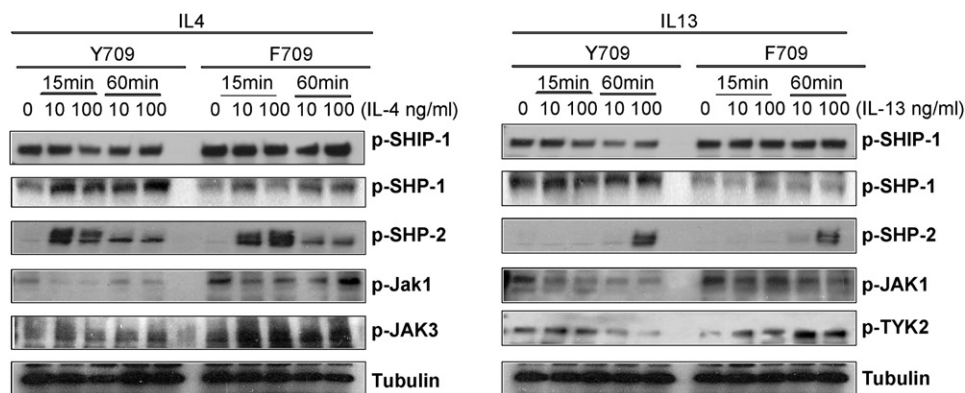


FIG 4. The F709 mutation impairs SHP-1 activation in BMDMs. WT or F709 mutant BMDMs were treated with IL-4 or IL-13 at 10 or 100 ng/mL for 15 or 60 minutes, as indicated. Cellular lysates were derived and probed for pSHP-1, pSHP-2, pSHIP-1, pJAK1, pJAK3, pTYK2, and β -tubulin, the latter as a protein loading control. Results are representative of 2 to 3 independent experiments per immunoblot panel.

