

An Interleukin 4 (IL-4) Mutant Protein Inhibits both IL-4 or IL-13-induced Human Immunoglobulin G4 (IgG4) and IgE Synthesis and B Cell Proliferation: Support for a Common Component Shared by IL-4 and IL-13 Receptors

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Summary

Interleukin 4 (IL-4) and IL-13 share many biological functions. Both cytokines promote growth of activated human B cells and induce naive human surface immunoglobulin D⁺ (sIgD⁺) B cells to produce IgG4 and IgE. Here we show that a mutant form of human IL-4, in which the tyrosine residue at position 124 is replaced by aspartic acid (hIL-4.Y124D), specifically blocks IL-4 and IL-13-induced proliferation of B cells costimulated by anti-CD40 mAbs in a dose-dependent fashion. A mouse mutant IL-4 protein (mIL-4.Y119D), which antagonizes the biological activity of mouse IL-4, was ineffective. In addition, hIL-4.Y124D, at concentrations of up to 40 nM, did not affect IL-2-induced B cell proliferation. hIL-4.Y124D did not have detectable agonistic activity in these B cell proliferation assays. Interestingly, hIL-4.Y124D also strongly inhibited both IL-4 or IL-13-induced IgG4 and IgE synthesis in cultures of peripheral blood mononuclear cells, or highly purified sIgD⁺ B cells cultured in the presence of anti-CD40 mAbs. IL-4 and IL-13-induced IgE responses were inhibited >95% at a ~50- or ~20-fold excess of hIL-4.Y124D, respectively, despite the fact that the IL-4 mutant protein had a weak agonistic activity. This agonistic activity was $1.6 \pm 1.9\%$ ($n = 4$) of the maximal IgE responses induced by saturating concentrations of IL-4. Taken together, these data indicate that there are commonalities between the IL-4 and IL-13 receptor. In addition, since hIL-4.Y124D inhibited both IL-4 and IL-13-induced IgE synthesis, it is likely that antagonistic mutant IL-4 proteins may have potential clinical use in the treatment of IgE-mediated allergic diseases.

IL-4 directs mouse and human B cells to produce, respectively, IgG1 and IgE or IgG4 and IgE (1-6). Recently, we (7) and others (8) cloned and expressed another T cell-derived cytokine (IL-13). As with IL-4, IL-13 induces human B cells to switch to IgG4 and IgE production in an IL-4-independent fashion (7-9, 10). IL-13 shares many other properties with IL-4. Like IL-4, it has growth-promoting effects on preactivated B cells (7, 9) and it suppresses the production of the proinflammatory cytokines (IL-1 α , β , IL-6, IL-8, and TNF- α) and other monokines (GM-CSF, M-CSF, G-CSF, IL-10, and IL-12) by activated monocytes, whereas, it enhances the production of the IL-1R antagonist (10a). In addition, IL-13 modulates the expression of various surface molecules

on B cells and monocytes associated with functions of these cells (9, 10a).

Despite having only ~30% homology with IL-4, IL-13 shares structural homologies with IL-4 and other cytokines, such as IL-2, growth hormone, GM-CSF and M-CSF, which all have a four-helix bundle motif, consisting of four antiparallel α helices (11-14). The receptors of these cytokines are also structurally related (15-17). Amino acid residues involved in receptor binding in several of the ligands of this receptor family have been determined (18-20). Extensive mutational substitution analysis of mouse IL-2 has indicated that replacement of one amino acid residue (Glu 141 \rightarrow Asp) at the COOH terminus of the fourth α helix results in a loss of receptor activation, whereas most of its receptor binding capacity is preserved (18). Based on the structural homology between IL-2 and IL-4 (12-14) a residue analogous to mIL-2 Glu 141 was found in human IL-4 (21, 22). Substitution of Tyr 124

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by Asp, at the COOH terminus of the fourth α helix of hIL-4, results in high affinity receptor binding, but not in receptor activation (21, 22). Because of these properties, hIL-4 Tyr 124 \rightarrow Asp (designated hIL-4.Y124D) was shown to antagonize IL-4-induced CD23 expression on human B cells (21).

