2003

Targeted inactivation of the IL-4 receptor α chain I4R motif promotes allergic airway inflammation

Frank Blaeser  
Washington University School of Medicine in St. Louis

Paul J. Bryce  
Harvard University

Nga Ho  
Washington University School of Medicine in St. Louis

Vidya Raman  
Washington University School of Medicine in St. Louis

Fatma Dedeoglu  
Harvard University

See next page for additional authors

Follow this and additional works at: http://digitalcommons.wustl.edu/open_access_pubs

Part of the Medicine and Health Sciences Commons

Recommended Citation
Blaeser, Frank; Bryce, Paul J.; Ho, Nga; Raman, Vidya; Dedeoglu, Fatma; Donaldson, Debra D.; Geha, Raif S.; Oettgen, Hans C.; and Chatila, Talal A., "Targeted inactivation of the IL-4 receptor α chain I4R motif promotes allergic airway inflammation." Journal of Experimental Medicine. 198,8. 1189-1200. (2003).  
http://digitalcommons.wustl.edu/open_access_pubs/667

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact engeszer@wustl.edu.
Targeted Inactivation of the IL-4 Receptor α Chain I4R Motif Promotes Allergic Airway Inflammation

Frank Blaeser,1 Paul J. Bryce,2 Nga Ho,1 Vidya Raman,1 Fatma Dedecoglu,2 Debra D. Donaldson,3 Raif S. Geha,2 Hans C. Oettgen,2 and Talal A. Chatila1,4

1 Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110
2 Department of Pediatrics, Harvard Medical School, Boston, MA 02115
3 Department of Respiratory Disease, Wyeth Research, Cambridge, MA 02140
4 Department of Pathology and Immunology and the Center for Immunology, Washington University School of Medicine, St. Louis, MO 63110

Abstract

The insulin/interleukin-4 (IL-4) receptor (I4R) motif mediates the association of insulin receptor substrate (IRS)-2 with the interleukin-4 (IL-4)Rα chain and transduces mitogenic signals in response to IL-4. Its physiological functions were analyzed in mice with a germline point mutation that changed the motif’s effector tyrosine residue into phenylalanine (Y500F). The Y500F mutation abrogated IRS-2 phosphorylation and impaired IL-4–induced CD4+ T lymphocyte proliferation but left unperturbed Stat6 activation, up-regulation of IL-4-responsive gene products, and Th cell differentiation under Th2 polarizing conditions. However, in vivo the Y500F mutation was associated with increased allergen-induced IgE production, airway responsiveness, tissue eosinophilia, and mucus production. These results define an important role for the I4R motif in regulating allergic inflammation.

Key words: interleukin 4 receptor • targeted mutagenesis • immunoglobulin E • insulin receptor substrate • asthma

Introduction

Interleukin-4 (IL-4) plays a key role in the development of Th2 responses and the evolution of immediate hypersensitivity reactions. IL-4 promotes the proliferation and survival of lymphocytes, Th2 lineage commitment of CD4+ T helper cells, and immunoglobulin isotype switching to IgE. It also exerts direct effects on antigen-presenting cells and on nonhematopoietic cells such as airway epithelial and smooth muscle cells. The actions of IL-4 are mediated by heterodimeric receptor complexes that have in common a 140-kD α subunit (IL-4Rα) that binds to and transduces growth-promoting and transcription-activating functions of IL-4 (1–3). In hematopoietic cells, IL-4Rα associates with the γc chain, a subunit common to several cytokine receptors (4–6; for review see reference 7). IL-4Rα also pairs with the IL-13Rα1, which is expressed on hematopoietic and nonhematopoietic cells to mediate signaling by both IL-4 and IL-13 (8). Targeted disruption of the IL-4Rα subunit in mice abrogates the IgE response, consistent with its central role in mediating allergic inflammation (9).

IL-4Rα, γc, and IL-13Rα1 physically associate with specific members of the Janus family of protein kinases, Jak1, Jak3, and Jak2 or TyK2, respectively, that serve to couple ligand binding to intracellular activation events (10). Binding of IL-4 to IL-4Rα is followed by receptor heterodimerization and activation of Jak kinases by transphosphorylation (11, 12). Activated Jak kinases mediate the phosphorylation of the cytoplasmic tail of IL-4Rα on conserved tyrosine residues that serve as docking sites for downstream Src homology domain (SH2) and phosphotyrosine-binding domain (PTB) signaling proteins. Three closely clustered tyrosine residues serve as docking sites for Stat6, an SH2 domain–containing transcription factor selectively coupled