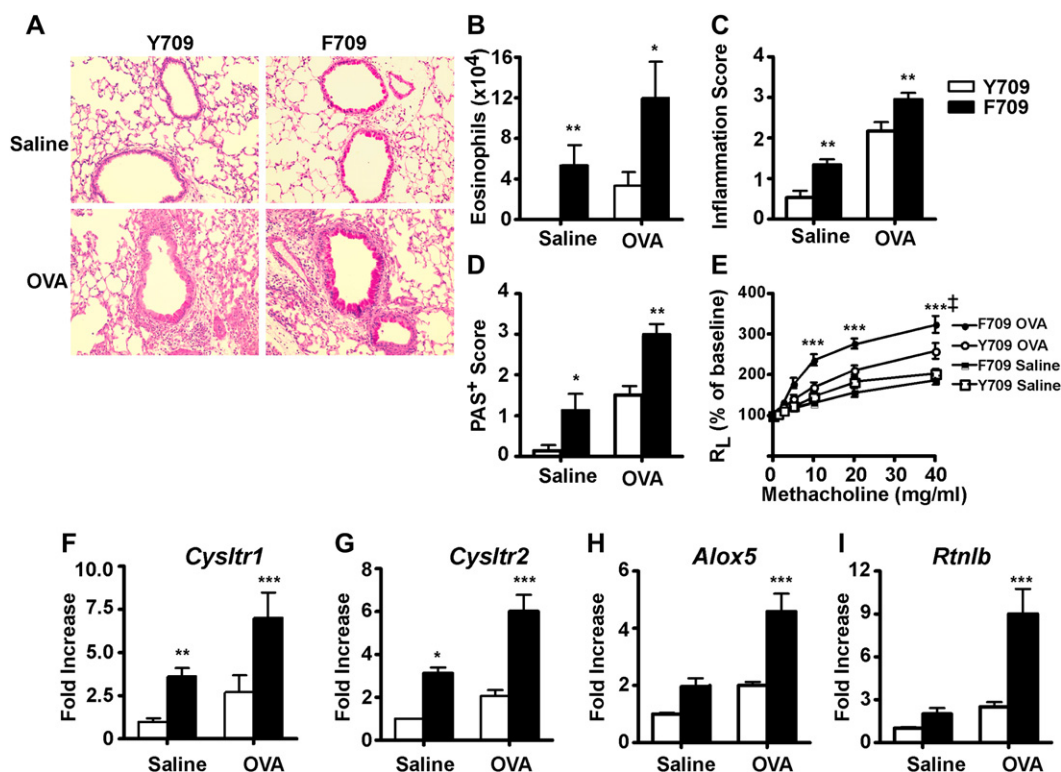


FIG 5. The Y709F substitution promotes acute antigen-induced allergic airway inflammation and AHR. Sham (saline/alum)- or OVA/alum-sensitized C.129.*Il4ra*^{Y709/Y709} (Y709) and C.129.*Il4ra*^{F709/F709} (F709) mice ($n = 10$ mice per group) were challenged intranasally with OVA on 3 consecutive days and then analyzed. **A**, Lung histology (PAS staining, magnification 20 $\times$). **B**, Peribronchial inflammation scores. **C**, PAS-positive goblet cells in the airways. **D**, Eosinophilia in bronchoalveolar lavage fluid of sham- and OVA-immunized mice challenged with OVA. **E**, AHR measurements. Changes in lung resistance (R_L) were measured in mice that had been either sham immunized or subjected to a limited protocol of OVA immunization ($n = 5$ -8 per group). **F-I**, Real-time PCR analysis of *Cysltr1*, *Cysltr2*, *Alox5*, *Alox15*, and *Rtnlb* mRNA levels in total lung extracts of OVA-sensitized and challenged mice versus control mice ($n = 3$ -6 per group). * $P < .05$, ** $P < .01$, *** $P < .001$, and † $P < .0001$ for genotype effect (Fig 5, B, Kruskal-Wallis test; Fig 5, C-I, 2-way ANOVA with post-test analysis).

persisted for the duration of cytokine treatment. However, the course of STAT6 dephosphorylation once the cytokine treatment was terminated remained unaltered. In contrast to the conclusions of a previous report, our results suggest that the ITIM regulates the induction, rather than termination, phase of IL-4R signaling,

at least in the cell types under study.²⁸ Earlier studies on other receptor systems and on the IL-4R itself indicate that the mechanism of action of ITIMs in regulating receptor signaling involves recruitment of the phosphatases SHP-1, SHP-2, and SHIP-1.¹³ Suppressor of cytokine signaling (SOCS) proteins

also bind at ITIMs and thus might contribute to signal regulation at the IL-4R α ITIM.²⁹ The identity of the respective intermediate recruited to the IL-4R ITIM in different cell types might vary depending on the level of expression. It is also likely that, in addition to signal regulation, the IL-4R α ITIM might mediate distinct effector functions that are dependent on the identity of the bound intermediate. The balance of intermediates bound at the IL-4R α ITIM in different tissues remains to be established, as does the nature of their distinct effector functions in response to IL-4 signaling, IL-13 signaling, or both.

The cytoplasmic domain of the IL-4R α possesses 3 STAT6 binding sites in tandem, which ensures amplification of the receptor signaling by means of STAT6 activation, and a single ITIM site, the function of which is demonstrably inhibitory.^{1,2,30} Although at baseline the receptor did not signal constitutively, the F709 mutation amplified IL-4R-related responses *in vivo*. Many of the phenotypes associated with the F709 mutation, including strikingly increased baseline IgE levels, differential augmentation of alternative macrophage activation by IL-13, and enhanced goblet cell metaplasia and allergic airway inflammation are attributes associated with increased signaling through the type II IL-4R.^{5,6} These results indicate an important function of the ITIM would be to increase the threshold for receptor activation, especially by the type II IL-4R, so as to suppress exaggerated *in vivo* responses to subthreshold immunogenic stimuli.

Both *in vitro* and *in vivo* studies revealed that the mechanism by which the IL-4R ITIM promotes signaling involves upregulation of JAK activation and STAT6 phosphorylation. This mechanism does not relate to an influence on IL-4 and IL-13 binding to the IL-4R extracellular domain because it remains operative when examined in a chimeric receptor composed of the EpoR extracellular domain and the IL-4R intracellular domain (see Fig E2). Rather, it appears to involve defective recruitment of SHP-1 to the mutant receptor. SHP-1 phosphorylation was decreased on IL-4 and (more so) IL-13 signaling in mutant BMDMs, which is indicative of impaired recruitment and activation of SHP-1 by the ITIM. The mechanism for the disproportionate effect of the ITIM mutation on signaling through the IL-4R type II receptor (by IL-13) versus the type I receptor (by IL-4) might involve residual recruitment of SHP-1 by the other components of the type I IL-4R, as evidenced by decreased, although not absent, phosphorylation of SHP-1 by IL-4 in ITIM mutant cells (Fig 4). A recent study has linked the enhanced efficacy of the type I IL-4R in inducing alternative macrophage activation to its differential capacity to activate the IRS/PI3-kinase pathway.³¹ However, there was no evidence that the ITIM mutant resulted in enhanced IRS/PI3-kinase activation, as monitored by the activation of the downstream kinase AKT.

Although the F709 mutation augmented STAT6 phosphorylation in B cells, it was not associated with enhanced IgE production by isolated B cells induced to switch to IgE production in a T cell-independent manner. Similarly, it did not upregulate the expression in B cells of the STAT6-responsive gene products CD23 and I-Ad. These results suggest that in addition to regulating STAT6 phosphorylation, intermediates recruited at the ITIM might provide additional signals that promote STAT6 function. The loss of such signals on ITIM mutagenesis might counterbalance the effects of upregulated STAT6 phosphorylation. Thus the striking increase in the level of IgE production in the ITIM mutant mice is likely to reflect increased IL-4 production secondary to favored T_H2 responses.

