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-4Rα) plays a critical role in the pathology of allergic diseases. The IL-4Rα 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-4Rα 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-4Rα, especially by IL-13. (J Allergy Clin Immunol 2010;125:1128-36.)

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-4Rα), which can pair with alternative subunits to assemble a complete receptor.[1-3] The IL-4Rα associates with the common γ c chain (γc) to form a type I IL-4 receptor complex that is found predominantly in hematopoietic cells and binds exclusively IL-4.[4] The IL-4Rα can also pair with the IL-13Rα1 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-4Rα.[4-6] Phosphorylation of Y575, Y603, and Y633 of the human IL-4Rα 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-4Rα, 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-4Rα (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-4Rα.[13] Global SHP-1 and SHIP-1 deficiency is associated with enhanced signaling through the IL-4Rα and enhanced allergic airway inflammation.[14-16] However, SHP-1, SHP-2,

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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-4Rα: 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
SHP-1/2: Src homology 2 domain–containing protein tyrosine phosphatase 1/2
STAT: Signal transducer and activator of transcription
WT: Wild-type

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-4Rα signaling difficult to discern. In this report we aimed to specifically examine the role of the ITIM in IL-4Rα signaling in mice whose IL-4Rα 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-4Rα (C.129Il4ra$^{F709/F709}$) and similarly manipulated control mice that retain the native Y709 residue (C.129.Il4ra$^{Y709/Y709}$) 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 phoshoproteins 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 μg of OVA mixed in aluminum potassium phosphate (alum) and then challenged intranasally with 50 μg 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 μg of OVA (Sigma-Aldrich) in 2 mg of alum administered intraperitoneally. A week later, the mice were challenged with intranasal antigen (50 μg 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