Abbreviations used in this paper: AHR, airway hyperresponsiveness; AID, activation-induced cytidine deaminase; BAL, bronchoalveolar lavage; CSR, class switch recombination; DC-PCR, digestion-circularization PCR; ES, embryonic stem; GLT, germline transcripts; I4R, insulin/IL-4 receptor; IL-4, interleukin-4; IRS, insulin receptor substrate; ITIM, immunoreceptor tyrosine-based inhibitory motif; PI3-K, phosphatidylinositol 3-kinase; PTB, phosphotyrosine-binding domain; SH, Src homology domain; SHP, SHP-tyrosine-containing phosphatase.
to the IL-4R, which is indispensable for the induction of gene expression by IL-4, including the γ1 and e heavy chain germline transcription (13). A distal tyrosine residue at the COOH terminus of IL-4Rα defines an immunoreceptor tyrosine-based inhibitory motif (ITIM) that binds SH2 domains of several phosphatases, including the SH2-containing tyrosine phosphatase (SHP)-1 and SHP-2 and inositol 5’ phosphate (14, 15).

The sequence surrounding the proximal tyrosine 500 (Y500) residue in the cytoplasmic tail of the murine IL-4Rα chain defines a motif that is highly homologous with sequences in the insulin and insulin-like growth factor 1 receptors (16). This motif, referred to as the insulin-IL-4 receptor (I4R) motif, interacts upon its phosphorylation at Y500 with several PTB-domain-containing adaptor proteins that couple the IL-4Rα to downstream signaling cascades. In hematopoietic cells, the insulin receptor substrate 1 (IRS-1) and IRS-2 are the most prominent phospho-I4R substrate (17, 18). However, phospho-I4Rα also interacts with other PTB-containing adaptors including IRS-3 (19), and Shc (20). Recruitment of IRS-2 to IL-4R containing adaptors including IRS-1 (16), FRIP/(IRS)-2 is the most prominent phospho-I4R substrate (17, 18). IRS-2 has been implicated in signal transduction via the insulin receptor (21–23). Studies using mutant IL-4Rα chains transfected into cells lines and IRS-2-deficient lymphocytes have implicated the I4R motif and the IRS-2 signaling pathway in transmitting mitogenic signals in response to IL-4 (16, 18, 23, 24). Furthermore, a role for this motif in human allergic disorders has been suggested by a polymorphism in the human receptor, S503P, that strongly associates with atopy, asthma, and airway hyperresponsiveness (AHR) (25–27). In this study, we addressed the role of the I4R motif in IL-4 receptor signaling in vivo by introducing a germline mutation in the murine IL-4Rα gene that results in replacement of Y500 with a phenylalanine.

Targeting plasmids were introduced by electroporation into RW4 embryonic stem (ES) cells and subjected to G418 selection. Homologous recombination was ascertained by Southern blotting using a probe corresponding to exon 4 of IL4RA, which lies 5’ to the homology sequence. Neomycin gene insertion is associated with the introduction of a new EcoRI site, leading to a decrease in the size of the genomic EcoRI fragment that normally hybridizes with the probe from 12.5 kb to 8.5 kb (see Fig. 1). Successfully targeted clones were transiently transfected with a Cre recombinase to remove the inserted neomycin gene, leaving in place one loxP sequence flanked by EcoRI sites.

Heterozygous ES cells were injected into C57BL/6 blastocysts, and resultant male chimeras were mated with BALB/c females. Offspring were screened for heterozygotes by Southern blotting and PCR analysis. Heterozygotes were further bred for 8–10 generations on BALB/c background. Homozygous mutant mice and wild-type littermate controls were generated by mating HET parents. WT BALB/c and IL-4Rα knock-out mice (BALB/c-Il4ra tm(1Sz) were obtained from the Jackson Laboratory. All protocols were in accordance with NIH guidelines and approved by the Animal Care and Use Committee of Washington University School of Medicine.

