

An Atopy-Associated Polymorphism in the Ectodomain of the IL-4R α Chain (V50) Regulates the Persistence of STAT6 Phosphorylation¹

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Several commonly occurring polymorphisms in the IL-4R α have been associated with atopy in humans; the Q576R and the S503P polymorphisms reside in the cytoplasmic domain, whereas the I50 to V50 polymorphism resides in the extracellular domain of the IL-4R α . The effects of these polymorphisms on signaling remain controversial. To determine the effect of the polymorphisms on IL-4 signaling in human cells, we stably transfected the human monocytic cell line U937 with murine IL-4R α cDNA bearing the I or V at position 50 and the P503/R576 double mutant. Each form of the murine IL-4R α mediated tyrosine phosphorylation of STAT6 in response to murine IL-4 treatment similar to the induction of tyrosine phosphorylation by human IL-4 signaling through the endogenous human IL-4R α . After IL-4 removal, tyrosine-phosphorylated STAT6 rapidly decayed in cells expressing I50 or P503R576 murine IL-4R α . In contrast, STAT6 remained significantly phosphorylated for several hours after murine IL-4 withdrawal in cells expressing the V50 polymorphism. This persistence in tyrosine-phosphorylated STAT6 was associated with persistence in CIS mRNA expression. Blocking IL-4 signaling during the decay phase using the JAK inhibitor AG490 or the anti-IL-4R α Ab M1 abrogated the persistence of phosphorylated STAT6 observed in the V50-IL-4R α -expressing cells. These results indicate that the V50 polymorphism promotes sustained STAT6 phosphorylation and that this process is mediated by continued engagement of IL-4R α , suggesting enhanced responses of V50 IL-4R when IL-4 is limiting. *The Journal of Immunology*, 2009, 183: 1607–1616.

The cytokine IL-4 plays a critical role in immune responses (1). IL-4 is produced by activated T lymphocytes, basophils, eosinophils, and mast cells and acts upon many cell types. For example, IL-4 acts on T cells to drive Th2 cell differentiation and it directs B lymphocytes to produce IgG4 and IgE in humans and IgG1 and IgE in mice (2). IL-4 induces expression of MHC class II on macrophages and induces the expression of genes associated with parasitic infections (2, 3). IL-4 also impacts nonhematopoietic cells. IL-4 increases the expression of VCAM-1 on endothelial cells while suppressing E-selectin and can induce the expression of eotaxin by lung epithelial cells (4–6). IL-4 mediates many of these responses by binding to and signaling through either the type I IL-4R complex, which is composed of the IL-4R α -chain and the common γ -chain, or the type II IL-4R com-

plex composed of the IL-4R α -chain and the IL-13R α_1 -chain (7–9). Binding of IL-4 to the IL-4R α -chain leads to the subsequent activation of JAK and downstream signaling pathways including the STAT6 pathway and the insulin receptor substrate pathway (3).

Single nucleotide polymorphisms leading to amino acid changes have been described for the mouse and human IL-4R α (10–12). Several polymorphisms in the IL-4R α -chain have been associated with allergy and asthma in humans (13–15). Two of the polymorphisms located in the cytoplasmic domain, S503 to P and the Q576 to R, are frequently linked (15). This double mutation (S503P/Q576R) has been associated with lower total IgE concentrations, similar to the single S503P polymorphism, and an increase in the phosphorylation of insulin receptor substrate molecules (15). An I50 in the extracellular domain of the IL-4R α -chain was linked with enhanced signal transduction culminating in an increase in the production of IgE (14, 16). In contrast, several other studies reported no correlation of this polymorphism with enhanced IgE levels in patients (17, 18). Furthermore, the V50 polymorphism has been linked with enhanced CD23 expression, an increase in atopic asthma, and an increase in allergic bronchopulmonary aspergillosis (19, 20). Experiments designed to analyze the effects of these polymorphisms on receptor signaling have been contradictory (14, 16, 19, 21–24).

Previous studies on the effects of the IL-4R α -chain polymorphisms have focused on linkage analyses in patients, functional differences in B cells or T cells isolated from patients, or the analysis of signaling by human IL-4R α expressed in the background of murine B or T cells (13, 19, 22, 24–28). The analysis of responses in patient-derived cells can be impacted by the variable genetic backgrounds of the patients and therefore such studies may provide confounding results. Furthermore,

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the effects of the polymorphisms may not be apparent in murine lymphocytes. Therefore, in this study we investigated signaling through mouse IL-4R α engineered to express the polymorphisms described for the human IL-4R α in human monocytic cells. We have found that the V50 polymorphism enhances the persistence of STAT6 phosphorylation.

Materials and Methods

Reagents

Recombinant murine IL-4 and recombinant human IL-4 were obtained from R&D Systems. AG490 was obtained from Calbiochem and was stored at -20°C in DMSO. Sodium orthovanadate (Na_3VO_4) was obtained from ICN. Cell lysis buffer (50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM sodium fluoride) was supplemented before each experiment with $1\times$ Protease Inhibitor Cocktail (10 mM benzamidine hydrochloric acid, 20 mM iodoacetamide, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 100 $\mu\text{g}/\text{ml}$ trypsin inhibitor, and 2 mM 1,10-phenanthroline), 1 mM Na_3VO_4 , and 1 mM PMSF. Geneticin (G418) was obtained from Life Technologies. Rat anti-murine IL-4R α (CD124) M1 blocking Ab was obtained from BD Pharmingen.

Cell culture and transfection

Human monocytic U937 cells were derived from malignant cells from a patient with histiocytic lymphoma (29) and were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM glutamine (complete medium). The murine stem cell virus 2.2 plasmids containing polymorphisms in the mouse IL-4R α -chain analogous to those described in human patients (I50-, V50-, Q576R-, S503P-, and P503R576-IL-4R α) linked to enhanced GFP by an IRES were previously described (22). For transfection, U937 cells were resuspended at 1.0×10^7 cells per 300 μl of PBS. U937 cells were mixed with murine stem cell virus 2.2 plasmids I50-, V50- (both are also S503 and Q576), or P503R576-IL-4R α and the pBH Neo^r plasmid (encoding the neomycin gene for chemical selection with G418) at a 10:1 ratio (in micrograms), respectively, and incubated on ice for 5 min. The U937 cells were electroporated at 950 μF , and 250 V using the Gene Pulser II (Bio-Rad) and incubated on ice for 5 min. Transfected U937 cells were cultured in complete medium for 24 h after which time the transfected U937 cells were collected by centrifugation, resuspended in medium containing G418 (800 $\mu\text{g}/\text{ml}$), and cultured in 24-well plates. Transfected U937 cells were grown through limiting dilution in selection medium. Transfected U937 cells that grew in selection medium were analyzed for the expression of GFP and the murine IL-4R α -chain by flow cytometry.

FACS analysis

U937 cells were prepared for flow cytometric analysis in the following manner. Cells were collected by centrifugation and washed three times in a 1X PBS/2% FBS solution (FACS buffer). The cells were resuspended in FACS buffer and pushed through a filter cap. Fc receptors were blocked at 4°C for 15 min using purified human IgG (Sigma-Aldrich). Cells were then incubated for 30 min at 4°C in the presence of rat anti-murine IL-4R α linked to PE (M1; BD Pharmingen). Cells were collected by centrifugation, washed three times, and resuspended in FACS buffer. Flow cytometric analysis was performed on BD Biosciences FACScan and FACSCalibur machines.

For the analysis of IL-4 binding to cell surface receptors, I50- and V50-IL-4R α U937 cells were treated with 10 ng/ml murine IL-4 for 15 min. Cells were collected by centrifugation and either immediately resuspended in FACS buffer supplemented with 1% sodium azide (Azide FACS buffer) or they were incubated an additional 30 min in RPMI 1640 at 37°C before washing three times and resuspending in Azide FACS buffer. Fc receptors were blocked as previously described. Cells were incubated for 30 min at 4°C in the presence of biotin rat anti-murine IL-4 (BD Pharmingen). Cells were incubated for 20 min at 4°C in the presence of PE-streptavidin (BD Pharmingen). Cells were collected by centrifugation, washed three times, and resuspended in Azide FACS buffer. Flow cytometric analysis was performed as previously described.