The Y709F mutation also mediated allergic airway inflammation both at baseline and in an antigen-dependent manner (OVA sensitization). In both cases the inflammation was associated with increased airway eosinophilia. 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 F709 allele promotes allergic airway inflammation.^{32,33} Gene expression analysis identified additional mechanisms by which the F709 allele can exert proinflammatory functions. In particular, the Y709F mutation upregulated the expression of components of the leukotriene pathway, which play a critical role in allergic airway inflammation: 5-lipoxygenase (*Alox5*) and cysteinyl leukotriene receptor 1 and 2 (*Cyslr1* and *Cyslr2*, respectively).³⁴ It also upregulated the expression of resistin-like β (*Retnlb*), which promotes allergic airway inflammation and the induction of subepithelial fibrosis.³⁵ Together, these findings reinforce intervention strategies at these pathways aimed to interrupt the evolution of airway inflammation associated with a strongly proatopic phenotype like that of the Y709F substitution.³⁵

Despite the potentiation by the F709 mutation of STAT6 phosphorylation in B cells, there was no observed difference in the intrinsic capacity of the mutant B cells to produce IgE in response to IL-4. These results suggest that the ITIM transmits STAT6-independent signals that promote IgE production in B cells and that this function of the ITIM is lost on its mutagenesis. They also indicate that the increase in IgE production observed *in vivo* reflects the increased supply of IL-4, which is in line with the observed T_H2 bias in the F709 mice both *in vitro* (Fig 2, A) and *in vivo* (see Fig E6, B).

Epidemiologic surveys have linked different IL-4R α polymorphisms to phenotypes of atopy and asthma both individually and in combination.³⁶⁻³⁹ It is remarkable that a mutation that results in dramatic proallergic responses, such as the F709 mutation, was nevertheless associated with a relatively modest increase in IL-4/IL-13-induced STAT6 phosphorylation (20% to 50% increase over WT that seen in controls). These findings indicate that even subtle changes in receptor signaling associated with some IL-4R polymorphisms can result in a profoundly atopic phenotype. The contribution of altered ITIM function to the proatopic and disease-promoting effects of different IL-4R α polymorphisms merits further investigation.

We thank Dr Raif Geha for his gift of the EpoR/IL-4R α cDNA construct, James J. Booth for animal care, and Maria Garcia-Lloret and Brigitte Gomperts for helpful discussions.

Key messages

- The IL-4R α ITIM negatively regulates signaling by IL-4 and especially by IL-13 *in vivo*.
- Polymorphisms that subtly promote IL-4R signaling amplitude might disproportionately skew the allergic response *in vivo*.

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METHODS

Cell lines

The EBV-negative human Burkitt lymphoma cell line BJAB was cultured in RPMI medium, and HeLa cells were maintained in Dulbecco modified Eagle medium. Cultures were supplemented with 10% FBS and maintained at 37°C in a 5% CO₂ atmosphere.

Plasmids and cDNA

Dominant-negative murine SHP-1 (C453S) was derived as previously described.^{E1} Mutations of individual or both SH2 domains were made as previously described.^{E2} All constructs were cloned into pBluescript (Stratagene, La Jolla, Calif) under the T7 promoter and expressed in HeLa cells by using the vaccinia virus expression system as previously described.^{E2} cDNA encoding the extracellular and transmembrane domains of murine EpoR fused to the intracellular domain of the human IL-4R α were generated and subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, Calif) containing the neomycin resistance gene as previously described.^{E3} For the retroviral construct, dominant-negative murine SHP-1 (C453S) with a myc tag at the 3' end was cloned into pLZR-BMN (provided by Dr G. Nolan) and propagated in Phoenix-Amphotrophic packaging cells.^{E4}

Derivation of EpoR/IL-4R α -expressing BJAB clones

BJAB cells were transfected with expression constructs encoded by means of electroporation with the respective vector followed by antibiotic selection with G418 (Gibco/BRL, Carlsbad, Calif). Antibiotic-resistant cells were seeded in 96-well plates at 0.5 cells per well, and individual clones were derived and analyzed. Expression of EpoR was assessed by immunoblotting with a rabbit anti-mouse EpoR antiserum (kind gift from Dr G. Longmore, Washington University, St Louis, Mo).

Immunoprecipitation and immunoblotting

BJAB cells were treated with the indicated agent and then rapidly placed in pellets and lysed on ice for 15 minutes in a lysis buffer.^{E5} Immunoprecipitation was carried out with anti-mouse IL-4R α and STAT6 antibodies (Santa Cruz Biotechnology) and anti-JAK1 antibodies (Sigma-Aldrich), all coupled with protein G agarose (Sigma-Aldrich). Immunoprecipitated samples were resolved by means of denaturing PAGE and then electroblotted onto nitrocellulose membranes. The blots were probed with the anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-STAT6 antibody (Santa Cruz Biotechnology), and anti-phospho-JAK1 (pY1022/pY1023) antibody (Sigma-Aldrich). The filters were washed and then developed with horseradish peroxidase-conjugated secondary reagent and the enhanced chemiluminescence system for peroxidase-based detection (Amersham).

