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An immune cell-selective interleukin 4 agonist

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ABSTRACT **Interleukin 4 (IL-4) is a pleiotropic cytokine. Of the cell types responsive to IL-4, T cells express one IL-4 receptor (IL-4R) type, IL-4Rα/IL-2Rγ (class I IL-4R), whereas endothelial cells express another type, IL-4Rα/IL-13Rα (class II IL-4R). It was hypothesized that IL-4 variants could be generated that would be selective for cell types expressing the different IL-4Rs. A series of IL-4 muteins were generated that were substituted in the region of IL-4 implicated in interactions with IL-2Rγ. These muteins were evaluated in T cell and endothelial cell assays. One of these muteins, containing the mutation Arg-121 to Glu (IL-4/R121E), exhibited complete biological selectivity for T cells, B cells, and monocytes, but showed no activity on endothelial cells. Receptor binding studies indicated that IL-4/R121E retained physical interaction with IL-2Rγ but not IL-13Rα; consistent with this observation, IL-4/R121E was an antagonist of IL-4-induced activity on endothelial cells. IL-4/R121E exhibits a spectrum of activities *in vitro* that suggest utility in the treatment of certain autoimmune diseases.**

Interleukin (IL) 4 exhibits a variety of biological activities *in vitro*; the range of cellular specificities leads to a number of physiologic effects *in vivo*, such as immunomodulation (T and B cells), anti-inflammatory action (monocytes), proinflammatory action (endothelial cells), and profibrotic action (fibroblasts) (1, 2). For instance, the immunomodulatory properties of IL-4, in particular its central role in the polarization of T helper (Th) cells to the Th2 phenotype (3, 4), suggest that it may be of benefit in the treatment of autoimmune diseases characterized by an imbalance of Th cells (5), such as multiple sclerosis (6) and insulin-dependent diabetes mellitus (type I diabetes) (7, 8). However, the therapeutic use of IL-4 in humans has been limited, caused in part by dose-limiting side effects observed in human clinical trials. Major side effects and toxicities include gastrointestinal disorders and vascular leak syndrome (9–12).

The receptor for IL-4 (IL-4R) consists of a primary binding subunit, IL-4Rα, which, together with one of at least two secondary components, forms the signaling complex (13). T cells express one IL-4R type, IL-4Rα/IL-2Rγ (14, 15) (class I IL-4R), whereas endothelial cells express another type, IL-4Rα/IL-13Rα (16–20) (class II IL-4R). The primary binding determinants of IL-4 for IL-4Rα are located in the A and C helices of IL-4 (21), whereas major interactions between the IL-4/IL-4Rα complex and IL-2Rγ appear to reside with residues Arg-121, Tyr-124, and Ser-125 in the D helix (22, 23). As the class II IL-4R also uses IL-4Rα, it is probable that this region of the D helix of IL-4 also interacts with IL-13Rα to form a signaling-competent IL-4/receptor complex. Thus, mutations in this region of IL-4 may result in the generation of an IL-4 agonist mutein selective for one receptor form over the other.

Potential proinflammatory effects of IL-4 include the induction of monocyte chemoattractant protein 1 (MCP-1) and IL-6 secretion by endothelial cells (24), as well as the upregulation of vascular cell-adhesion molecule 1 (25) (VCAM-1). These effects can result in inflammatory responses, such as the recruitment of monocytic cells (24) and the adhesion and transmigration of lymphocytes at sites of activation (26). IL-4 muteins (22, 23) tested *in vivo* suggested that activation of endothelium leads to the side effects associated with IL-4 administration (R.G., J.M.G., and A.B.S., unpublished work). This observation, coupled with the characterization of unique IL-4R on T cells and endothelial cells, suggested that a T cell-selective IL-4 variant could be generated that would exhibit the immunomodulatory activity of IL-4 in the absence of dose-limiting toxicity. A cell-selective IL-4 agonist lacking the proinflammatory activities of wild-type IL-4 thus may possess an enhanced therapeutic index.