Cell signaling

The U937 cell lines expressing various forms of murine IL-4R α were treated in the absence or presence of various concentrations of murine IL-4 for 15 min. The reactions were stopped by adding 25 ml of ice-cold 1X PBS. Cells were collected by centrifugation, resuspended in 500 μl of cell lysis buffer, and incubated on ice for 20 min. The cells were centrifuged at

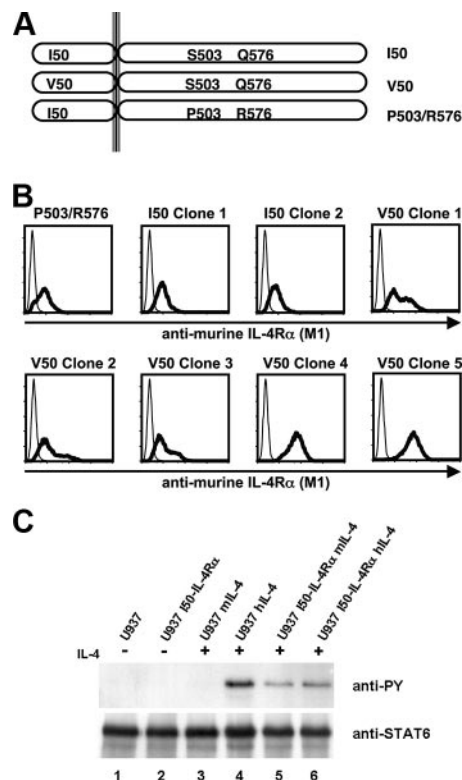


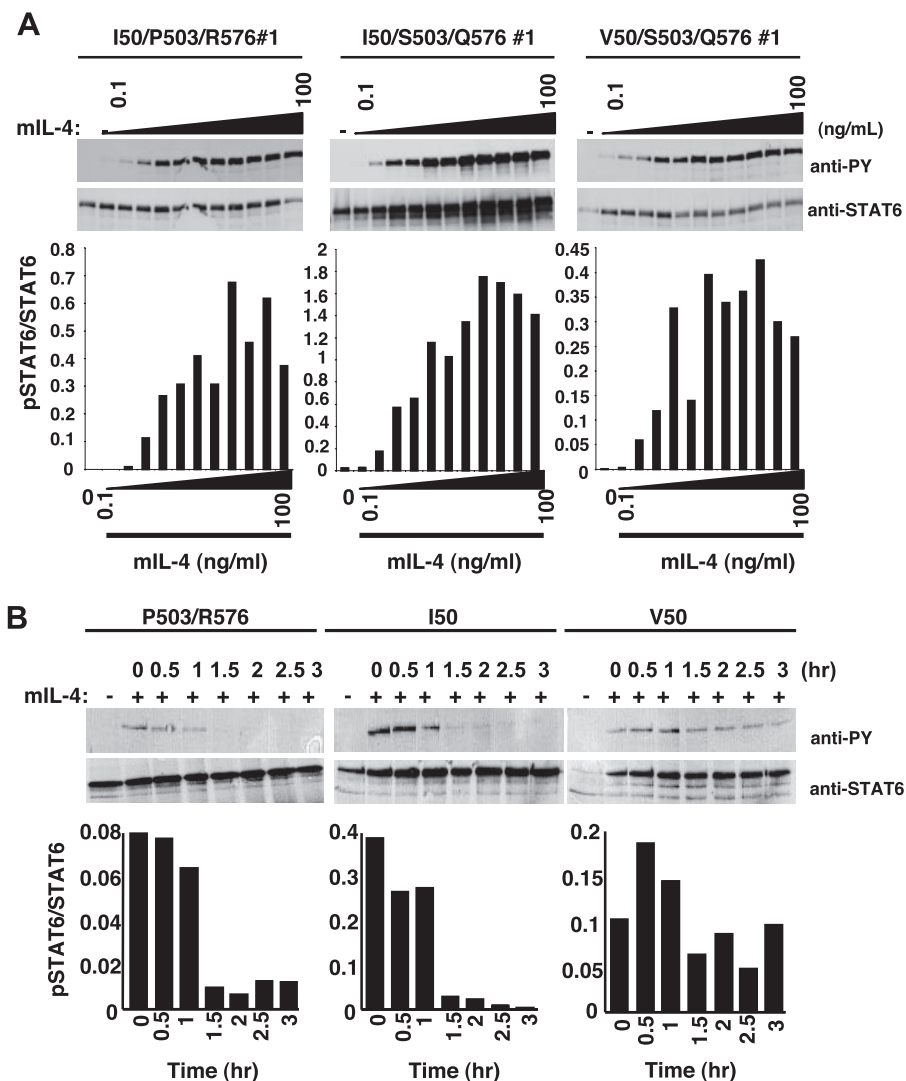
FIGURE 1. Murine IL-4R α expression on transfected U937. **A**, U937 cells were transfected with cDNA encoding various polymorphic forms of the murine IL-4R α and neomycin resistance to allow selection of positive clones in G418. **B**, Following selection in G418, cells were screened by FACS for murine IL-4R α expression using the M1 Ab. **C**, Parental U937 cells and U937 expressing the murine I50-IL-4R α were stimulated in the absence or presence of 10 ng/ml murine and 10 ng/ml human IL-4 for 15 min. The cells were subsequently lysed, and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab to detect phosphotyrosine. The blot was stripped and re-probed with an anti-STAT6 Ab to detect STAT6.

14,000 rpm for 10 min at 4°C . Cleared lysates were transferred to a new microcentrifuge tube and stored at -70°C . For the washout experiments, the cells were stimulated in the absence or presence of 10 ng/ml murine IL-4 for 15 min. The reactions were stopped by adding 25 ml of ice-cold 1X PBS. Cells were collected by centrifugation, washed three times in plain RPMI 1640, and resuspended in RPMI 1640 and incubated at 37°C . Every 30 min over the course of 3 h, the cells were mixed, and aliquots of the cells were collected by centrifugation and lysed. Where indicated, the cells were treated in the absence or presence of M1 anti-murine IL-4R α blocking Ab or the JAK inhibitor AG490 before the addition of IL-4 or after IL-4 treatment during the washout phase. Every 30 min over the course of 3 h an aliquot of cells from the indicated time points was removed, mixed with trypan blue, and analyzed for viable cell number.

Real-time PCR

The U937 cell lines expressing various forms of murine IL-4R α were treated in the absence or presence of murine IL-4 (10 ng/ml) for 15 min. The reactions were stopped by adding 25 ml of 1X PBS. Cells were collected by centrifugation, washed three times in plain RPMI 1640, and resuspended in RPMI 1640 and incubated at 37°C . Every 30 min over the course of 3 h, the cells were mixed, and aliquots of the cells were collected by centrifugation. Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's protocol, and cDNA was generated using the SuperScript III First Strand Synthesis System (Invitrogen). Real-time PCR was performed in triplicate with specific primer sets for human CIS (cytokine-inducible Src homology region 2 domain-containing protein) (forward) 5'-GCTGTGCATAGCCAAGACCTT-3' and (reverse) 5'-CTG GCATCTTCTGCAGGTGTT-3' synthesized by Invitrogen on an Applied Biosystems 7900HT machine. Relative mRNA levels for specific genes are reported as fold induction over background levels detected in untreated

FIGURE 2. Analysis of STAT6 phosphorylation and dephosphorylation in the presence or absence of IL-4. **A**, U937 cells expressing the murine P503/R576-, I50-, or V50-IL-4R α were stimulated with various concentrations of murine IL-4 (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 ng/ml) for 15 min. The cells were lysed, and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab to detect phosphotyrosine. The blots were stripped and reprobed with an anti-STAT6 Ab to detect STAT6. The film was scanned and the NIH Image 1.63 program was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and graphed. **B**, U937 cells expressing the murine P503/R576-, I50-, or V50-IL-4R α were stimulated in either the absence or presence of murine IL-4 for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured at 37°C in selection RPMI 1640. The cells were lysed at the indicated time points and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine Ab. The blot was stripped and reprobed with an anti-STAT6 Ab to detect STAT6. The film was scanned and the NIH Image 1.63 was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and graphed.



samples, with HPRT (hypoxanthine guanine phosphoribosyl transferase) as the internal reference gene ($2^{-\Delta\Delta Ct}$ method).