Peptide conjugation, binding assays, and immunoblotting

The following synthetic peptides (plain and biotinylated) corresponding to the sequences flanking amino acids Y575, Y603, and Y713 of IL-4R α were synthesized: unphosphorylated WT Y575, SAPTSGYQEFVHAVE-NH₂; WT phospho-Y575 (pY575wt), SAPTSG(pY)QEFVHAVE-NH₂; mutant phospho-Y575 (pY575mut), SAPTSG(pY)REFVHAVE-NH₂; phospho-Y603 (pY603; high-affinity STAT6 site), SSGEEG(pY)KPFQDLIP-NH₂; and phospho-Y713 (pY713), SLGSGIV(pY)SALTCHL. Peptides were purchased from Quality Controlled Biochemicals, Inc (Hopkinton, Mass), and the Keck facility at Yale University.

Binding of SHP-1 to biotinylated IL-4R α peptides was carried out by incubating 20 μ g of the indicated biotinylated peptide with a 1-mL lysate of HeLa cells (equivalent to 5×10^6 cells), which expressed myc-tagged SHP-1 (C453S) or mutant derivatives. Peptide-protein complexes were precipitated with Streptavidin beads (Sigma), resolved on SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with rabbit anti-SHP-1 antibodies or with a monoclonal murine anti-myc tag antibody (clone 9E10). Binding assays with SHP-1₁₋₂₂₂ glutathione-S-transferase fusion protein (prepared as described elsewhere^{E6}) were carried out by incubating 20 μ L of peptide-conjugated agarose beads (1 mg of peptide/mL of agarose) with

0.1 μ g of purified fusion protein in 1 mL of lysis buffer for 2 hours at 4°C. Peptide-bound SHP-1 proteins were resolved by means of SDS-PAGE and visualized by means of immunoblotting with a previously described polyclonal rabbit anti-mouse SHP-1 antiserum.^{E1}

SHP-1 phosphatase assays

Baculovirus-expressed full-length SHP-1 was purified by means of conventional chromatography and HPLC.^{E6} The reaction mixture contained 100 μ mol/L peptide, 250 ng of SHP-1, 50 mmol/L HEPES (pH 7), 5 mmol/L EDTA, 1 mmol/L dithiothreitol, and 5 mmol/L p-nitrophenyl phosphate as substrate. Reactions were incubated for 30 minutes at 37°C, and relative phosphatase activity was measured based on absorbance at 405 nm. Results are expressed as the fold increase over basal phosphatase activity of SHP-1 in the absence of peptide. A phosphorylated CD22 peptide corresponding to tyrosine 825 was used as a positive control for the phosphatase assay.

Derivation of genetically manipulated mice

Genomic DNA used to construct the targeting vector were isolated from a bacterial artificial chromosome clone derived from the 129X1Sv/J murine strain that contained the murine IL-4R α gene (Genome Systems).^{E7} The replacement-type targeting vector (pKO; Lexicon) contained 13.5 kb of *IL4RA* sequences that extended from a *Xho*I site proximal to exon 9 to a *Kpn*I site distal to exon 12 of the gene. An AC $\rightarrow$ TT substitution was introduced by means of site-directed mutagenesis (QuickChange, Stratagene) into the second and third positions of codon Y709 (corresponding to bp 2362-63 of the murine IL-4R α cDNA^{E8}), changing it to F709 (TTT). 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 embryonic stem cells and subjected to G418 selection. Successfully targeted clones were transiently transfected with a Cre recombinase to remove the inserted neomycin gene.

Heterozygous embryonic stem cells were injected into C57BL/6 blastocysts, and resultant male chimeras were mated with BALB/c females. Offspring were screened for heterozygotes by means of Southern blotting and PCR analysis. Heterozygous animals were further bred for 10 generations on a BALB/c background (Taconic) to generate the congenic strain C.129/*Il4ra*^{F709/F709}. A targeted clone in which the floxed neo cassette was incorporated (later removed by Cre recombinase action) but that retained the WT Y709 codon was used to generate the control C.129/*Il4ra*^{Y709/Y709} congenic murine strain. C.129/*Il4ra*^{Y709/Y709} mice were used as control animals, except for specifically indicated experiments in which native BALB/c mice were used.

Mouse genotyping

Screening of WT and mutant *Il4ra* alleles was carried out by means of PCR amplification with genomic DNA and the following primers: common forward primer, 5'-ATCCTGTTCCTAACCAGTCC-3'; WT-specific reverse primer, 5'-GGCAAGTGAGGGAAGAGT-3'; and mutant specific reverse primer, 5'-GGCAAGTGAGGGAACGAAA-3'. Primers used for PCR screening of the residual *LoxP*-containing allele were as follows: forward, 5'-GGTGTCTATT TAGGTGCC-3'; reverse, 5'-TCTTCTCTTACTCTGTGCT-3'.