In this study, we investigated the capacity of hIL-4.Y124D to antagonize two major biological functions of IL-4 and IL-13, namely their B cell growth-promoting effects and their induction of IgG4 and IgE synthesis. We demonstrated that the IL-4 mutant Y124D blocks both IL-4 and IL-13-induced B cell proliferation and IgG4 and IgE synthesis, in a dose-dependent fashion. The capacity of hIL-4.Y124D to inhibit the biological activities of both IL-4 and IL-13 has important consequences for the therapeutic potential of this antagonist in the treatment of IgE-mediated allergic diseases.

Materials and Methods

Cells. Highly purified B cells were isolated from normal human spleens obtained from cadaver donors, as previously described (9, 23). Briefly, splenocytes were stained for 30 min on ice with the following PE-conjugated mAbs: anti-CD3, -CD4, -CD8, -CD14, -CD16, and -CD56 (Becton Dickinson & Co., San Jose, CA). The cells were then washed once in medium, centrifuged over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO), and washed twice in PBS. The stained splenocytes were negatively sorted with a FACStar Plus[®] (Becton Dickinson & Co.). An aliquot of the sorted cells was reanalyzed by FACS[®] after staining with CD20-FITC (anti-Leu16) or an isotype control mAb. The sorted cells were always >98% CD20⁺. In other experiments, surface (s)IgD⁺ B cells were obtained by two-color sorting of splenocytes labeled for negative sorting as above, followed by anti-hIgD-FITC mAb (Nordic, Tilburg, The Netherlands). sIgD⁺ cells were obtained by sorting the PE-negative and FITC-positive cells, as described (9). Reanalysis of the sorted cells showed that they were >99.5% sIgD⁺. Blood samples were obtained from healthy volunteers, and PBMC were isolated by centrifugation over Histopaque 1077. The cells were then washed twice and resuspended in Yssel's medium supplemented with 10% FCS.

Reagents. The mutant human IL-4 (hIL-4.Y124D) which has a single amino acid substitution at position 124 (Tyr \rightarrow Asp), and a mutant mouse IL-4 protein, in which Tyr at position 119 was replaced by Asp, were generated and purified as described (22). mIL-4.Y119D protein was prepared using the same protocol as the hIL-4.Y124D protein (22). Human IL-13 (sp act, 5.2×10^6 U/mg) was produced and purified as described (7). Recombinant human IL-4 (sp act 10^7 U/mg) and IL-2 were provided by Schering-Plough Research (Bloomfield, NJ). The anti-CD40 mAb 89 was a gift from Dr. J. Banchereau (Schering-Plough, Dardilly, France). Pansorbin[™] heat-killed *Staphylococcus aureus* Cowan strain I cells (SaC) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and used at a final concentration of 0.05%.

B Cell Proliferation Assay. Highly purified B cells were cultured in U-bottom 96-well Linbro plates (Flow Laboratories Inc., McLean, VA) at a density of 5×10^4 cells/well in Yssel's medium supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), in a final volume of 200 μ l. Native and mutant cytokines, anti-CD40 mAb and SaC, were added in concentrations as indicated, and the cultures were incubated at 37°C for 5 d. 1 μ Ci of tritiated thymidine (³H]TdR) was added to each well during the last 16 h of culture, and the cells were harvested by a cell harvester (Skatron In-

struments AS, Oslo, Norway) onto a filter mat. [³H]TdR activity was measured by a beta counter (Beckman Instruments Inc., Fullerton, CA), after addition of scintillation fluid. Results are expressed as mean \pm SD of triplicate cultures.