Immunoprecipitation and Western blotting

Frozen cell lysates were thawed on ice. To precipitate STAT6, polyclonal rabbit anti-human STAT6, S-20 (Santa Cruz Biotechnology) was added to 500 μ l of whole cell lysate and incubated at 4°C for 2 h. To precipitate the murine IL-4R α , monoclonal anti-murine IL-4R α was used. The precipitates were collected by adding 80 μ l of a 50% slurry of protein G-Agarose (Life Technologies) in cell lysis buffer. Samples were placed in a slow rotation device and incubated for 1 h at 4°C. The precipitates were washed in lysis buffer and solubilized in SDS sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride membrane. The membranes were then probed with an anti-phosphotyrosine mAb, RC20, or polyclonal anti-murine IL-4R α . The bound Abs were detected using ECL (Amersham Biosciences). The blots were stripped and reprobed as necessary. The film was scanned and band intensity was analyzed using the public domain software NIH Image. Band intensity was used to determine the phosphorylated STAT6 to total STAT6 ratio. The Student *t* test was used to determine the significance of differences between groups. The relative mobility of the mature IL-4R α was calculated as the distance migrated (in centimeters) by the Molecular Weight Markers (Amersham) divided by the total distance migrated by the dye front. These results were graphed. The distance migrated by the major species of the IL-4R α was divided by the total distance migrated by the dye front. These relative mobility values were plotted against the Molecular Weight Markers to calculate a predicted molecular weight. *N*-linked oligosaccharides were removed from the IL-4R α by enzymatic digestion. The precipitated samples were incubated with 2000 U of peptide:N-glycosidase

F (PNGase F)³ (New England Biolabs) or 5000 U of endoglycosidase H (Endo H; New England Biolabs) for 1 h at 37°C. The samples were separated on a 4–20% SDS-polyacrylamide gel before transfer to polyvinylidene difluoride membrane. The membrane was probed with a polyclonal anti-murine IL-4R α Ab. The bound Abs were detected using ECL (Amersham Biosciences) and the relative mobility of the receptors were calculated as previously described.

Results

To analyze the effects of IL-4R α polymorphisms on signal transduction in human cells, we stably transfected U937 cells with cDNA encoding the various IL-4R α -chain polymorphisms (Fig. 1A). Posttransfection and selection, the U937 cells were analyzed for the expression of the murine IL-4R α -chain using flow cytometric analysis (Fig. 1B). U937 clones transfected with murine stem cell virus cDNA encoding the P503/R576-, I50-, or V50-IL-4R α demonstrated comparable staining with the anti-murine IL-4R α mAb M1. To determine whether the introduced murine receptor was responsive to murine IL-4, U937 cells and U937 cells expressing the murine I50-IL-4R α were stimulated with murine IL-4 or human IL-4. Tyrosine phosphorylation of STAT6 was analyzed by

³ Abbreviations used in this paper: PNGase F, peptide:N-glycosidase F; Endo H, endoglycosidase H.

immunoprecipitation and immunoblotting (Fig. 1C). Stimulation of parental U937 cells with human IL-4 resulted in the induction of STAT6 phosphorylation, whereas stimulation with mouse IL-4 failed to do so. Stimulation of U937 cells expressing the murine I50-IL-4R α with either human or mouse IL-4 resulted in the induction of STAT6 phosphorylation. These results indicate that murine IL-4 is incapable of stimulating U937 cells through the endogenous human IL-4R α . The results also show that the introduced murine I50-IL-4R α signals appropriately in response to murine IL-4, and at a level comparable to the response elicited by human IL-4. In contrast to IL-4, both human and murine IL-13 stimulated the tyrosine phosphorylation of STAT6 in parental and transfected U937 similarly (data not shown). Thus, this model was not suitable to analyze the effects of IL-4R α polymorphisms on IL-13 signaling.

To determine the effects of the IL-4R α polymorphisms on the activation of STAT6, a dose response was performed. P503R576-, I50-, and V50-IL-4R α U937 clones were stimulated with increasing concentrations of murine IL-4 followed by analysis of STAT6 tyrosine phosphorylation (Fig. 2A). Murine IL-4 stimulated the tyrosine phosphorylation of STAT6 with similar sensitivity in P503R576-, I50-, and V50-IL-4R α U937 clones reaching half-maximal phosphorylation at 0.5–1 ng/ml. In contrast to studies in lymphocytes (22, 24), these results indicate that P503R576-, I50-, and V50-IL-4R α do not exert their effects by substantially increasing sensitivity to IL-4. However, these results do not indicate whether the IL-4R α polymorphisms regulate STAT6 activation levels through other means.

Studies analyzing the effects of a Y713F mutation in the IL-4R α α -chain on IL-4 signaling revealed a decrease in the rate of dephosphorylation of STAT6 after excess, free IL-4 was removed from the culture (30). These results were supported by studies comparing signaling in cells from viable moth-eaten mice and cells from WT mice, in which there was a decrease in the rate of dephosphorylation of STAT6 in cells from the viable moth-eaten mice after IL-4 removal (30). These findings suggested the possibility that the polymorphisms may impact the decay of STAT6 phosphorylation and not the efficiency of STAT6 activation per se. To determine whether the IL-4R α polymorphisms exerted an effect on the decay of STAT6 phosphorylation, P503R576-, I50-, and V50-IL-4R α U937 clones were stimulated with murine IL-4, the excess cytokine was washed away, and the cells were cultured in the absence of cytokine. Tyrosine phosphorylation of STAT6 was analyzed by immunoprecipitation and immunoblotting every 30 min over 3 h (Fig. 2B). After removal of IL-4, the levels of tyrosine-phosphorylated STAT6 declined over time. Interestingly, the loss of tyrosine-phosphorylated STAT6 occurred at ~1.5 h after removal of IL-4 in both P503R576- and I50-IL-4R α U937 clones. Similar results were obtained when parental U937 cells were first stimulated with human IL-4 (data not shown). However, the levels of tyrosine-phosphorylated STAT6 remained elevated up to 3 h after removal of murine IL-4 in V50-IL-4R α U937 clones. Additional clones expressing the I50 or V50-IL-4R α were also analyzed (Fig. 3). The enhanced phosphorylation of STAT6 observed in the V50-IL-4R α -expressing cells was highly significant at 1.0, 1.5, and 2.0 h after removal of IL-4.