Real-time PCR analysis

Total RNA was extracted from splenocytes of WT and IL-4R α 709F mutant mice with TRIzol (Gibco/BRL). The RNA was treated with Dnase I to remove residual genomic DNA contamination and then reverse transcribed. *Il4ra* transcripts were amplified by using the following pair of primers (both on *Il4ra* exon 12): forward, 5'-GAGCAGCCTTCACACCAGG-3'; reverse, 5'-TGA CATGAGGTTGGCTTCTG-3'. For glyceraldehyde-3-phosphate dehydrogenase transcripts, the primers were as follows: forward, 5'-ACCACAGTCCATGCCAT CAC-3'; reverse, 5'-TCCACCACCCTGTGTGCTGTA-3'. For real-time PCR analysis, cDNA was reverse transcribed from 2 μ g of RNA per sample and used in real-time PCR assays that used commercially available oligonucleotides and Taqman-based reagents (Applied Biosystems, Foster City, Calif). The comparative Ct (threshold cycle) method normalized to glyceraldehyde-3-phosphate

dehydrogenase was used to analyze relative changes in gene expression. The fold change was calculated as $2^{-\Delta\Delta Ct}$ for negative $\Delta\Delta Ct$ values (indicating a fold change increase) and $-2^{\Delta\Delta Ct}$ for positive $\Delta\Delta Ct$ values (indicating a fold change decrease).

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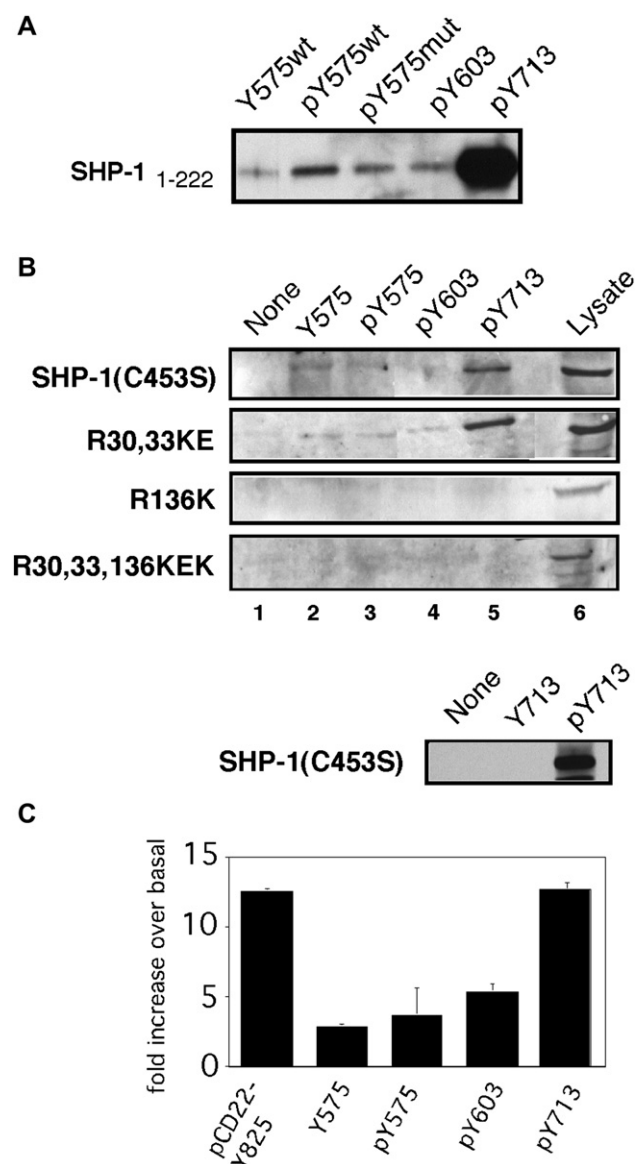


FIG E1. Human IL-4R α -pY713 phosphopeptide binds to and activates SHP-1. **A**, Binding of IL-4R α phosphopeptides to a recombinant SHP-1 fragment spanning the SHP-1 SH2 domains (SHP-1₁₋₂₂₂). Lane 1, Unphosphorylated Y575 (Y575wt); lane 2, pY575 (pY575wt); lane 3, pY575/R576 (pY575mut); lane 4, pY603; and lane 5, pY713. **B**, upper panel, Binding of IL-4R α phosphopeptides to phosphatase-deficient SHP-1 (C453S); N-terminal SH2 domain mutant R30,33KE; C-terminal SH2 domain mutant R136K; and double mutant R30,33,136KEK. Lane 1, No peptide; lane 2, unphosphorylated Y575 (Y575); lane 3, pY575; lane 4, pY603; and lane 5, pY713. Lane 6 shows expression of the respective SHP-1 protein in the HeLa cell lysates used in the binding assays. Lower panel, Association of SHP-1 (C453S) with pY713, but not Y713, peptide. **C**, pY713 activates SHP-1 catalysis. Recombinant SHP-1 was incubated with the indicated IL-4R α peptides or CD22 Y825 ITIM phosphopeptide (positive control) and assayed for phosphatase activity.