METHODS

Generation and Production of Muteins. Muteins were generated by site-directed mutagenesis using the method of Kunkel *et al.* (27) using human IL-4 cDNA as template. IL-4 mutein cDNA from M13 (replicative form) DNA containing the correct mutated sequence was subcloned to the plasmid vector pFastBac1 (GIBCO/BRL). After subcloning, recombininant baculovirus DNA (hereafter referred to as Bacmid) was generated by transforming pFastBac1 containing the mutein cDNA to the *Escherichia coli* strain DH10Bac (GIBCO/BRL) as described by the manufacturer. Muteins were produced in *Spodoptera frugiperda* 9 (*Sf* 9) insect cells by using the Bac-to-Bac (GIBCO/BRL) baculovirus expression system as per manufacturer's protocol. Supernatent containing IL-4 mutein was harvested and sterilized by filtration through a sterile 0.2-μm filter. Muteins were purified by using an anti-human IL-4 mAb affinity matrix followed by reverse-phase liquid chromotography, lyophilized, and resuspended in sterile PBS for assays. Mutein so purified was typically a single band by SDS/PAGE (silver stain) and was quantitated by amino acid analysis (accuracy typically >90%).

T Cell Proliferation. Phytohemagglutinin (PHA) blasts were obtained by isolating human T cells from fresh blood from normal donors and were prepared essentially as described by Kruse *et al.* (22). PHA blasts (5×10^4 per well) were incubated with varying amounts of IL-4 or mutein in RPMI medium 1640/fetal bovine serum (FBS) in 96-well plates for 48 hr at 37°C, pulsed with 1 μCi of [^{3}H]thymidine (DuPont/NEN)/well for 6 hr, and harvested, and radioactive incorporation was

Abbreviations: IL, interleukin; IL-R, IL receptor; MCP-1, monocyte chemoattractant protein 1; VCAM-1, vascular cell-adhesion molecule 1; PHA, phytohemagglutinin; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; BBE, bovine brain extract.

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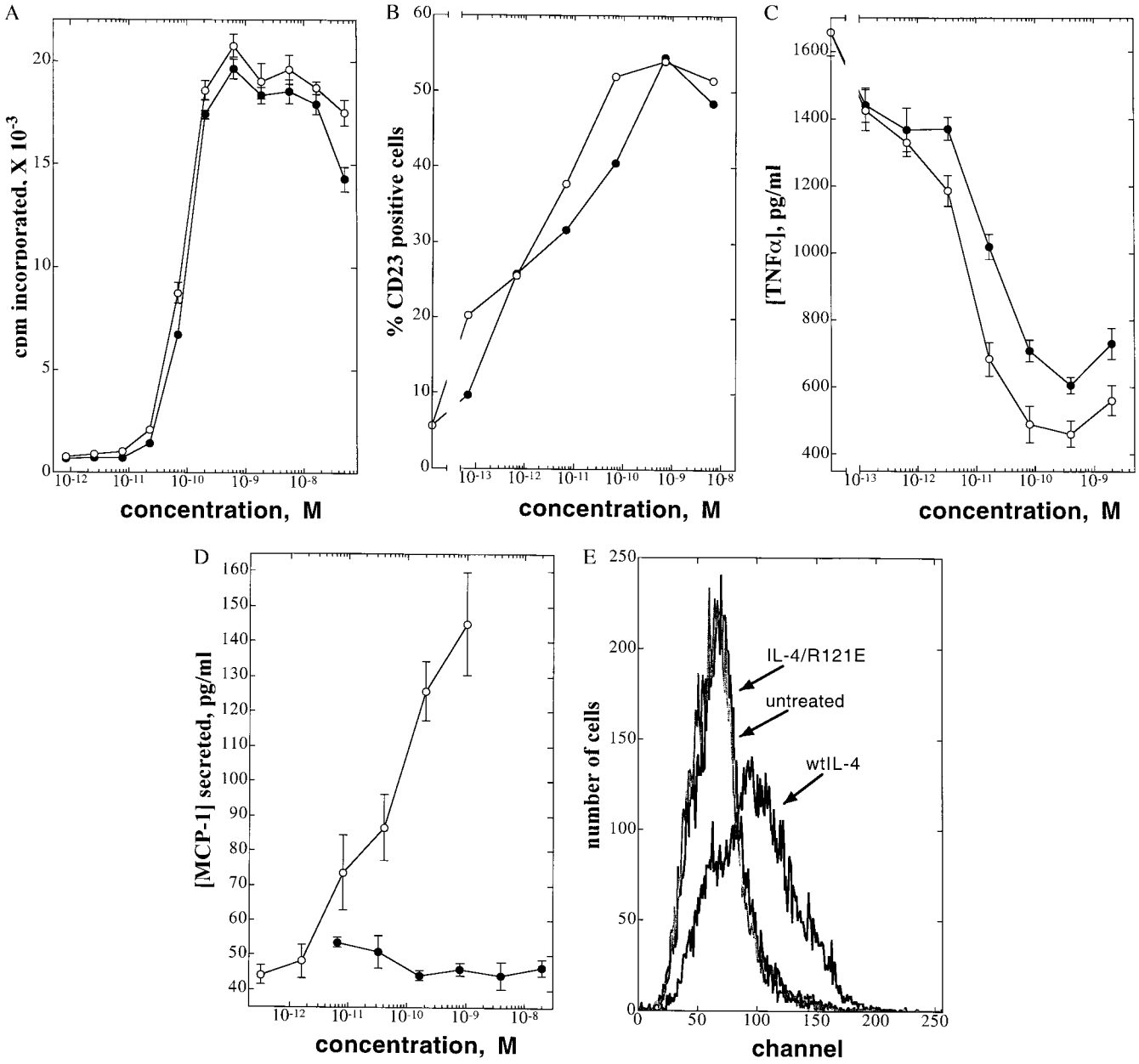