PCR Analysis. Screening of WT and mutant IL4RA alleles was performed by PCR amplification using genomic DNA and the following allele-specific forward (F) primers: 5’-TTGCAGACCAATTCCTGGCTA-3’ (WT-specific) and 5’-TTCGAGACACATCCTGGCTT-3’ (mutant-specific), and a common reverse (R) primer: 5’-ACTGCGCTGAACACTCTC-3’. Primers used for PCR screening of the residual loxP-containing allele were: F, 5’-GGTTGCTCTATTTTTAGGTGCC-3’ and R, 5’-TCTTCTTTCTTACTCTGT-3’. For RT-PCR analysis, total RNA was extracted from spleenocytes of WT and IL-4Rα Y500F mutant mice using 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 a two-step process using the following pairs of nested primers: outer pair F (exon 11), 5’-CAGAACCGGAAAGCAGATCAACC-3’ and R (exon 12), 5’-CCCTGCTTCTACGCCTTCGACAACAG-3’; inner pair F (both exon 12), 5’-GAGGCAGGCTTCCACCAAGC-3’ and R, 5’-ACTGCGCTGAACACTCTC-3’. For GAPDH transcripts, the primers were F, 5’-ACCAGACTCCATGCCCATAC-3’ and R, 5’-TCCACACACTTCTTGCAG-3’. For class switch recombination (CSR) studies, RNA was isolated from splenocytes that have been either left untreated or treated for 48 h with IL-4 at 50 ng/ml (R&D Systems) or with anti-CD40 mAb (clone HM40-3; BD Biosciences) plus IL-4. Primers used for RT-PCR amplification of germline, postswitch, and activation-induced cytidine deaminase (AID) transcripts were described previously (29). GAPDH transcripts were amplified as an internal control using the following primers: F, 5’-ACCAGACTCCATGCCCATAC-3’ and R, 5’-TCCACACACTTCTTGCAG-3’. All PCR reactions were performed on various dilutions of cDNA to ensure that the products measured were in the linear range. Depending on the PCR product being measured, 5–30 ng of cDNA fell within this range.

Digestion–circularization (DC)–PCR. Genomic DNA was isolated from cultured splenic B cells stimulated for 6 d with anti-CD40 mAb IL-4. DNA was digested with EcoRI, circularized, and used as template for PCR using primers as reported previously for the β2-Sy1 and nicotinic acetylcholine receptor unit (ACHR) (30) and for β2-Se (31).
**Flow Cytometry Analysis.** Single cell suspensions were stained with the indicated antibodies and analyzed on a FACSCalibur® cytomter (Becton Dickinson). FITC- or PE-conjugated mAbs used were anti-TCRβ (H57), CD3 (145–2C11), CD4 (L3T4), CD8 (53–67), CD23 (B3B4), CD124 (mIL-4R-M1), B220 (RA3–6B2), and anti I-A^b^ (AMS-32.1) (BD Biosciences).

**Lymphocyte Cultures and In Vitro Th Cell Differentiation.** Splenocytes were fractionated into CD4^+^ T cells or B cells by magnetic-activated cell sorting using isolation kits for the respective lymphocyte population (Miltenyi Biotec). Purified B cells were >97% B220^+^ and <1% TCR^β^, and CD4^+^ T cells were >85% TCR^β^+/CD4^+^ and <1% B220^+^. T and B lymphocyte cultures (5 × 10^5^ cells/well in 96-well flat-bottomed plates) were treated with the indicated mitogens for 48 or 72 h, respectively. The following mitogens were used: anti-TCRβ mAb and anti-CD40 mAb (BD Biosciences), F(ab')2 goat anti-mouse IgM antibodies (Jackson ImmunoResearch Laboratories), PMA and ionomycin (Sigma–Aldrich), recombinant IL-4 (Peprotech), and IL-2 (Biological Resources Branch, National Cancer Institute). The cultures were pulsed with 0.4 μCi/well of ^3^H-dThd for 18 h and then harvested. Proliferation was measured as counts per minute incorporated.

T cell blasts were generated by culturing splenocytes on plates coated with anti-TCRβ mAb (10 μg/ml) for 3 d followed by expansion of cell cultures with IL-2 (100 U/ml) for 7 d. Induction of in vitro IgE production was accomplished by culturing splenocytes for 7 d with anti-CD40 mAb in the presence of IL-4 at 100 ng/ml. For in vitro Th cell differentiation, splenocytes were cultured for 3 d on anti-TCRβ–coated plates in the presence of IL-4 (100 ng/ml) and anti-IFN-γ mAb (10 μg/ml) (Th2 conditions) or IL-12 (1 ng/ml) and anti-IL-4 mAb (10 μg/ml) (Th1 conditions) (R&D Systems). The cultures were expanded for an additional 4 d with IL-2 (100 U/ml) after which the cells were washed and restimulated with plate-coated anti-TCRβ mAb for 48 h. Culture supernatants were assayed for IL-4, IL-5, IL-13, and IFN-γ by ELISA (IL-4 and IFN-γ; PharMingen and IL-5 and IL-13; R&D Systems).

**Immunoblotting and Immunoprecipitation.** Cells were stimulated with IL-4 (100 ng/ml) for the indicated periods of time and then lysed with either 2X Laemmlı gel loading buffer or 0.5% NP-40 detergent buffer (32). Whole cell lysates or immunoprecipitates derived from NP-40 detergent extracts were resolved by SDS/Lyter buffer (32). Whole cell lysates or immunoprecipitates lyised with either 2

**Statistical Analysis.** Student's t test was used to compare the groups of mice. Where appropriate, Penh results were analyzed by two-way ANOVA. A P value smaller than 0.05 was considered statistically significant.