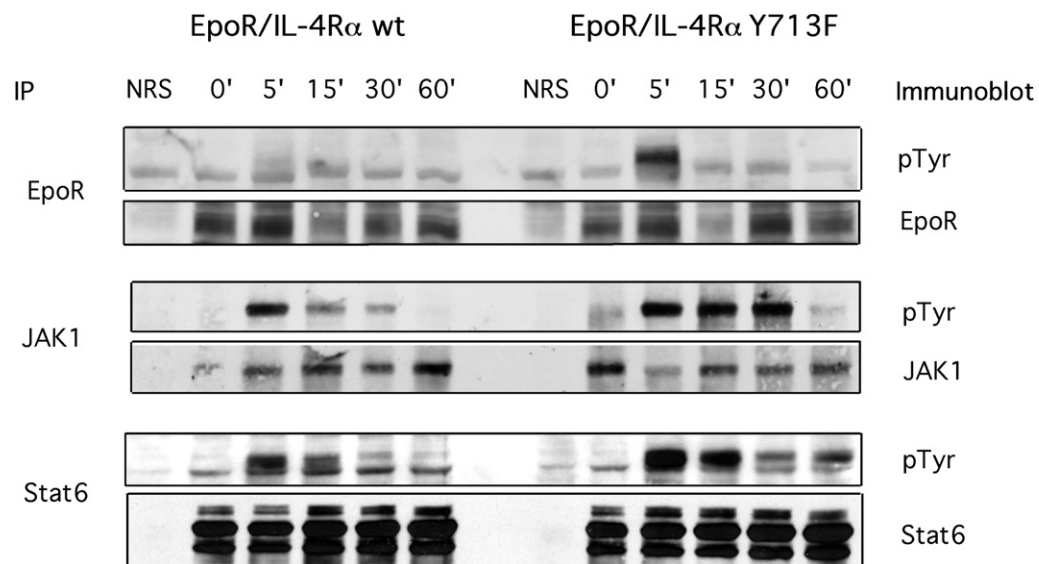


FIG E2. Mutagenesis of the human IL-4R α Y713 ITIM enhances receptor signaling. BJAB cells expressing either WT or mutant EpoR/IL-4R α chimera were treated with erythropoietin for the indicated time periods. The cells were then lysed, and EpoR, JAK1, and STAT6 immunoprecipitates (*IP*) were resolved by means of SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (pTyr). Blots were then reprobed with antibodies against the respective protein species. Similar results were found in 2 other experiments. *NRS*, Immunoprecipitates obtained with normal rabbit serum treatment of extracts of unstimulated cells, to detect non-specifically-bound proteins.

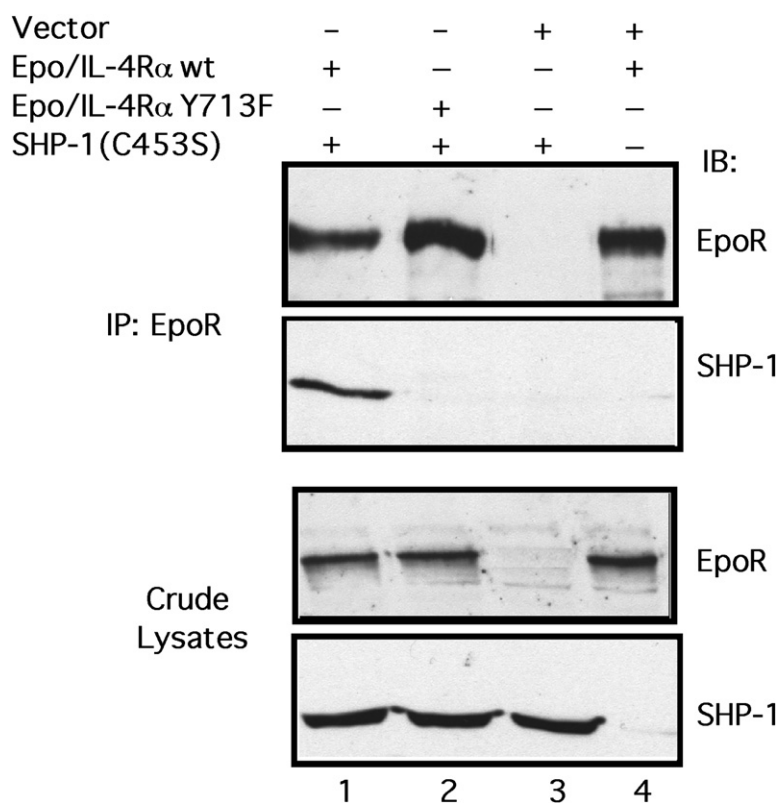


FIG E3. Association of SHP-1 with the EpoR/IL-4R α chimera in a HeLa cell overexpression system. Cells were cotransfected with the vectors SHP-1 (C453S), EpoR/IL-4R α wt, and EpoR/IL-4R α Y713F or the indicated vector controls. The cells were lysed, and the chimeric receptors were immunoprecipitated (IP) with an anti-EpoR antiserum. Immunoprecipitates were subjected to immunoblotting (IB) with anti-EpoR antiserum followed by anti-SHP-1 mAb to detect SHP-1 (C453S). Similar results were found in 2 other experiments.