Culture Conditions for Ig Production. 50,000 highly purified B cells or 10^5 PBMC were cocultured in Yssel's medium supplemented with 10% FCS, in a final volume of 0.2 ml. Recombinant human IL-4 and IL-13, human or mouse mutant IL-4 and anti-CD40 mAb (20 μ g/ml) were added as indicated. Cultures were set up in six replicates in U-bottom 96-well Linbro plates and incubated 12–14 d at 37°C in 5% CO₂. At the end of the incubation period, the supernatants from each of the six wells were harvested and pooled for isotype determination.

Isotype Determination. Ig isotype production was determined by ELISA as previously described (5, 9). The sensitivities of the ELISA were determined with calibrated standards from Behringwerke Ag (Marburg, Germany), and found to be 0.2 ng/ml for IgE and IgG4, and 0.5–1 ng/ml for total IgG and IgM.

Results and Discussion

hIL-4.Y124D Inhibits both IL-4 and IL-13-induced B Cell Proliferation. The effects of hIL-4.Y124D on IL-4 and IL-13-induced human B cell proliferation were determined in a culture system, in which highly purified B cells were costimulated by anti-CD40 mAbs. Maximal B cell proliferation in these cultures was obtained at saturating concentrations of 280 pM of IL-4 and 540 pM of IL-13 (Fig. 1). Although IL-4 seemed to be slightly more potent than IL-13, the proliferative responses were generally in the same range. Addition of various concentrations of hIL-4.Y124D to these cultures resulted in inhibition of the proliferative responses in a dose-dependent fashion. 50% inhibition of IL-4 and IL-13-induced B cell proliferation was generally observed at 5–10-fold excess of hIL-4.Y124D ($n = 3$), whereas both IL-4 and IL-13-induced B cell proliferation were consistently inhibited by $\geq 95\%$ at a 20–50-fold excess of hIL-4.Y124D. mIL-4.Y119D, which antagonizes mouse IL-4 activities (Zurawski, S., unpublished observations) used as control, was ineffective (Fig. 1). In addition, hIL-4.Y124D tested at concentrations of up to 40 nM failed to inhibit IL-2-induced proliferation in the presence of anti-CD40 mAbs and SaC (Fig. 2). hIL-4.Y124D has been shown to have significant agonistic activity in inducing CD23 expression on human B cells (21), varying from 8 to 45% of the maximal CD23 content and the maximal number of CD23⁺ expressing B cells, respectively (21). However, hIL-4.Y124D, even at concentrations of up to 100 nM, was unable to induce significant B cell proliferation in the presence of anti-CD40 mAbs (data not shown).

hIL-4.Y124D Inhibits IL-4 and IL-13-induced IgG4 and IgE Synthesis. In Fig. 3 it is shown that hIL-4.Y124D inhibits, in a dose-dependent fashion, IgG4 and IgE synthesis by PBMC induced by various concentrations of either IL-4 or IL-13. hIL-4.Y124D was equally effective in inhibiting IL-4- and IL-13-induced IgG4/IgE synthesis, despite the fact that IL-13 is less potent than IL-4 in inducing Ig synthesis. Maximal inhibition of IgE responses (>95%, $n = 4$) in the presence of optimal concentrations of IL-4 and IL-13 occurred at ~ 100 - and ~ 20 -fold excess of hIL-4.Y124D, respectively. However,