**Results**

**Generation of IL-4Rα Y500F Mutant Mice by Targeted Knock-in Mutagenesis.** A targeting construct was designed to replace exon 12 of IL4Rα of ES cells with another bearing an A→T single base pair substitution at the second position of the Y500 codon that changed the codon specificity from tyrosine to phenylalanine (28) (Fig. 1 A). This mutation has previously been demonstrated to abrogate pY500-dependent binding of PTB domain–containing adaptor proteins to the IR4 motif and consequently to uncouple downstream signaling pathways activated via pY500 (16, 18). Successfully targeted ES clones were used to derive murine male chimera that transmitted the Y500F mutation into the germline (Fig. 1 B). Matings of IL-4Rα Y500F heterozygotes resulted in generation of Y500F homozygous mutant mice in numbers consistent with autosomal recessive Mendelian inheritance. Homozygous mutant mice of both sexes were phenotypically indistinguishable from their WT and heterozygous 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. 1, B and C).

RT-PCR analysis revealed that the mutant allele was transcribed at levels equivalent to those of the WT allele (Fig. 1 D). The presence of the A→T substitution in mu-
tant transcripts was directly confirmed by sequencing of the RT-PCR products. (Fig. 1 D). Expression of mutant receptor protein was verified by flow cytometry to be closely matched to WT controls (Fig. 1 E). Analysis revealed the T and B lymphocytes of the IL-4R\textsubscript{/H9251}Y500F mutant mice to be normal in number and phenotype (unpublished data).

The Y500F Mutation Abrogates IRS-2 Activation. The impact of the Y500F mutation of IL-4R\textsubscript{/H9251} signaling events was examined in T and B lymphocyte populations treated with IL-4. Treatment of splenic T lymphoblasts with IL-4 revealed that Jak1 kinase activation was normally induced (Fig. 2 A). However, phosphorylation of IL-4R\textsubscript{α} was markedly decreased in the Y500F mutant lymphocytes compared with WT controls, consistent with Y500 being a major target of phosphorylation by Jak kinases (Fig. 2 A). Significantly, the Y500F mutation abrogated the tyrosine phosphorylation of IRS-2 in response to IL-4 treatment, indicating failure to recruit IRS-2 to the mutant receptor (Fig. 2 B). In contrast, IL-4–induced phosphorylation of Dok-R (FRIP), a PTB domain protein reported previ-
ously to associate with phospho-Y500/I4R (19), was unaffected. This indicated that phosphorylation of Dok-R by IL-4 can proceed in a phospho-Y500–independent manner. IRS-2 phosphorylation results in the recruitment and activation of phosphatidylinositol 3-kinase (PI3-K) and activation of the downstream kinase Akt, which play an important role in IL-4–induced proliferation (35). The latter event is associated with the phosphorylation of Akt on serine 473. In Y500F T lymphocytes, Akt activation by IL-4 was abrogated as evidenced by the failure of IL-4 treatment to induce Akt phosphorylation on serine 473 (Fig. 2 C). In contrast to the failure to activate the IRS-2/Akt pathway in Y500F, activation of Stat-6 was unaffected. Tyrosine phosphorylation of Stat-6 in response to IL-4 proceeded equally well in WT and mutant T lymphocytes (Fig. 2 D).

Impairment of the IRS-2 signaling cascade was also noted in IL-4–treated Y500F primary splenic B cells in which induction of IRS-2 phosphorylation by IL-4 was abolished (Fig. 2 E). The specificity of this finding was demonstrated using another PTB domain adaptor protein, Dok1, which was found constitutively phosphorylated in B cells. When normalized for protein content, its phosphorylation level was marginally increased after IL-4 treatment but without appreciable difference between WT and mutant B cells (Fig. 2 E).

B cells express the p46/p52 isoforms of Shc, a PTB domain–containing adaptor that exhibits low affinity binding to IL-4Rα and constitutively pY500 relative to IRS proteins (36). IL-4 treatment induces Shc phosphorylation in some tissues including B cells (37), prompting examination of Shc phosphorylation in response to IL-4 treatment of WT and Y500F B cells. In WT B cells, IL-4 treatment induced sustained phosphorylation of the p46 isoform (Fig. 2 F). IL-4 also induced p46 Shc phosphorylation in Y500F mutant B cells but with reduced intensity. In contrast, the p52 isoform was constitutively phosphorylated in both cell types and was not affected by IL-4 treatment. These results indicated partial dependence of p46 Shc phosphorylation by IL-4 on the Y500 residue.
Failure of IL-4 treatment to induce IRS-2 phosphorylation in Y500F mutant B cells was associated with abrogation of downstream Akt activation (Fig. 2 G). In contrast, induction by IL-4 of Stat-6 phosphotyrosine phosphorylation was not impaired (Fig. 2 H). These results established that in both T and B lymphocytes the Y500F mutation abrogated the activation by IL-4 of the IRS-2-dependent signaling cascade, whereas sparing other cascades activated via IL-4Rα.