To determine whether this enhanced persistence of STAT6 phosphorylation observed in the V50-IL-4R α U937 clones had biological implications, we analyzed the expression of a STAT6-dependent gene that would be regulated during this time frame. In previous studies, we found that the SOCS family member CIS was rapidly induced by IL-4 in U937 cells (30). To determine whether the IL-4R α polymorphisms exerted an effect on the induction and decay of CIS mRNA, I50- and V50-IL-4R α U937 clones were stimulated with murine IL-4 for 15 min, the excess cytokine was

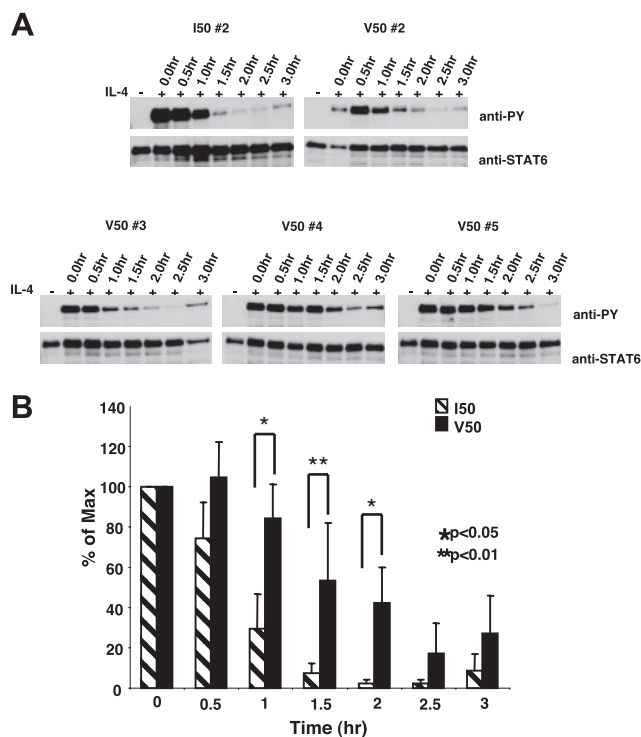


FIGURE 3. Cytokine washout analysis of STAT6 phosphorylation in IL-4-stimulated I50- and V50-IL-4R α U937 clones. **A**, I50- and V50-IL-4R α U937 clones were stimulated in either the absence or presence of murine IL-4. Poststimulation, the IL-4 was washed out and the cells were recultured at 37°C in selection RPMI 1640 for varying times. The cells were lysed at the indicated time points and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab. The blot was stripped and reprobed with an anti-STAT6 Ab to detect STAT6. **B**, The film was scanned and the NIH Image 1.63 program was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and the maximum percentage was determined and graphed using Microsoft Excel. The average of three I50-IL-4R α clones and five V50-IL-4R α clones is shown $\pm$ the SEM. The Student *t* test was used to calculate statistical significance.

washed away, and the cells were cultured in the absence of cytokine. The relative abundance of CIS mRNA was analyzed by quantitative PCR (Fig. 4). In both the I50- and V50-IL-4R α U937 clones, we observed the induction of CIS mRNA after 1 h of further culture, with maximal induction occurring after 1.5 h. As expected, these kinetics represented a lag between the phosphorylation of STAT6 (maximal after 15 min of IL-4 treatment) and the induction of CIS mRNA (Fig. 4A). In absolute terms, the fold enhancement in CIS mRNA was typically greater in the V50-IL-4R α -expressing cells. The relative abundance of CIS mRNA remained elevated for 2.5 h in the V50-IL-4R α expressing cells, whereas it declined in the I50-IL-4R α expressing cells. The induction of CIS mRNA in several clones expressing the I50- or V50-IL-4R α was analyzed in independent experiments and expressed as a percentage of maximal response (Fig. 4B). Using this representation, which controls for the differential levels of CIS mRNA induction, the persistence of CIS mRNA observed in the V50-IL-4R α -expressing cells was significantly greater at 2.0 and 2.5 h after removal of IL-4.

The persistence of STAT6 phosphorylation observed in V50-IL-4R α U937 clones compared with I50-IL-4R α U937 clones and the location of these polymorphisms in the extracellular domain of

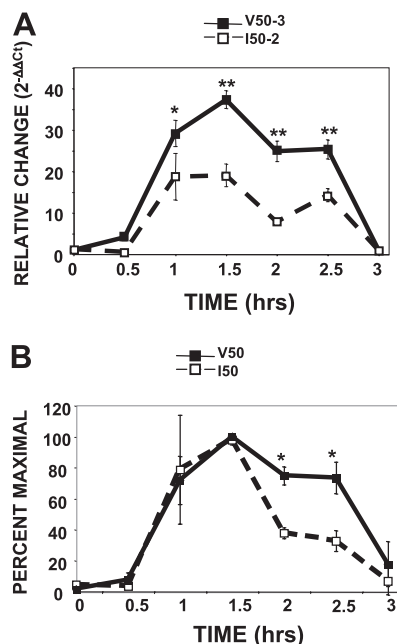


FIGURE 4. U937 cells expressing the V50-IL-4R α show prolonged elevation of CIS mRNA after a short IL-4 treatment followed by cytokine washout. I50- and V50-IL-4R α U937 clones were stimulated in either the absence or presence of 10 ng/ml murine IL-4 for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured for various times. Total RNA was harvested and cDNA was generated. Real-time PCR analysis using specific primers for human CIS was performed in triplicate. *A*, Results are expressed as average fold increase $\pm$ SD of CIS mRNA compared with that of unstimulated cells. HPRT was used as the internal reference. A representative experiment analyzing the two I50 and the three V50 clones is shown. The Student *t* test was used to calculate statistical significance. *, *p* < 0.05; **, *p* < 0.001. *B*, Experiments similar to *A* were performed using two different clones of each variant multiple times. The percentage of maximal CIS induction was calculated for each clone in individual experiments. The average percentage $\pm$ SD of maximal induction (*n* = 4 experiments) is shown. The Student *t* test was used to calculate statistical significance. *, *p* < 0.05.

the IL-4R α -chain suggested that the differences in STAT6 phosphorylation could be due to repeated binding of IL-4 to the polymorphic IL-4R α -chain and continual signaling. The binding of murine IL-4 to the murine IL-4R α can be blocked using the M1 anti-IL-4R α blocking Ab and vice versa (31). To determine the optimal concentration of the M1 blocking Ab that results in complete inhibition of IL-4-induced STAT6 phosphorylation, V50-IL-4R α U937 clones were pretreated for 60 min with various concentrations of the M1 anti-IL-4R α blocking Ab followed by treatment with murine IL-4 (Fig. 5*A*). The optimal concentration of the M1 anti-IL-4R α blocking Ab that induced complete inhibition of STAT6 phosphorylation in V50-IL-4R α U937 clones was 10 μ g/ml. To determine whether renewed IL-4 binding to the V50-IL-4R α was necessary for the prolonged phosphorylation of STAT6, V50-IL-4R α U937 clone no. 5 was stimulated in the absence or presence of 10 ng/ml murine IL-4. The cytokine was washed away and the cells were cultured in the absence or presence of M1 anti-IL-4R α blocking Ab. Tyrosine phosphorylation of STAT6 was analyzed by immunoprecipitation and immunoblotting over time (Fig. 5, *B* and *C*). IL-4-stimulated V50-IL-4R α U937 cells treated with the M1 blocking Ab showed an increase in the rate of dephosphorylation of STAT6 in comparison to untreated V50-IL-4R α U937 cells. We further compared the rate of STAT6 dephosphorylation in I50- and V50-IL-4R α U937 clones