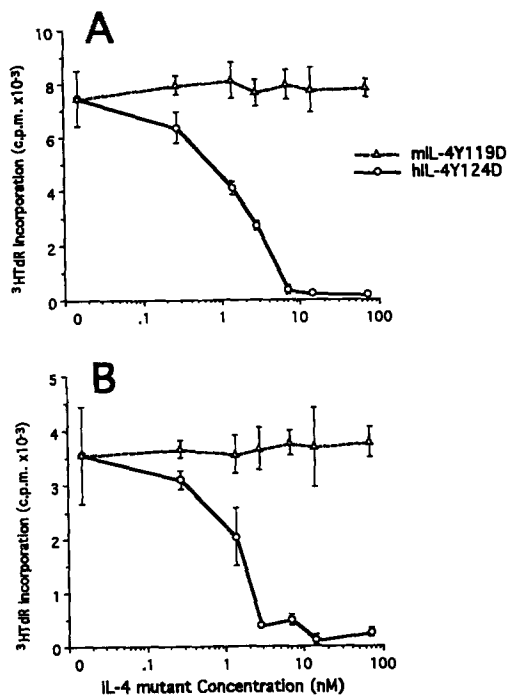


Figure 1. Inhibition of B cell proliferation by hIL-4.Y124D. Highly purified B cells were cultured in the presence of anti-CD40 mAb and IL-4 (280 pM) (A); or IL-13 (540 pM) (B). hIL-4.Y124D and mIL-4.Y119D were added at the onset of the cultures at the concentrations indicated. Results are expressed as mean \pm SD of triplicate cultures. Background proliferation of cells (834 ± 52 cpm) in the presence of anti-CD40 mAbs is deducted. One of four experiments shown.

IL-4 and IL-13-induced IgG4 synthesis by PBMC, was less effectively blocked by hIL4.Y124D. This may be due to the considerably higher agonistic activity of the IL-4 mutant protein on IgG4 versus IgE production. IgE production induced by hIL-4.Y124D at concentrations of up to 20 nM never exceeded 5% of that induced by optimal concentrations of IL-4 (mean 1.6 ± 1.9 , $n = 4$). However, the agonistic activity of hIL-4.Y124D could be as high as 25% (15.7 ± 11.4 , $n = 4$) of IL-4-induced IgG4 production. These differences in the IgG4 versus the IgE-inducing capacity of hIL-4.Y124D are probably attributable to the observation that lower concentrations of IL-4 are required for induction of IgG4 synthesis than for IgE production. Although considerable donor variation has been observed (23), the concentrations of IL-4 or IL-13 necessary for induction of IgG4 synthesis generally were fourfold lower than those required for induction of IgE synthesis by PBMC (Punnonen, J., and J. E. de Vries, unpublished observations), indicating that induction of IgG4 synthesis in cultures of PBMC is a more sensitive assay. Similarly, concentrations of IL-4 required for induction of optimal IgG1 synthesis by mouse B cells were lower than those required for optimal IgE synthesis (24). In addition, it may be possible that other costimulatory factors in addition to IL-4 or IL-13 are involved in induction of IgG4 synthesis, as has been shown for induction of IgG1 synthesis in mice