The Y500F Mutation Impairs IL-4–induced CD4+ T Cell Proliferation. The role of the Y500 residue in transducing mitogenic signals in lymphocytes was examined in splenic CD4+ T cells and B cells of WT and Y500F mutant mice. Fig. 3 A shows that in contrast to WT CD4+ T cells, which exhibited a modest proliferative response to IL-4, the response of mutant T cells was substantially decreased. Significantly, the Y500F mutation abrogated the comitogenic function of IL-4 in supporting T cell proliferation induced by anti-TCR antibodies. This effect was specific in that the response to stimulation with anti-TCR antibodies and IL-2 or with phorbol ester PMA and the calcium ionophore ionomycin was not affected.

IL-4 also induced a modest proliferative response in purified B cells that, unlike the case in CD4+ T cells, was not compromised by the Y500F mutation (Fig. 3 B). Furthermore, the Y500F mutation did not impact the capacity of IL-4 to support B cell proliferation induced by B cell mitogens including anti-CD40 and anti-IgM antibodies (Fig. 3 B). These results established that the Y500 pathway is selectively required to support IL-4 mitogenic function in T but not B lymphocytes.

The impact of the Y500F mutation on the up-regulation of classical IL-4 responsive genes was examined by determining the expression levels of CD23 and the MHC class II antigen I-A^d in IL-4–treated WT and Y500F B cells. Fig. 3 C demonstrates that the Y500F mutation had no effect on the up-regulation by IL-4 of CD23 and I-A^d. This is consistent with previous observations that the up-regulation by IL-4 of CD23 and MHC class II antigens proceeds in a Stat-6–dependent manner (38, 39).

Impact of Y500F Mutation on Th Cytokine Production. The impact of the Y500F mutation on Th cell differentiation was examined by comparing the in vitro differentiation of WT and Y500F T cells into Th1 or Th2 effector lymphocytes in the presence of IL-12 and IL-4, respectively. There was no significant difference in IL-4–, IL-5–, or IL-13 production between WT and Y500F T cells that have been differentiated under Th2 polarizing conditions and then stimulated by T cell receptor engagement (Fig. 4 A). Similarly, there was no significant difference in IFN-γ production between WT and Y500F T cells that have been differentiated under Th1 conditions. However, the residual production of all three Th2 cytokines was significantly decreased in Y500F T cell cultures that have been differentiated under Th1 polarizing conditions compared with their WT counterparts. This suggests that the Y500 pathway modulates Th cell differentiation by promoting the persistence of Th2 cells in the context of Th1 skewing conditions.

The impact of Y500F mutation on Th cytokine production was further analyzed in mice immunized with OVA mixed with alum, a Th2-promoting adjuvant. Lymphocytes of WT and IL-4Rα Y500F mutant mice that have been immunized with OVA proliferated equally well upon in vitro stimulation with OVA (unpublished data). Furthermore, splenocytes and LN cells of OVA-immunized WT and IL-4Rα Y500F mutant mice produced similar amounts of IL-4, IL-5, IL-13, and IFN-γ upon stimulation with OVA in vitro (Fig. 4 B). These results indicated that the Y500F mutation did not compromise Th2 cell differentiation in the context of a Th2-biased in vivo immune response.

The Y500F Mutation Enhances Antibody Responses. The impact of the Y500F mutation on humoral immunity was
examined by determining the total and antigen-specific antibody responses after immunization with OVA mixed with alum adjuvant to promote Th2-type responses (40), or with saline/alum as immunization control. Fig. 5 A reveals that Y500F mutant mice exhibited substantially higher total IgE levels than WT controls both after immunization with OVA/alum and after control injection of saline/alum (2.5- and 7-fold, respectively). In contrast, the levels of IgM, IgG1, 2a, 2b, and 3 isotypes were similar. Significantly, OVA-specific antibody responses of several isotypes, including IgG1, IgG2a, and IgE, were three- to five-fold higher in the IL-4R

/\H9251

Y500F mutant mice compared with control (Fig. 5 B). In contrast, levels of OVA-specific IgM, IgG2b, IgG3, and IgA were not significantly different in WT and mutant mice.