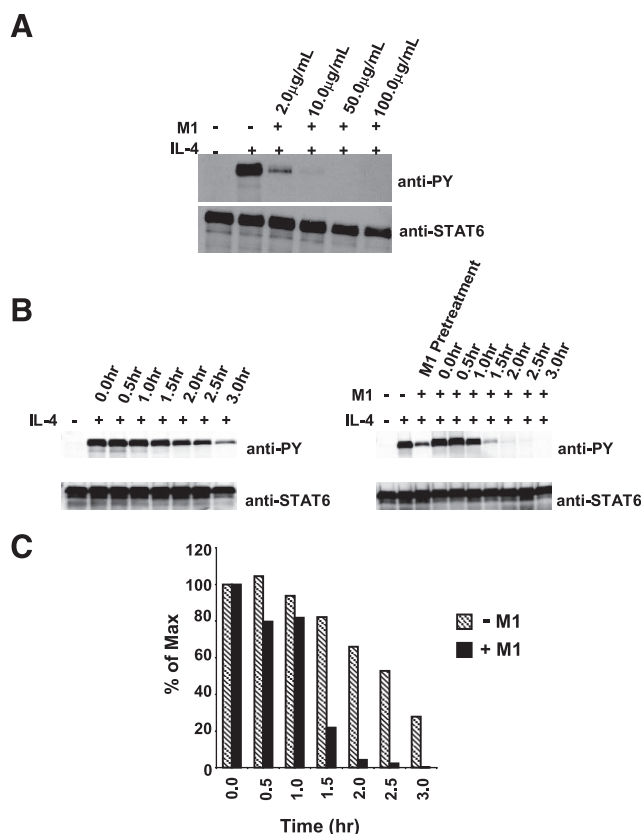


FIGURE 5. M1 blocking of murine IL-4-stimulated V50-IL-4R α U937 clones. *A*, V50-IL-4R α U937 cells were treated with various concentrations of the M1 anti-murine IL-4R α blocking Ab (2.0, 10.0, 50.0, and 100.0 μ g/ml) for 60 min. The cells were then stimulated with 10 ng/ml murine IL-4 for 15 min. The cells were lysed and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab. The blot was stripped and reprobed with an anti-STAT6 Ab to detect STAT6. *B*, V50-IL-4R α U937 clone no. 5 was stimulated in either the absence or presence of 10 ng/ml murine IL-4 for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured in the presence or absence of 10 μ g/ml anti-murine IL-4R α blocking Ab M1. The cells were lysed at the indicated time points and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine Ab. The blots were stripped and reprobed with an anti-STAT6 Ab to detect STAT6. *C*, Results from *B* were scanned and the NIH Image 1.63 program was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and the percentage of maximal phosphorylation was determined and graphed.

treated with the M1 blocking Ab (Fig. 6). The rate of dephosphorylation of STAT6 in IL-4-stimulated V50-IL-4R α U937 cells treated with the M1 blocking Ab was similar to the rate of STAT6 dephosphorylation observed in I50-IL-4R α U937 clones. These observations indicate that the effect of the V50 polymorphism on the persistence of STAT6 phosphorylation is dependent on renewal of IL-4 binding to unoccupied receptors.

To determine whether the continual activation of JAK is necessary to support the prolongation of STAT6 phosphorylation in V50-IL-4R α U937 clones, the JAK inhibitor AG490 was added after the removal of IL-4 to abrogate JAK activity during the wash-out stage. To determine the optimal concentration of AG490 that results in complete inhibition of IL-4 induced STAT6 phosphorylation, U937 cells were pretreated for 60 min with various concentrations of AG490 followed by stimulation with human IL-4

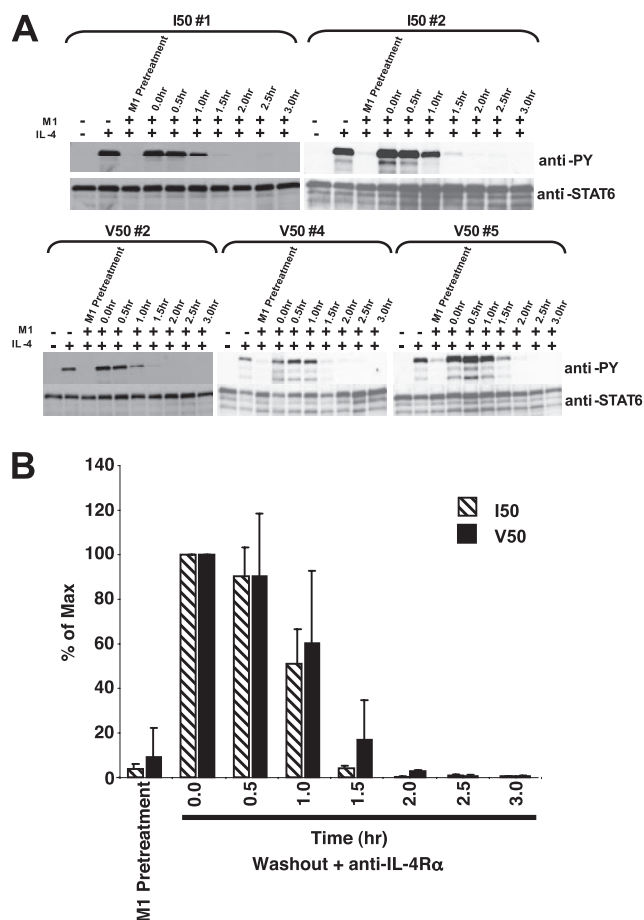


FIGURE 6. Cytokine washout analysis of STAT6 phosphorylation levels in IL-4-stimulated and M1-treated I50- and V50-IL-4R α U937 clones. **A**, I50- and V50-IL-4R α U937 clones were stimulated in either the absence or presence of 10 ng/ml murine IL-4 and M1 (10 μ g/ml) as indicated for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured in the presence of 10 μ g/ml anti-murine IL-4R α blocking Ab M1. The cells were lysed at the indicated time points and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab. The blots were stripped and reprobed with an anti-STAT6 Ab to detect STAT6. **B**, The film was scanned and the NIH Image 1.63 program was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and the percentage of maximal phosphorylation was determined and graphed using Microsoft Excel. The average $\pm$ SEM of three I50-IL-4R α clones and three V50-IL-4R α clones is shown.

(Fig. 7A). The optimal concentration of AG490 that induced complete inhibition of STAT6 phosphorylation in U937 cells was 1.0 mM. This concentration is considerably higher than that needed to block STAT6 phosphorylation in the murine B lymphoma cell line M12.4.1 (50 μ M), and may be a property of these leukemic U937 cells (30). To assess STAT6 tyrosine phosphorylation during the cytokine washout stage while in the presence of AG490, V50-IL-4R α U937 clone no. 5 was stimulated with murine IL-4. The cytokine was washed away, and the cells were cultured in the absence or presence of 1.0 mM AG490. Tyrosine phosphorylation of STAT6 was analyzed by immunoprecipitation and immunoblotting over time (Fig. 7, B and C). Treatment of V50-IL-4R α U937 cells with AG490 resulted in an increase in the rate of dephosphorylation of STAT6 in comparison to untreated V50-IL-4R α U937 cells. Again, we compared the rate of STAT6 dephosphorylation in I50- and V50-IL-4R α U937 clones treated with AG490 (Fig. 8). There was no loss of viable cell number under these con-

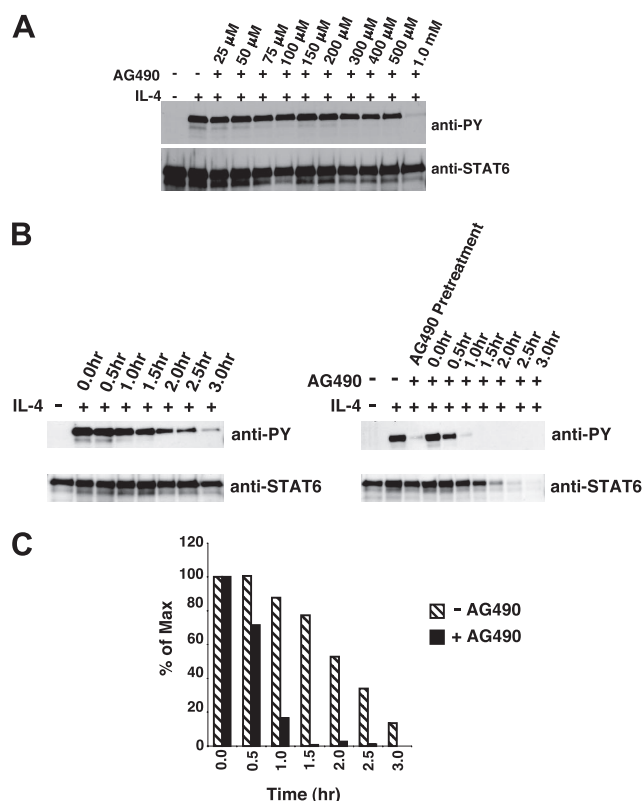


FIGURE 7. Cytokine washout analysis of STAT6 phosphorylation in V50-IL-4R α U937 clones stimulated with IL-4 in the absence or presence of AG490. **A**, U937 cells were treated with various concentrations of the JAK inhibitor AG490 for 60 min. The cells were then stimulated with 10 ng/ml IL-4 for 15 min. The cells were lysed and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab. The blot was stripped and reprobed with an anti-STAT6 Ab to detect STAT6. **B**, V50-IL-4R α U937 clones were stimulated in either the absence or presence of 10 ng/ml murine IL-4 for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured at 37°C in the presence or absence of 1.0 mM AG490 for various time points. STAT6 was immunoprecipitated from cell lysates and subjected to Western blot analysis using an anti-phosphotyrosine Ab. The blots were stripped and reprobed with an anti-STAT6 Ab to detect STAT6. **C**, Results in **B** were scanned and the NIH Image 1.63 program was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and the percentage of maximal phosphorylation was determined and graphed.