FIG. 1. Cell-selective activity of IL-4/R121E. Similar to wild-type IL-4 (●), IL-4/R121E (○) stimulates T cell proliferation (PHA blasts) (*A*), up-regulates CD23 expression on B cells ($CD19^+$ cells) (*B*), and suppresses lipopolysaccharide induction of tumor necrosis factor α secretion from monocytes (freshly isolated PBMC) (*C*). However, it is unable to induce either MCP-1 secretion (*D*) or VCAM-1 surface staining (*E*) on HUVEC. Similar results were obtained by measuring IL-6 secretion from HUVEC, and MCP-1 and IL-6 secretion from human pulmonary arterial endothelial cells (data not shown).

measured in a TopCount scintillation counter (Packard). For antagonist assays, the conditions were identical except that varying concentrations of mutein were added simultaneously with a constant concentration of 100 pM IL-4.

Up-Regulation of CD23 on B Cells. Human peripheral blood mononuclear cells were prepared from buffy coats from normal individuals by centrifugation through Ficoll-Paque. Cells were incubated for 3 days at 10^6/well in 6-well plates (Costar, no. 25820) containing 1 ml of RPMI medium 1640/FBS with varying concentrations of IL-4 or IL-4/R121E. After harvest, cells were incubated with fluorescein isothiocyanate-coupled anti-CD19 (Dako) and phycoerythrin-coupled anti-CD23 (Becton Dickinson), then analyzed on a FACScan (Becton Dickinson). A minimum of 5,000 cells/sample were analyzed.

Secretion of Tumor Necrosis Factor α by Lipopolysaccharide-Activated Mononuclear Cells. Human peripheral blood mononuclear cells were obtained from normal donor blood and purified by centrifugation by using Ficoll–Paque Plus (Pharmacia) essentially as described by Kruse *et al.* (22). Cells were resuspended in RPMI medium 1640/FBS and seeded to 48-well plates at a density of 3×10^5 cells/well in combination with 0.5 μg/ml of lipopolysaccharide (Sigma) and varying concentrations of IL-4 or mutein and allowed to incubate for 24 hr at 37°C. Supernatants were harvested and evaluated neat by a human tumor necrosis factor α ELISA (Cistron, Pine Brook, NJ).