Enhanced IgE production was also observed upon in vitro stimulation of naive Y500F splenocytes with anti-CD40 mAb and IL-4, which induce isotype switching to IgE and IgG1 independent of T cell help. Under optimal stimulation conditions, the Y500F splenocytes produced about threefold higher amount of IgE compared with splenocytes of WT littermate controls. In contrast, both WT and Y500F cells produced similar amounts of IgG1 (Fig. 6 A). These results indicated that the enhanced IgE antibody responses in the IL-4R

/\H9251

Y500F mutant mice reflected, at least in part, altered B cell responsiveness to IL-4.

To elucidate the mechanism of enhanced IgE production, we analyzed the induction of molecular events associated with CSR. These include expression of Ce and Cy1 germline transcripts (GLT), expression of the gene for AID followed by S\n\n→S

/\H9255

1

and S\n\n→S

/\H9253

1 deletional switch recombination, and expression of mature I\n\n-Ce and I\n\n-Cy1 transcripts (41). Fig. 6 B demonstrates that the Y500F mutation was associated with enhanced induction of ce germ-line transcripts (GLT) in splenic B cells stimulated for 48 h with anti-CD40 mAb + IL-4. γ1 GLT were only modestly increased with IL-4, whereas µ GLT were unaffected. In contrast, both ce and γ1 postswitch transcripts (µ\n\n→Cy1 and µ\n\n→Ce, respectively) were markedly increased in Y500F splenic B cells stimulated with anti-CD40 mAb +
The functional consequences of Y500F mutation were further analyzed in a model of antigen-induced airway inflammation. Mice immunized i.p. with OVA then challenged by OVA inhalation develop an IL-4Rα–dependent allergic inflammatory airway response characterized by AHR to methacholine, eosinophilic infiltration, and goblet cell hyperplasia. Accordingly, mice were immunized with OVA then subjected to inhalation challenge with OVA. Fig. 7 A reveals that at baseline, both WT and mutant mice immunized with OVA exhibited similar Penh. OVA-sensitized IL-4Rα Y500F mutant mice exposed to aerosolized OVA exhibited significantly enhanced bronchial responses to methacholine compared with similarly treated WT littermates (P = 0.03). In contrast, both WT and mutant mice sham immunized with saline/alum and then exposed to aerosolized OVA exhibited an equally modest increase in Penh upon methacholine challenge.

The enhanced bronchial responsiveness observed in OVA-treated Y500F mutants was accompanied by an exaggerated allergic inflammatory response. Examination of lung tissue of WT and mutant mice stained with hematoxylin and eosin revealed patchy peribronchial inflammatory infiltrates, composed primarily of eosinophils and lymphocytes, that were substantially more intense in OVA-sensitized and challenged Y500F mutant mice compared with WT controls (Fig. 7 B). In contrast, sham-immunized mice exposed to aerosolized OVA showed normal lung histology (unpublished data). Analysis of BAL fluid of OVA-sensitized and challenged mice revealed that OVA-sensitized Y500F mutants exhibited greater recruitment of eosinophils into the airways compared with similarly treated WT mice (Fig. 7 C). In contrast, the two strains of mice exhibited similar numbers of eosinophils in the peripheral blood (unpublished data).

Allergen-driven inflammation of the airways is often accompanied by goblet cell metaplasia. IL-4 and IL-13 are mediators of this response, which proceeds in an IL-4Rα–dependent manner (43–46). Significantly, the IL-4Rα Y500F mutants exhibited markedly increased goblet cell metaplasia with mucus overproduction, revealed by periodic acid–Schiff staining, compared with controls (Fig. 7 D). These results indicated that the Y500F mutation exaggerated antigen–induced airway allergic inflammatory responses.

To determine whether the airways of Y500F mutant mice are intrinsically more responsive to signaling via IL-4Rα, we examined their response to direct IL-4 and IL-13 instillation compared with WT control mice. AHR measured after intranasal instillation of IL-4 was not significantly different between WT and the IL-4Rα Y500F mutant mice (Fig. 7 E). AHR induced by IL-13 instillation was significantly decreased at higher concentrations of methacholine challenge in Y500F mutant mice compared

---

**Figure 6.** The Y500F mutation augments isotype switching. (A) Production of IgG1 and IgE by cultured splenocytes treated with an anti–CD40 mAb + IL-4. Levels of IgG1 and IgE antibodies present in IgG1 and IgE in supernatants of unstimulated cells were <8 ng/ml, respectively. Results are means ± SE antibody (4 mice/group). Similar results were found in three separate experiments. (B) Molecular events in isotype switching. RT-PCR analysis of μ, γ1, and ε germline (GLT) and γ1 and ε postswitch transcripts (μ→γ1 and ε→ε, respectively) in splenic B cells treated for 48 h with IL-4, anti–CD40 mAb, or both. Results are representative of four pairs of WT and mutant mice from two independent experiments. (C) Sp-Sy1 and Sp-Şε deletional switch recombination measured by DC-PCR at day 6 after treatment with anti–CD40 mAb + IL-4. PCR was performed using 5 ng (lanes 1 and 3) or 20 ng (lanes 2 and 4) of template DNA.