ditions for up to 2.5 h (data not shown). In the presence of AG490, there was no significant difference in the levels of STAT6 phosphorylation found in V50- or I50-IL-4R α expressing cells after IL-4 removal (Fig. 8). These results indicate that the prolongation of STAT6 phosphorylation observed in V50-IL-4R α U937 clones is a result of new binding of IL-4 to the V50-IL-4R α and new JAK-dependent signaling.

Because the excess-free IL-4 was washed away after the initial stimulation phase, any new IL-4 binding to unoccupied receptors must come from IL-4 remaining at the cell surface during the secondary culture. To determine whether murine IL-4 was detectable on the surface of the cells, I50- and V50-IL-4R α U937 clones were stimulated in the absence or presence of murine IL-4 for 15 min. The cytokine was washed away and the cells were cultured for an additional 30 min at 37°C. The presence of murine IL-4 on the surface of the cells was analyzed by FACS using an anti-IL-4 Ab that is not influenced by binding to the IL-4R α (Fig. 9). The combination of murine IL-4 followed by anti-IL-4 detected cell surface

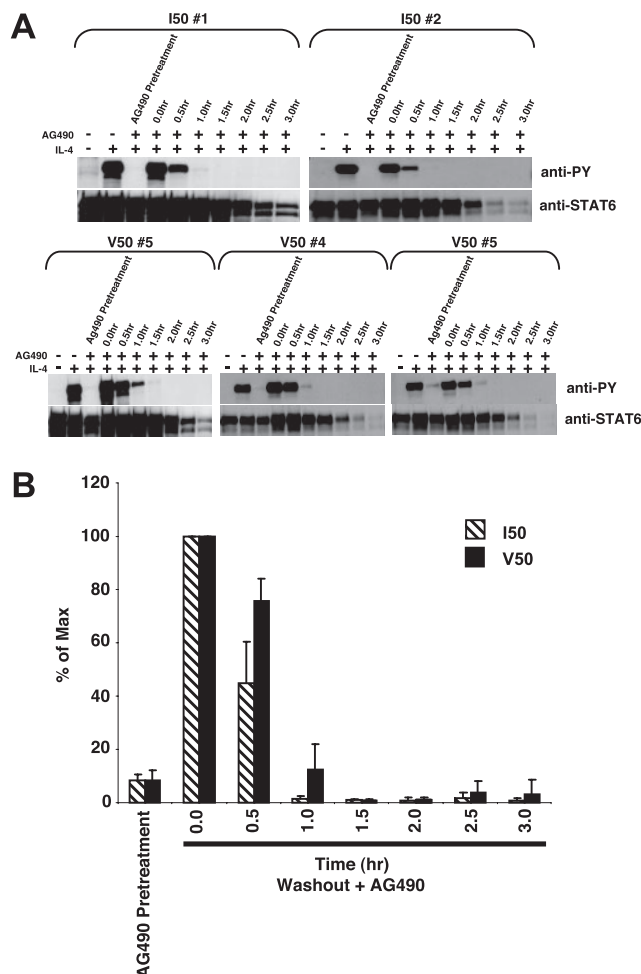


FIGURE 8. Cytokine washout analysis of STAT6 phosphorylation in IL-4-stimulated and AG490-treated I50- and V50-IL-4R α U937 clones. **A**, I50- and V50-IL-4R α U937 clones were stimulated in either the absence or presence of 10 ng/ml murine IL-4 or 1 mM AG490 as indicated for 15 min. Poststimulation, the IL-4 was washed out and the cells were recultured in the presence of 1.0 mM AG490. The cells were lysed at the indicated time points and STAT6 was immunoprecipitated and subjected to Western blot analysis using an anti-phosphotyrosine (anti-PY) Ab. The blots were stripped and reprobed with an anti-STAT6 Ab to detect STAT6. **B**, The film was scanned and NIH Image 1.63 was used to determine the density of the bands developed on the Western blots. The ratio of phosphorylated STAT6 to total STAT6 was calculated and the percentage of maximal phosphorylation was determined and graphed. The average $\pm$ SEM of three I50-IL-4R α clones and three V50-IL-4R α clones is shown.

IL-4 on I50-IL-4R α - and V50-IL-4R α -expressing cells (Fig. 9A); the staining intensity was similar to the intensity observed using the M1 Ab to directly detect IL-4R α on the surface of these cells. Interestingly, murine IL-4 was not detected on I50-IL-4R α U937 cells after the cytokine washout or secondary culture. However, murine IL-4 was detected on the surface of V50-IL-4R α U937 cells immediately after washing and after an additional 30 min of culture at 37°C (Fig. 9B).

The prolongation of STAT6 phosphorylation observed in V50-IL-4R α U937 cells appears to require new binding of murine IL-4 to the IL-4R α and JAK activation. These results suggest differences in interactions with the I50- and V50-IL-4R α . It has been suggested that binding of IL-4 to the IL-4R α may be affected by the glycosylation of either IL-4 or IL-4R α (10, 32). Expression of Ile49 in murine IL-4R α either as a natural polymorphism found in BALB/c mice or as an induced mutation leads to the abrogation of