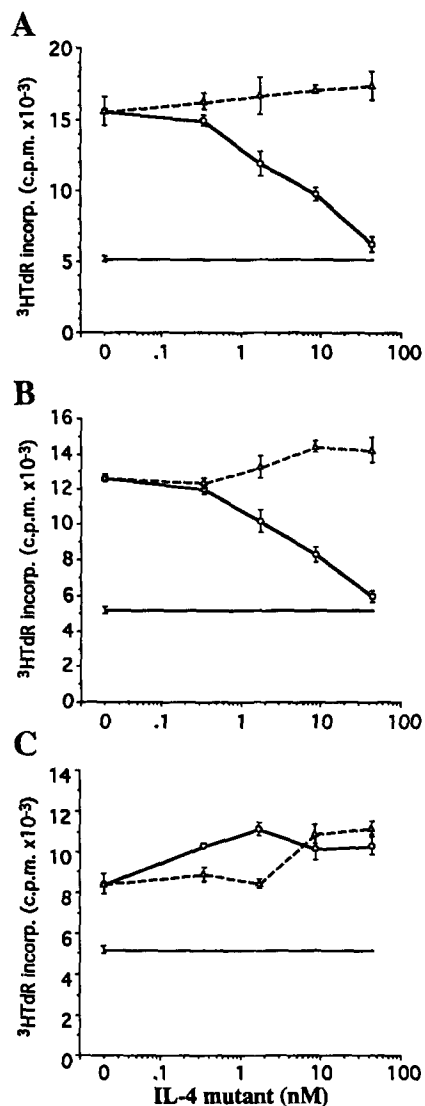


Figure 2. Effects of hIL-4.Y124D on IL-4 and IL-13-induced B cell proliferation. Highly purified B cells were stimulated with SaC, anti-CD40 mAbs, and IL-4 (280 pM) (A); IL-13 (140 pM) (B), or IL-2 (65 pM) (C). Baseline B cell proliferation ($5,200 \pm 200$ cpm) in response to SaC and CD40 mAbs in the absence of cytokines is represented by the horizontal line; (—○—) hIL-4.Y124D; (—△—) mIL-4.Y119D.

with disrupted IL-4 genes. These mice failed to produce IgE, but still produced IgG1, although at lower levels (25).

Comparable data were obtained in culture systems, in which highly purified sIgD⁺ B cells were induced to produce IgG4 and IgE by IL-4 or IL-13 in the presence of anti-CD40 mAbs. hIL-4.Y124D blocked both IL-4 and IL-13-induced IgG4 and IgE synthesis (Fig. 4). Again, strong inhibition of both IL-4 and IL-13-induced IgG4 and IgE synthesis was observed. Interestingly, hIL-4.Y124D had no detectable agonistic activity in this culture system, not even on IgG4 synthesis (data not shown).

IL-4 and IL-13 share structural and functional properties (7, 9, 10, 22). In addition, IL-4 and IL-13 have no additive or synergistic effects on induction of IgG4 or IgE synthesis

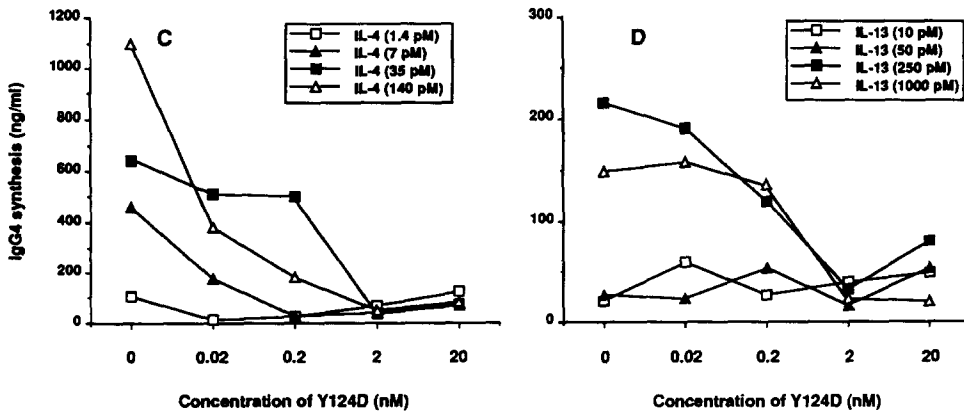
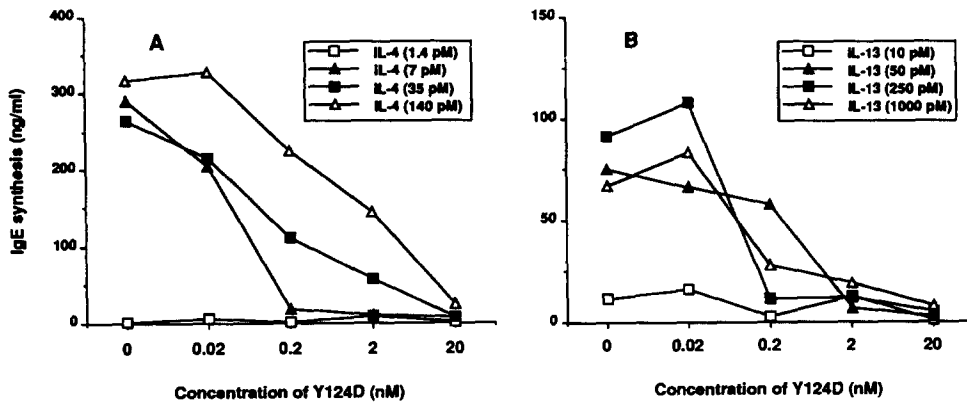