IL-4 compared with WT control B cells. The increased levels of ε and γ1 postswitch transcripts in Y500F splenic B cells were associated with heightened expression of AID, an enzyme that plays an obligate role in CSR and somatic hypermutation (29). Treatment with IL-4 alone or with anti–CD40 + IL-4 induced higher levels of AID transcripts in mutant compared with WT B cells.

Further evidence of enhanced CSR to the γ1 and ε heavy chain loci in Y500F mutant B cells was obtained by DC-PCR amplification. The results showed enhanced Sp-Sy1 and Sp-Şε deletional switch recombination in anti–CD40 mAb + IL-4–treated Y500F B cells compared with similarly treated WT B cells (Fig. 6 C). Neither stimulus induced these events by itself (unpublished data). Overall, these results indicated that the Y500F mutation potentiated molecular mechanisms involved in switching to both γ1 and ε loci. The observation that in vitro IgE but IgG1 synthesis was increased in Y500F B cells treated with anti–CD40 + IL-4 in the face of an early surge in both γ1 and ε postswitch transcripts is most likely due to subsequent sequential switching from γ1 to ε (42).

Potentiation of Allergen-induced Airway Inflammation by the Y500F Mutation. The functional consequences of Y500F mutants exhibited greater recruitment of eosinophils into the airways compared with similarly treated WT mice (Fig. 7 C). In contrast, the two strains of mice exhibited similar numbers of eosinophils in the peripheral blood (unpublished data).
with WT controls. Treatment with both cytokines was associated with a modest, predominantly neutrophilic inflammatory cell population in the BAL fluid that was not significantly different between WT and IL-4RΔH5251Y500F mutant mice (Fig. 7F). These results indicated that the enhanced AHR observed in OVA-sensitized and challenged IL-4RΔH5251Y500F mutant mice was likely due to the exaggerated allergic inflammatory response rather than to increased responsiveness of resident airway cells to IL-4 and IL-13.

**Discussion**

By using mice with a point mutation in the germline that targets the effector tyrosine residue of the I4R motif, an important role for this motif was revealed in the regulation of IL-4Rα-dependent immune responses in vivo. Mutagenesis of the Y500 residue resulted in the uncoupling of IL-4Rα from the IRS-2 pathway and its downstream components PI3-K/Akt. It also resulted in impaired CD4+ T cell proliferation in response to IL-4. However, rather than dampening IL-4Rα-dependent responses in vivo, the Y500F mutation enhanced antigen-specific antibody responses and allergic airway inflammation, consistent with a previously unrecognized negative regulatory function of the I4R motif in vivo allergic responses.

The targeting strategy specifically inactivated Y500-dependent signaling while leaving unperturbed signaling via other tyrosine residues in the cytoplasmic domain of IL-4Rα. Of the adaptor molecules implicated previously in docking at the I4R motif, only IRS-2 was completely uncoupled from IL-4Rα by Y500F mutagenesis. IL-4–induced tyrosine phosphorylation of Dok-R was not impaired, suggesting a redundant function of Y500 in its recruitment. Shc phosphorylation was attenuated but not abrogated, indicating partial dependence on Y500 for IL-4–induced Shc activation. Other signaling events that are mediated by IL-4Rα in a Y500-independent manner proceeded unperturbed, including Jak kinase activation and Stat6 tyrosine phosphorylation.

Consistent with studies implicating signaling pathways downstream of IL-4Rα Y500 in cell proliferation, the mitogenic response of IL-4Rα Y500F CD4+ T cells to IL-4 was greatly decreased and comitogenesis with anti-TCR antibodies abolished. A previous observation that IRS-2–deficient lymphocytes exhibit impaired IL-4–induced proliferation implicates failure of IRS-2 activation in the defective IL-4–mitogenic response of IL-4Rα Y500F T cells (23). Surprisingly, however, a proliferative defect was not observed in B cells treated with IL-4 alone or in combination with anti-CD40 or anti-IgM antibodies. IL-4–induced
IRS-2 and Akt phosphorylation was similarly abrogated in IL-4Rα Y500F T and B cells, ruling out both signaling intermediates as the immediate locus of this discrepancy. The rescue of IL-4 mitogenic function in IL-4Rα Y500 B cells suggests compensation by a currently unknown adaptor or signaling intermediate that is activated in a Y500-independent manner.