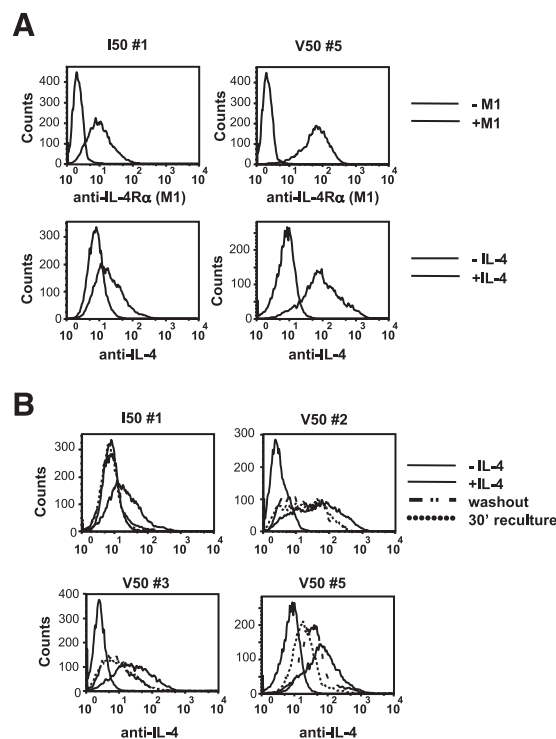
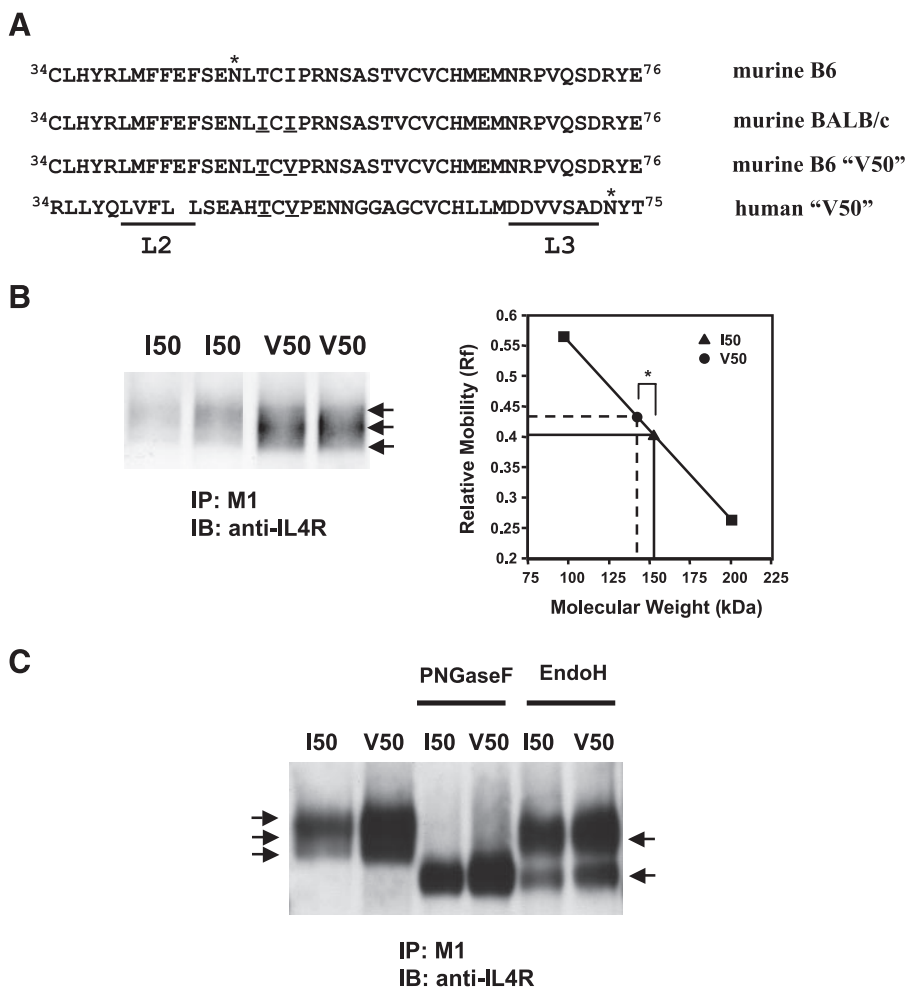


FIGURE 9. Prolonged binding of IL-4 to V50-IL-4R α . **A**, U937 clones expressing the I50- or V50-IL-4R α were analyzed for expression of IL-4R α by M1 staining or by IL-4 binding. Cells were treated with 10.0 ng/ml murine IL-4 for 15 min at 4°C. The cells were isolated and stained for murine IL-4 bound to the receptors using the biotin rat anti-mouse IL-4 mAb. **B**, U937 clones expressing the I50- or V50-IL-4R α were treated with 10.0 ng/ml murine IL-4 for 15 min. The cells were isolated and separated into three groups: IL-4 treatment, IL-4 removal, and 30-min postremoval of IL-4. The cells were stained for murine IL-4 bound to the polymorphic receptors using the biotin rat anti-mouse IL-4 mAb.

an *N*-glycosylation site at Asn⁴⁷, a marked enhancement of mobility on SDS gels, and a 7- to 16-fold increase in the dissociation rate when compared with murine IL-4R α -expressing Thr⁴⁹ (10). Based on the crystal structure of human IL-4R α , this region of the receptor is not predicted to make direct contact with IL-4 but it is near the binding loop 2 (33) (Fig. 10A). Although the conservative change from isoleucine to valine at position 50 in the extracellular domain of the murine IL-4R α would not directly affect the consensus glycosylation site Asn47XThr, the position of the residue is near this glycosylation site and could influence oligosaccharide processing (Fig. 10A). Therefore, to determine whether there are glycosylation differences between I50- and V50-IL-4R α , the murine IL-4R α was immunoprecipitated from I50- and V50-IL-4R α U937 clones using M1 and analyzed by immunoblotting. Both forms of murine IL-4R α migrated as broad diffuse bands as is expected for a cell surface glycoprotein with complex and heterogeneous oligosaccharides. However, the major species of V50-IL-4R α precipitated from U937 cells migrated significantly faster when compared with the migration of I50-IL-4R α (Fig. 10B). Similar results were obtained using a rabbit anti-C-terminal specific Ab or wheat germ agarose for the immunoprecipitation (data not shown). This difference correlates with a smaller predicted molecular mass for V50-IL-4R α in comparison with the predicted molecular mass of ~150 kDa for I50-IL-4R α (Fig. 10B). The enhanced mobility of the V50-IL-4R α on SDS gels suggests an alteration in glycosylation patterns.

To determine whether the smaller predicted molecular mass of the V50-IL-4R α is due to an alteration in glycosylation, the murine

FIGURE 10. Alteration of mobility of V50-IL-4R α on SDS-PAGE. **A**, The amino acid residues of the extracellular domain of the murine and human IL-4R α surrounding position 50 are shown. The threonine and isoleucine polymorphic residues located at position 49 are underlined. The IL-4 binding regions of the human IL-4R α , loop 2 (L2) and loop 3 (L3), are underlined. The asterisk for Asn⁴⁷ in the C57BL/6 IL-4R α sequence and for Asn⁷³ in the human IL-4R α sequence denotes sites of confirmed glycosylation. The Asn⁴⁷ site is not glycosylated in the BALB/c-IL-4R α . **B**, Lysates were prepared from several clones of U937 expressing I50- or V50-IL-4R α . The IL-4R α was immunoprecipitated with M1 anti-IL-4R α mAb. The samples were separated by SDS-PAGE and Western blotted with rabbit anti-IL-4R α . Arrows indicate three different migrating species of the IL-4R α . The relative mobility (R_f) for the major band in each lane was calculated as described in *Materials and Methods*. The average $\pm$ SEM ($n = 3$ clones for I50, $n = 5$ clones for V50) of the relative mobility values from several clones run in two independent experiments is shown. *, $p < 0.05$. SEM is too low to be seen in the graph. **C**, Lysates were prepared from several clones of U937 expressing I50- or V50-IL-4R α . IL-4R α was immunoprecipitated with M1 anti-IL-4R α mAb and subjected to enzymatic digest with either PNGase F or Endo H. The samples were separated by SDS-PAGE and Western blotted with rabbit anti-IL-4R α .



IL-4R α was immunoprecipitated from I50- and V50-IL-4R α U937 clones and subjected to enzymatic digest of the glycoproteins using PNGase F or Endo H (Fig. 10C). Digestion of murine IL-4R α from I50- or V50-IL-4R α U937 cells with PNGase F, an enzyme that cleaves all *N*-linked oligosaccharides, resulted in a single band that migrated similarly on SDS-Gels. The predicted molecular mass of the digested receptor was ~ 130 kDa, indicating that ~ 20 kDa of the IL-4R α is comprised of *N*-linked sugars. Digestion of both receptor types with Endo H, an enzyme that only cleaves high mannose *N*-linked oligosaccharides, resulted in two bands indicating the IL-4R α does contain some high mannose oligosaccharide chains. The similar migration patterns of PNGase F- or Endo H-digested murine IL-4R α from I50- and V50-IL-4R α U937 suggest that the enhanced mobility of V50-IL-4R α is due to glycosylation differences. Taken together, these results suggest that subtle glycosylation differences may modulate IL-4 binding parameters leading to alterations in the persistence of STAT6 tyrosine phosphorylation.