Cytokine Secretion from Endothelial Cells. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego) and maintained as per supplier's protocols, except that EGM media (Clonetics) containing 10% FBS was used (EGM/FBS). Cells (passage 3–6) were harvested by incubation with trypsin/EDTA, washed, and plated at subconfluent densities in 48-well plates in EGM/FBS

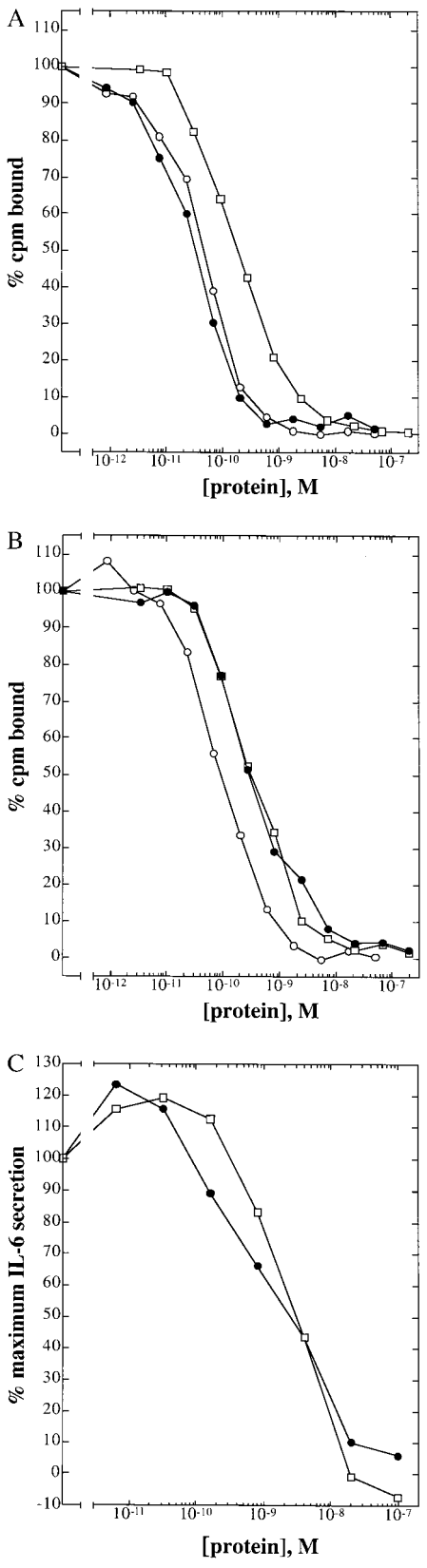

FIG. 2. Competitive receptor binding and antagonism of IL-4 activity. IL-4 (○), the IL-4 antagonist IL-4/R121D/Y124D (23) (□), and IL-4/R121E (●) were evaluated for their ability to compete ^{125}I-IL-4 on (*A*) T cells and (*B*) HUVEC. IL-4/R121E had an affinity for T cells ≈3-fold greater than that of IL-4 (5 pM vs. 16 pM, respectively), but had affinity similar to IL-4/R121D/Y124D on HUVEC (135 pM vs. 115 pM). The K_d values for the muteins in the receptor binding assays were calculated by using the LIGAND program (28). For T cells: IL-4, 16 pM; IL-4/R121D/Y124D, 53 pM; and IL-4/R121E, 5 pM. For HUVEC: IL-4, 31 pM; IL-4/R121D/Y124D, 115 pM; and IL-4/R121E, 135 pM (percentage coefficient of variation values ranged from 7% to 27%). Differences in absolute binding affinity of IL-4/R121D/Y124D may be an artifact of the specific conditions used for each cell type; conclusions were based only on relative comparisons. (*C*) As predicted by the receptor binding results, IL-4/R121E (●) behaves as a functional antagonist of IL-4 on endothelial cells, antagonizing IL-4-induced IL-6 secretion by HUVEC at least as well as the IL-4 antagonist IL-4/R121D/Y124D (□). Similar results were obtained for IL-4-induced MCP-1 secretion and VCAM-1 up-regulation (data not shown).

containing bovine brain extract (BBE; Clonetics). At confluency (3–4 days at 37°C), the medium was removed and replaced with EGM/FBS without BBE. Twenty-four hours later, varying concentrations of IL-4 or mutein were added to the cells in fresh EGM/FBS without BBE and allowed to incubate an additional 24 hr. For antagonist assays, the conditions were identical except that varying concentrations of mutein were added simultaneously with a constant concentration of 100 pM IL-4. Supernatants were harvested, and the concentration of MCP-1 or IL-6 was analyzed by using ELISA.