Given the critical function of Y500-coupled pathways in IL-4–induced T cell mitogenesis, the Y500F mutation would have been expected to impair evolution of a Th2-type, IL-4Rα–dependent immune response by restricting IL-4–dependent expansion of antigen–specific lymphocyte populations. However, differentiation into Th2 cells proceeded unimpaired both in vitro in response to Th2 polarizing signals and in vivo in response to a Th2-promoting antigenic stimulus (OVA mixed with alum) (40). This indicated that the Y500F mutation did not restrict Th2 cell differentiation under these experimental paradigms in agreement with previous studies on CD4+ T cells made to express 14R mutant IL-4Rα chains by retroviral transduction (47). However, decreased residual Th2 cytokine secretion was observed in Y500F that have been differentiated under Th1 polarizing conditions. This suggests a role for the Y500-coupled pathways in supporting Th2 cell differentiation and/or expansion that becomes limiting only under polarizing Th1 conditions. Further studies will be required to verify this proposition.

The Y500F mutation augmented the production of antigen-specific IgG1 and IgE antibodies associated with Th2 responses and antigen–specific IgG2a antibodies, which are normally associated with Th1-type immunity but are suppressed in Th2-type responses (40, 48). The up-regulation by the Y500F mutation of antigen-specific antibody responses was not associated with altered Th cytokine production, since the magnitude of the antigen–induced IL-4, IL-5, IL-13, and IFN-γ production was similar in WT and mutant lymphocytes. In contrast, the Y500F mutation up-regulated in vitro IgE production and augmented molecular events associated with CSR in response to treatment with anti-CD40 mAb and exogenously added IL-4. These results suggest negative regulatory function of IL-4Rα Y500-coupled signaling pathways in B cell antibody production. On the other hand, the enhanced antigen-specific IgG2a antibody responses in the context of a Th2-type immune response implicate the 14R motif in the suppression of Th1–associated antibody isotypes. Switching to IgG2a is dependent on the transcription factor T-bet (49). 14R signaling may alter the activity of this pathway and/or other pathways involved in switching to IgG2a.

The Y500F mutation enhanced allergen–induced AHR, tissue eosinophilia, and goblet cell metaplasia, consistent with a regulatory function of the 14R motif in allergic inflammation. Induction of AHR and inflammation is an IL-4Rα–dependent process involving hematopoietic and resident airway cells. In particular, induction of AHR, goblet cell metaplasia and mucin production is a direct attribute of IL-4Rα signaling in airway epithelial cells. Both IL-4 and IL-13 act directly on resident epithelial cells to induce goblet cell metaplasia in a Stat-6–dependent manner but independent of recruitment of hematopoietic inflammatory cells (46). AHR induced by cytokine instillation in the airways was either similar (IL-4) or decreased (IL-13) in IL-4Rα Y500F mutants compared with WT controls, indicating that the enhanced allergen–induced AHR and goblet cell metaplasia in the Y500F mutants was not due to heightened IL-4Rα responses in airway tissues. Rather, it incriminates the augmented allergic inflammatory response as the most likely mechanism. This may involve enhanced production by mast cells and basophils of IL-4 and IL-13 due to increased antigen–specific IgE antibody levels and/or altered recruitment to the airways of inflammatory cells including eosinophils and allergen–specific Th2 T cells.

In the human IL-4Rα, there exists a serine to proline polymorphism in the 14R motif (S503P), six amino acids downstream of the effector tyrosine residue (Y497). The presence of the more common serine residue is associated with elevated IgE levels, atopy, and asthma in humans, a phenotype that strongly overlaps with that of mice with targeted inactivation of the 14R motif (25–27). An adverse effect of the S503 relative to the P503 substitution on the function of the 14R motif of the human IL-4Rα chain would provide a mechanism by which this and perhaps other IL-4Rα polymorphic amino acid residues promote human allergic disorders.

We thank Michael White for ES cell injection, Mendy Miller for expert technical assistance, and Traian Lupo for animal care. This work was supported by National Institutes of Health grants HD35694 (to T.A. Chatila), AR47417 (to R.S. Geha), and AI05471 (to H.C. Oettgen) and a grant from the March of Dimes (to T.A. Chatila).

Submitted: 25 March 2003
Revised: 8 August 2003
Accepted: 12 September 2003

References
14. Wang, H.Y., W.E. Paul, and A.D. Keegan. 1996. IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor α chain. Immunity. 4:113–121.
The Journal of Experimental Medicine

Targeted Mutagenesis of the IL-4Rα 14R Motif

1200