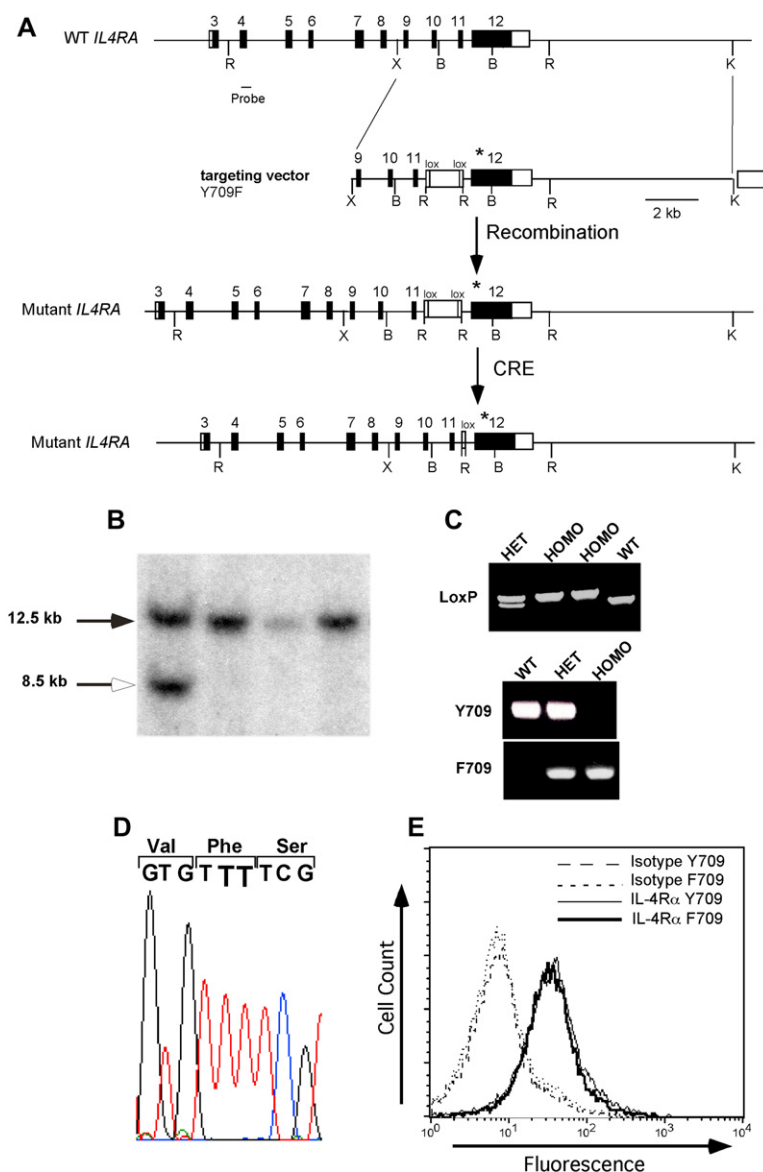


FIG E4. Targeted knock-in mutagenesis of the murine *IL-4Rα* ITIM. **A**, Targeting strategy. WT exon 12 was replaced with one carrying an AC→TT substitution (*) in the second and third positions of codon Y709, changing it to F709. **B**, Southern blot analysis of *EcoRI*-digested murine tail genomic DNA of WT Y709 and heterozygous (*HET*) and homozygous (*HOMO*) F709 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 murine tail DNA with Y709 and F709 allele-specific primers (upper and middle panels, 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 (Y709) and F709 mutant mice. **E**, Flow cytometric analysis of *IL-4Rα* expression in *IL-4Rα* Y/F576 lymphocytes. Splenic B cells of WT C.129.*Il4ra*^{Y709/Y709} (Y709) and homozygous C.129.*Il4ra*^{F709/F709} mutant (F709) mice were stained with phycoerythrin-conjugated rat anti-mouse *IL-4Rα* mAb (mIL4R-M1). Control staining was carried out on WT splenic B cells by using a phycoerythrin-conjugated, isotype-matched antibody.