Up-Regulation of VCAM-1 on HUVEC. HUVEC (passage 3–6) were harvested by incubation with trypsin/EDTA and washed, and cells were plated in T25 flasks (Falcon) at a density of 5×10^5 cells/flask in EGM/FBS containing BBE. After 48 hr, the media were removed and replaced with EGM/FBS without BBE. Twenty-four hours later, human IL-4 (1 nM) or IL-4/R121E (50 nM) was added to the cells in fresh EGM/FBS and allowed to incubate an additional 24 hr. For antagonist assays, the conditions were identical except that human IL-4 (1 nM) and IL-4/R121E (50 nM) were incubated simultaneously with cells. Cells were washed with PBS, harvested after incubation with PBS containing 0.2% glycerol, 20 mM EDTA, 0.2% BSA (PGEB solution), washed with PBS, and resuspended in 100 μl of PBS/1% BSA at a density of 1×10^6 cells/ml. Cells were stained with an anti-VCAM-1 mAb or mouse IgG1 isotype control (Sigma), followed by phycoerythrin-coupled anti-mouse IgG (Sigma). Cells were analyzed by using a FACStar Plus (Becton Dickinson) fluorescence-activated cell sorter.

Competitive Receptor Binding. Binding to endothelial cells was performed by using passage 3–6 HUVEC harvested by using PGEB solution, washed with PBS, and resuspended in RPMI medium 1640 containing 10 mM Hepes, 1% BSA, and 0.2% NaN_3 (binding buffer). Binding to T cells was performed by using PHA blasts prepared as described above. The cells were washed with PBS and resuspended in binding buffer. Cells (1×10^6) were incubated with 25 pM ^{125}I-IL-4 (DuPont/NEN) and varying concentrations of IL-4 or IL-4 mutein at 22°C for 3 hr. Nonspecific binding was determined by including 1 μM of unlabeled IL-4. Cell-bound ^{125}I-IL-4 was separated from free ^{125}I-IL-4 by centrifugation at 22°C (1 min at 12,000 × *g*) through dioctyl phthalate/dibutyl phthalate (2:3). Bound and total radioactivity were measured in a 1282 Compugamma CS γ-counter (LKB/Wallac). The equilibrium binding data were analyzed by using the LIGAND program (28).

RESULTS AND DISCUSSION

Substitutions in the sequence of IL-4 were designed to discriminate between IL-4Rs containing IL-2Rγ or IL-13Rα, and thus differentiate the activity of IL-4 between T cells and endothelial cells. IL-4 binding to IL-4Rα likely is mediated through contacts with its A and C helices (21). Interaction with IL-2Rγ likely is mediated through contacts in the D helix (22, 23), in particular the residues Arg-121, Tyr-124, and Ser-125, as substitution with aspartate individually at these positions resulted in the generation of partial agonist or antagonist IL-4