Discussion

Since the first report in 1997 (13), there have been numerous studies linking IL-4R α polymorphisms to allergic phenotypes in patient populations. However, a direct causal link between a specific polymorphism and an effect on IL-4-induced signaling has proved difficult to demonstrate and the literature is rife with controversial reports (13–16, 18–20, 22, 24–28, 34, 35). One of the strongest associations is that of a hydrophobic residue in the ectodomain of the IL-4R α ; this association is particularly challenging to under-

stand in conceptual terms due to the uncertainty of how such a change could alter allergy-related signaling. In this study, we have shown a direct effect of the V50 polymorphism in the IL-4R α on the persistence of STAT6 activation in response to IL-4 and in the persistence of a STAT6-dependent gene. Previous studies focused on the effects of polymorphisms on STAT6 phosphorylation in the continuous presence of saturating concentrations of IL-4. The induction of STAT6 phosphorylation under these conditions analyzes differences in STAT6 phosphorylation at equilibrium and does not account for the regulation of STAT6 dephosphorylation. STAT6 is rapidly phosphorylated and this activity remains elevated in cells cultured in the continuous presence of IL-4 for 24 h (36). However, Hanson et al. (30) showed that STAT6 is dephosphorylated in a time-dependent manner after the removal of excess free IL-4. Under such conditions, we observed enhanced longevity of phosphorylated STAT6 in V50-IL-4R α U937 cells as compared with I50-IL-4R α or endogenous human IL-4R α in U937 cells. This enhanced longevity of STAT6 phosphorylation over time indicates that the cycle of STAT6 activation/deactivation is altered in the V50-IL-4R α U937 cells. This alteration was associated with enhanced induction and persistence of CIS mRNA in U937 cells suggesting the V50 polymorphism may indeed influence biological responses.

STAT6 dephosphorylation is regulated through several different mechanisms including the action of tyrosine phosphatases, induction of SOCS family members, and the action of the proteasome. Because the V50 polymorphism is located in the extracellular domain of the IL-4R α -chain, we suspected that the prolongation of

STAT6 phosphorylation in V50-IL-4R α U937 cells was likely not due to modulation of these largely intracellular mechanisms. We found that the prolongation of STAT6 phosphorylation in V50-IL-4R α U937 cells was dependent upon continued JAK activity. This result is in contrast to the effect of JAK inhibition using AG490 on the prolonged STAT6 phosphorylation seen in cells expressing a Y713F-IL-4R α (30). This mutation abrogates a cytoplasmic Src homology region 2 domain-containing phosphatase-1 docking site in the IL-4R α , preventing the dephosphorylation of STAT6 by the protein tyrosine phosphatase-1 thus enhancing the persistence of STAT6 phosphorylation. AG490 had no effect on this phenotype, indicating that the effect of the Y713F mutation was independent of continued JAK activation.

The prolongation of STAT6 phosphorylation in V50-IL-4R α U937 cells and its abrogation by a JAK inhibitor suggested that the prolongation of STAT6 phosphorylation could be a result of renewed IL-4 binding to cell surface receptors. Although most of the free IL-4 was removed, IL-4 bound to a receptor could dissociate and bind to a nearby unoccupied receptor starting a new signaling cascade. Indeed, IL-4 binding to the IL-4R α is characterized by a fast on and fast off rate. Several investigators previously proposed that this fast off rate of IL-4 could result in new binding and new signaling (10, 37). We found that culturing V50-IL-4R α U937 cells in the presence of the M1 anti-IL-4R α blocking Ab led to the abrogation of the persistence of STAT6 phosphorylation and a similar rate of decrease to that observed in I50-IL-4R α U937 cells. This occurred in all clones regardless of the expression levels of receptor. Because M1 cannot bind to an occupied receptor, these results suggest that the persistence of STAT6 phosphorylation in V50-IL-4R α U937 cells requires new IL-4 binding to an unoccupied IL-4R inducing new JAK and STAT6 activation. This effect became apparent when excess free IL-4 was washed away. In support of this hypothesis, cell surface-bound IL-4 was detected on cells expressing V50-IL-4R α 30 min after free IL-4 was washed away, whereas bound IL-4 was undetectable on I50-IL-4R α -expressing cells at the same time point.

In addition to activating signal transduction, IL-4 binding to its receptor induces receptor-mediated internalization of IL-4 followed by its degradation (37). The receptors are then recycled to the plasma membrane. Galizzi et al. (37) reported that the rate of IL-4 internalization in the human B cell line Jijoye was twice as fast as the rate of IL-4 dissociation from the cell surface ($t_{1/2}$ = 13 min vs 25 min, respectively). Furthermore, mutation of IL-4 in the region that makes contact with common γ chain resulted in less IL-4 internalization due to an increased dissociation rate (38). Several investigators have proposed that alterations in dissociation rates could lead to a reduction in internalization and degradation of IL-4, allowing IL-4 to remain available for new receptor binding on the surface of the cells (10, 37). Although this model remains controversial, our results in this transfectant U937 cell system support the possibility. However, in vivo studies in allergic IL-13 $^{-/-}$ mice do not support such a model. The T49I polymorphism in the extracellular domain of the IL-4R α derived from BALB/c mice eliminates the *N*-linked glycosylation site at N47, a site present in the IL-4R α derived from C57BL/6 mice; this change in glycosylation was associated with an increase in the dissociation rate of IL-4 (10). Webb et al. (39) reported that the ability of IL-4 to promote allergic lung inflammation in the absence of IL-13 was substantially greater in mice expressing the C57BL/6 polymorphism (T49) with the slower dissociation rate.

The question remains as to the mechanism by which the conservative substitution of an amino acid that is not in the IL-4 binding domain (I to V at position 50) might influence ligand binding parameters and signal transduction (33, 40). Such a change in the

murine or human IL-4R α would not be expected to directly impact the addition of the core oligosaccharide to the closest *N*-linked site (N47 or N73, respectively). However, the processing of an individual *N*-linked sugar is determined by the configuration of the protein and its accessibility to the processing enzymes as the nascent protein travels through the endoplasmic reticulum and the Golgi in a particular cell type. We identified differences in the mobility of I50-IL-4R α and V50-IL-4R α derived from U937 cells on SDS gels; these mobility differences were abrogated by removal of *N*-linked oligosaccharides suggesting that the polymorphisms influence the processing of glycosylation.

However, the nature of the differences is unclear; it does not appear to be limited to differences in terminal sialic acid residues because neuraminidase treatment did not alter the persistence of phosphorylated STAT6 in I50- or V50-IL-4R α -expressing cells (data not shown). Characterization of the processing of the carbohydrate side-chains will require extensive analyses of the carbohydrate biochemistry of the I50-IL-4R α and V50-IL-4R α .

Although glycosylation of IL-4 or IL-4R α is not required for binding, it has been shown that the glycosylation state of either the IL-4R α -chain or IL-4 can influence ligand binding. Unglycosylated murine IL-4 was more active than its glycosylated counterpart in stimulating the D10 cell line (32). As discussed, the abrogation of an *N*-glycosylation site in the murine IL-4R α near loop 2 lead to an increase in the dissociation rate of IL-4 (10). Therefore it is possible the differences in signaling between the I50- and V50-IL-4R α are ultimately a result of glycosylation differences between the receptors. Early equilibrium binding studies did not detect any differences in overall binding affinity of human IL-4 for the human I50- or V50-IL-4R α (14, 16, 19). A detailed evaluation of glycosylation patterns has not been performed on the polymorphic human IL-4R α . It is interesting to note that the human IL-4R α has an *N*-linked site at position 73, a site confirmed to be glycosylated (41). This site is adjacent to loop 3, which contains amino acids important for interacting with IL-4. Our comparison of the I50 vs V50 polymorphisms was performed in a defined system using the murine IL-4R α expressed in a human cell line U937. This system certainly does not recapitulate the more complex genetic background found in the human population. Nevertheless, we speculate that the V50 polymorphism may also impact glycosylation of the human IL-4R α and that the relative impact may be cell type-specific. This glycosylation could influence IL-4 binding parameters leading to alterations in persistence of IL-4 signaling and gene expression in human patients. We are currently designing model systems to evaluate these possibilities and to test the contribution of the V50 polymorphism on IL-13-induced signaling.

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Disclosures

The authors have no financial conflict of interest.

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