Figure 3. Effect of IL-4 mutant protein Y124D on IL-4 and IL-13-induced IgE and IgG4 synthesis by human PBMC. PBMC were cultured in the presence of various concentrations of IL-4 (A and C) or IL-13 (B and D), and hIL-4:Y124D was added to the cultures at the concentrations indicated. IgE (A and B) and IgG4 (C and D) levels in the culture supernatants were measured by ELISA after a culture period of 12 d. The data represent mean Ig levels of six replicates obtained in a representative of four experiments.

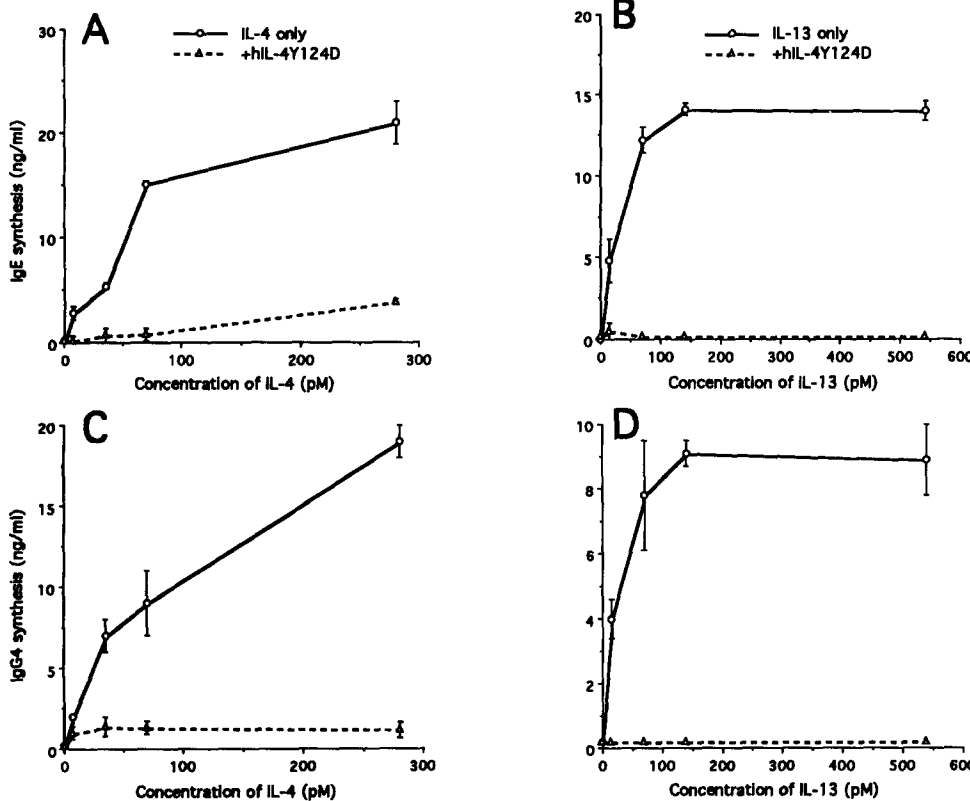


Figure 4. Effects of hIL-4:Y124D on IgG4 and IgE synthesis by B cells stimulated by anti-CD40 mAbs and IL-4 or IL-13. Highly purified sIgD⁺ B cells were cultured with anti-CD40 mAbs and IL-4 (A and C) or IL-13 (B and D). hIL-4:Y124D (7 nM) was added at the onset of the cultures. IgE (A and B) and IgG4 (C and D) concentrations were measured at day 12. mIL-4:Y119D tested at 7 nM failed to block Ig synthesis induced by IL-4 (280 pM) or IL-13 (540 pM), data not shown.