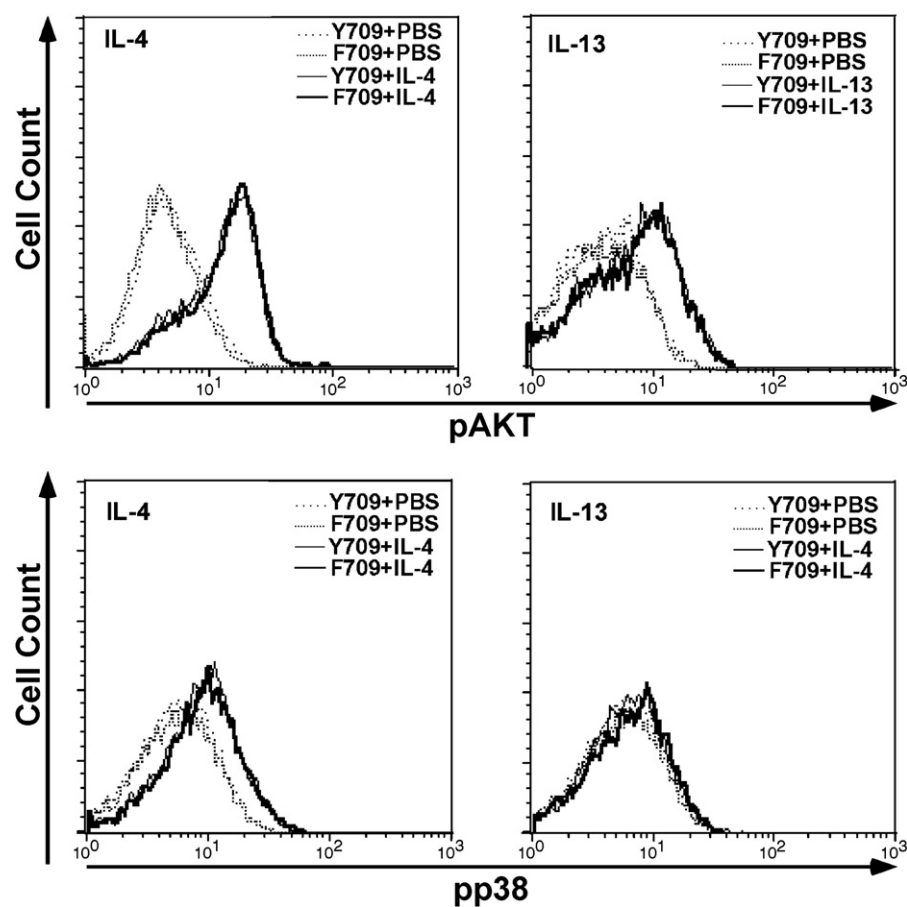


FIG E5. Intracellular staining profile of pAKT (*upper panels*) and pp38 MAP kinase (*lower panels*) in WT (Y709) and F709 BMDMs stimulated for 15 minutes at 37°C with IL-4 or IL-13 at 10 ng/mL. Results are representative of 2 independent experiments.

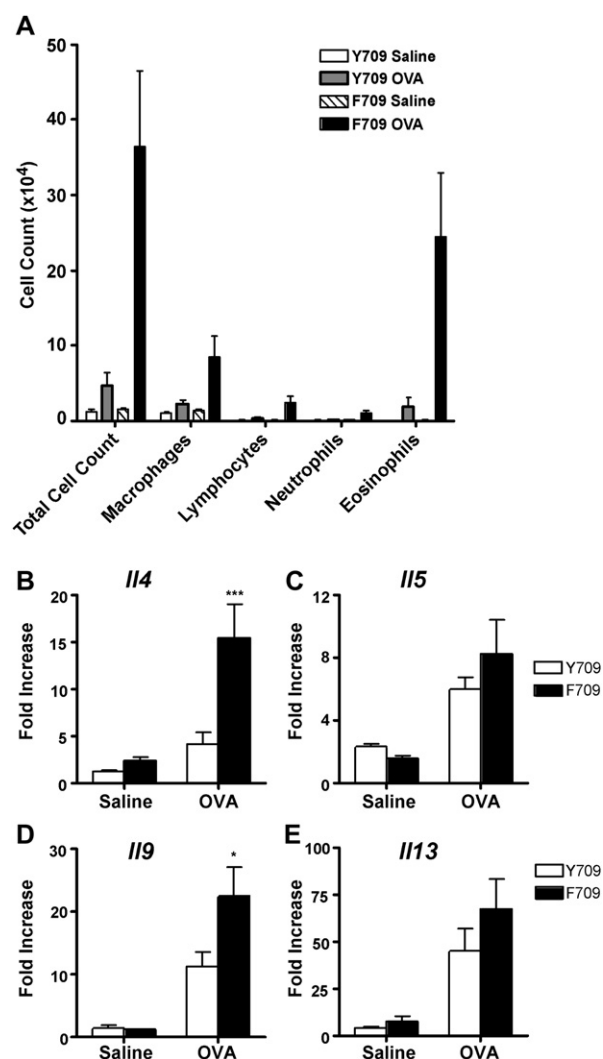


FIG E6. A, Differential count of inflammatory cells found in bronchoalveolar lavage fluid of C.129.*Il4ra*^{Y709/Y709} (Y709) and C.129.*Il4ra*^{F709/F709} (F709) mice subjected to a limited protocol of OVA sensitization followed by OVA challenge (see Fig 5, E). Results represent 5 to 8 mice per group. B-E, Real-time PCR analysis of *IL4*, *IL5*, *IL9*, and *IL13* mRNA levels in total lung extracts of OVA-sensitized and challenged mice versus control animals (n = 10-14 per group). * $P < .05$ and *** $P < .001$ (2-way ANOVA with Bonferroni post-test analysis of groups)