muteins when assayed on T cells (23). It recently has been reported that mutations in this region of the D helix of IL-4 exhibit reduced activity on renal carcinoma cells and the monocytic cell line THP-1 (29). In particular, substitution of Arg-121 with aspartate resulted in a mutein that exhibited weak agonist activity on peripheral lymphocytes and functioned as a renal cell carcinoma-specific IL-4 antagonist. The renal carcinoma cells did not express IL-2Rγ, suggesting that residues in this region of the D helix of IL-4 may be important for interactions with other IL-4R subunits in the context of Il-4Rα. It was surmised that these residues thus would interact with IL-13Rα in the presence of IL-4Rα (forming the class II IL-4R). Muteins that contained a series of individual substitutions at residues Arg-121, Tyr-124, or Ser-125 were generated and were evaluated for activation of T cells (human PHA blasts; proliferation) and endothelial cells (HUVEC) and secretion of IL-6 and MCP-1. Several IL-4 muteins were identified with T cell-selective activity (A.B.S., C.P.F., J.J.K. and J.M.G., unpublished work). One of these muteins, containing the substitution of Arg-121 with glutamate (IL-4/R121E), had essentially wild-type IL-4 activity on T cells and no detectable activity on endothelial cells (Fig. 1 *A* and *D*, respectively). This mutein also exhibited essentially wild-type activity on B cells, inducing CD23 expression (Fig. 1*B*) and CD40-dependent proliferation (data not shown). IL-4 has an anti-inflammatory effect on macrophages/monocytes, down-regulating the expression of tumor necrosis factor α, IL-1, and IL-8 (30). IL-4/R121E retains this activity (Fig. 1*C*), as expected from the presence of the class I IL-4R on monocytes (31). IL-4/R121E also did not induce another parameter of endothelial cell activation, the up-regulation of VCAM-1 on endothelial cells (Fig. 1*E*). These data indicate that IL-4/R121E will stimulate any cell expressing IL-4Rα/IL-2Rγ (e.g., T cells, B cells, and monocytes) in a manner similar to that of wild-type IL-4, yet be inactive on all cells that do not express IL-2Rγ despite the presence of IL-13Rα (e.g., endothelial cells and fibroblasts).

Competitive receptor binding studies with wild-type IL-4, IL-4/R121E, and the IL-4R antagonist IL-4/R121D/Y124D (32) were performed to determine the physical basis of the selective action of IL-4/R121E. IL-4/R121D/Y124D binds to IL-4Rα, but does not interact with IL-2Rγ in the context of IL-4Rα (32). On PHA blasts, IL-4 and IL-4/R121E both bound to a high affinity site (K_d = 16 pM and 5 pM, respectively), whereas IL-4/R121D/Y124D bound to a lower affinity site (K_d = 53 pM) (Fig. 2*A*). In contrast on HUVEC, only IL-4 bound to a high affinity site (K_d = 31 pM), whereas IL-4/R121E and IL-4/R121D/Y124D bound to lower affinity sites (K_d = 135 pM and 115 pM, respectively) (Fig. 2*B*). These data imply that IL-4/R121E retains physical interaction with IL-2Rγ (in the context of IL-4Rα) on T cells (Fig. 2*A*; affinity similar to wild type), but does not physically interact with IL-13Rα on HUVEC (Fig. 2*B*; affinity comparable to the IL-4 antagonist IL-4/R121D/Y124D, and thus binding to only IL-4Rα). The selective physical interaction of IL-4/R121E with IL-2Rγ, but not IL-13Rα, provides a physical explanation for its selective cellular specificity. Another prediction of this model is that IL-4/R121E also should serve as a selective antagonist of IL-4 action on endothelial cells. Indeed, IL-4/R121E was a potent antagonist of IL-4 activation of endothelial cells (Fig. 2*C*).

IL-4 itself has not yet proven clinically useful, because of, in part, dose-limiting toxicity. Identification of the cell types contributing to IL-4 toxicity and the generation of an appropriate cell-specific agonist may allow testing of such IL-4 muteins in human disease. For instance, the potential for IL-4 in the treatment of multiple sclerosis and type I diabetes has been demonstrated in mouse models of experimental autoimmune encephalitis (33, 34) and in the nonobese diabetic mouse (35) and has been postulated to function by balancing the autoimmune response away from a Th1 state through the induction of a Th2-like response (5). The mutein IL-4/R121E has a spectrum of cellular specificities distinct from any other known cytokine, suggesting potential therapeutic utility: it activates T cells and B cells (immunomodulatory activity), it down-regulates lipopolysaccharide-induced tumor necrosis factor α secretion by monocytes (anti-inflammatory activity), and it does not activate, and in fact antagonizes IL-4 activation of, endothelial cells. Further characterization of IL-4/R121E *in vivo* will allow testing of such therapeutic concepts.

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