(9). These data, and the observation that hIL-4.Y124D blocks both IL-4 and IL-13-induced B cell proliferation and Ig synthesis, suggest that IL-4 and IL-13 may bind to the same receptor. However, recent studies by Zurawski et al. (22) have shown that IL-13 does not bind to the cloned 130 kD IL-4 receptor, transfected into either mouse BaF3 cells, or monkey COS7 cells, but it inhibits competitively the binding of hIL-4 to functional human IL-4 receptors expressed on the IL-4-responsive erythromyeloid leukemic cell line TF-1 (22). In addition, in contrast to IL-4, IL-13 does not act on activated T cells that express high levels of hIL-4R (22). This information, together with the present data, demonstrates that the hIL-4R and IL-13R are distinct, complex receptors that likely share common component(s).

One model to explain the mode of action of hIL-4.Y124D could be that this mutant protein binds to the IL-4 receptor, but fails to induce dimerization of IL-4 receptors. However, IL-4 binds with a 50–100-fold higher affinity to functional IL-4 receptors as compared to the 130 kD IL-4 binding protein expressed on heterologous cells, whereas the IL-4 mutant protein binds to both receptor types with the same, lower, affinity (22). These observations argue against the lack of dimerization of the cloned 130-kD IL-4R as a model for the action of hIL-4.Y124D. They are rather in support of the presence of an additional component(s) on cells with functional IL-4R that is shared with IL-13R, and which is required for signaling (22). Therefore, the antagonistic effects of hIL-4.Y124D on IL-13 responses could be best explained by assuming that binding of hIL-4.Y124D to functional IL-4R maintains a nonproductive association between the IL-4 binding protein and the additional component(s). This nonproductive association serves to limit the supply of this additional component(s), which is required for the formation of productive complexes with the putative IL-13 binding protein.

It is well established that a ligand can elicit varying degrees of agonistic effects in different tissues (26). The basis for this

phenomenon is that different tissues with the same functional receptor can vary not just in receptor numbers, but also in how efficiently they can transmit a signal, as has also been shown for the IL-2R system (27). The expression levels of the IL-4R and IL-13R and their signal transducing capacity may also differ depending on the mode of B cell activation. Such activation-induced variations could possibly account for the lack of measurable agonistic activity of hIL-4.Y124D on proliferation of purified B cells costimulated by anti-CD40 mAbs. In addition, they may explain why hIL-4.Y124D has agonistic activity for IgG4 production in cultures of PBMC, but not for IgG4 synthesis by B cells costimulated by anti-CD40 mAbs. Based on the above model of action of hIL-4.Y124D, the potency of its antagonistic effects on IL-13 versus IL-4 responses will depend on the relative affinities of IL-4 and IL-13 for their receptors, but they may also reflect a possible diminution in the affinity of the functional IL-13R for IL-13 via the sequestration of the additional component(s) to a nonproductive IL-4R complex. The magnitude of this latter effect will largely depend on the relative contributions of the putative IL-13 binding protein and the additional component(s) to binding of IL-13.

The observation that IL-13, in addition to IL-4, efficiently directs IgE switching in naive human B cells suggests that specific IL-4 antagonists, such as anti-IL-4 mAbs or soluble IL-4R, will be insufficient for the treatment of IgE-mediated allergic diseases. hIL-4.Y124D antagonizes both IL-4 and IL-13-induced B cell proliferation and IgE synthesis. Therefore, inhibitory IL-4 mutants may provide the most effective way to induce IgE synthesis in atopic patients, and therefore may have therapeutic potential for the treatment of allergies. This will, however, require more information, not only about the in vivo effects of antagonistic mutant IL-4 proteins such as hIL-4.Y124D, but also about the biological effects of IL-13 and its relative role in the regulation of the human IgE response.

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