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Design of human interleukin-4 antagonists inhibiting interleukin-4-dependent and interleukin-13-dependent responses in T-cells and B-cells with high efficiency

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Human interleukin-4 possesses two distinct sites for receptor activation. A signalling site, comprising residues near the C-terminus on helix D, determines the efficacy of interleukin-4 signal transduction without affecting the binding to the interleukin-4 receptor α subunit. A complete antagonist and a series of low-efficacy agonist variants of human interleukin-4 could be generated by introducing combinations of two or three negatively charged aspartic acid residues in this site at positions 121, 124, and 125. One of the double variants, designated [R121D,Y124D]interleukin-4, with replacements of both Arg121 and Tyr124 by aspartic acid residues was completely inactive in all analysed cellular responses. The loss of efficacy in [R121D,Y124D]interleukin-4 is estimated to be larger than 2000-fold. Variant [R121D,Y124D]interleukin-4 was also a perfect antagonist for inhibition of interleukin-13-dependent responses in B-cells and the TF-1 cell line with a K_i value of approximately 100 pM. In addition, inhibition of both interleukin-4-induced and interleukin-13-induced responses could be obtained by monoclonal antibody X2/45 raised against interleukin-4R_{ex}, the extracellular domain of the interleukin-4 receptor α subunit. These results indicate that efficient interleukin-4 antagonists can be designed on the basis of a sequential two-step activation model. In addition, the experiments indicate the functional participation of the interleukin-4 receptor α subunit in the interleukin-13 receptor system.

Human interleukin-4 (IL-4) activates its cognate receptor system by sequential binding to two different receptor subunits. The first high-affinity binding step (K_d 100 pM) involves the interaction between IL-4 receptor α subunit (Beckmann et al., 1992; Galizzi et al., 1990; Idzerda et al., 1990) and IL-4 amino acid residues located on helices A and C (Kruse et al., 1993; Ramanathan et al., 1993). The subsequent second binding step involves a signalling site on helix D of IL-4 (Kruse et al., 1992) and a further receptor subunit, which according to recent evidence (Kondo et al., 1993; Russell et al., 1993) is probably the γ chain of the IL-2 receptor system, now designated γ_c.

Such a sequential two-step binding mechanism leading to receptor activation suggests a rationale for the design of antagonistic cytokine variants (Cunningham et al., 1991; De Vos et al., 1992). If the first high-affinity binding site is retained and the second signalling site is either destroyed by mutations (Fuh et al., 1992; Kruse et al., 1992) or blocked by monoclonal antibodies (Reusch et al., 1994), the oligomerisation process stops at the level of the unproductive 1:1 complex between the cytokine and the first receptor subunit. The signalling site of human IL-4 comprises side chains of Arg121, Tyr124 and Ser125. Previous results have shown that variants [R121D]IL-4 and [S125D]IL-4 have approximately 30% partial agonist activity and variant [Y124D]IL-4 has less than 1% partial agonist activity for T-cell proliferation compared to wild-type IL-4. High-affinity binding to the IL-4 receptor α subunit is retained in these variants as shown by *in vitro* binding experiments (Kruse et al., 1992). According to high-resolution three-dimensional NMR spectroscopy (Müller et al., 1994; Müller, T., unpublished results) the conformational changes produced by replacement of IL-4 Tyr124 by glycine or aspartic acid are confined to the immediate environment of the side chain. No changes in the secondary or tertiary structures of these IL-4 variants compared to the wild-type protein could be detected.

The variant [Y124D]IL-4 behaves as a complete antagonist for IL-4-dependent T-cell proliferation *in vitro*. It has, however, a partial agonist activity of up to 40% for the induction of CD23 expression in B-cells. The reason for this difference is not clear. Therefore, it was investigated as to how far this residual partial agonist activity on B-cells can be abolished by introducing combinations of two or three aspartic acid residues at positions 121, 124 and 125. More than additive effects could be observed if double-mutant and

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Abbreviations. IL-4, interleukin-4; IL-13, interleukin-13; EC_{50}, effector concentration resulting in half-maximal response; R_{max}, maximal response obtained at saturation levels; IC_{50}, concentration resulting in half-maximal inhibition; IL-4R_{ex}, soluble IL-4 receptor; [R121D,Y124D]IL-4, [R121D,S125D]IL-4, [Y124D,S125D]IL-4 and [R121D,Y124D,S125D]IL-4, variants of IL-4 where combinations of Arg121, Tyr124 or Ser125 have been replaced by aspartic acid.

triple-mutant proteins were analysed for partial agonist and antagonist activity in B-cells and T-cells. One of the double variants, [R121D,Y124D]IL-4, behaved as a complete antagonist during all analysed cellular responses.

Remarkably, not only IL-4-dependent but also IL-13-dependent B-cell responses were completely antagonized by the novel IL-4 variant [R121D,Y124D]IL-4. Similar results have recently been reported for variant [Y124D]IL-4 (Aversa et al., 1993; Zurawski et al., 1993). Furthermore, a monoclonal antibody X2/45 raised against the recombinant extracellular domain of the IL-4 receptor was also found to inhibit both IL-4-dependent and IL-13-dependent B-cell responses. These results provide independent conclusive evidence that the cloned IL-4 receptor α subunit is an essential constituent of the IL-13 receptor system.

MATERIALS AND METHODS

Recombinant human IL-13, produced and purified from *Escherichia coli*, was from IC Chemikalien GmbH.

Human IL-4 variants were generated by *in vitro* mutagenesis, expressed in *E. coli*, renatured and purified (Kruse et al., 1991, 1992). Variants were designated [R121D,Y124D]IL-4, [Y124D,S125D]IL-4, [R121D,S125D]IL-4, and [R121D, Y124D,S125D]IL-4, indicating the replacement of Arg121, Tyr124, or Ser125 by aspartic acid.

Monoclonal antibodies to IL-4R_{ex}, the extracellular domain of the IL-4 receptor α chain, were generated by standard procedures using recombinant human IL-4R_{ex} produced in Chinese hamster ovary cells as antigen (Kruse et al., 1993).

Assays for competition with radioligand binding, human T-cell proliferation, and induction in B-cells of CD23 (Fcε RII), the low-affinity receptor for IgE, have been described (Kruse et al., 1992; Kruse et al., 1993).

Statistical evaluations

All measurements were evaluated by means of the GraFit program (Erithacus Software) using the equation $y = a/(1+[X/I]\exp S) +$ back (IC_{50}-4 parameter logistic).

RESULTS

Binding properties of double and triple IL-4 mutant proteins

In previous experiments (Kruse et al., 1992), the variants [R121D]IL-4, [Y124D]IL-4 and [S125D]IL-4 were shown to retain high-affinity binding to the IL-4 receptor α chain. Table 1 shows that the occurrence of two or three negatively charged residues in the double and triple variants does not significantly alter the binding to the recombinant extracellular domain of the IL-4 receptor α subunit (IL-4R_{ex}). The K_d values are in the range 50–300 pM. These values are similar to those displayed by the single mutants (Kruse et al., 1993). These findings are consistent with recent structural data (Müller et al., 1994) showing that the conformation of the IL-4 protein is not altered by the amino acid replacement causing only local perturbations at the site of the changed side chain. Thus, it is reasonable to conclude that for the variants with multiple changes described in this study, the secondary and tertiary structures also remain grossly unaltered.

Table 1. Dissociation constants K_d between IL-4R_{ex} and IL-4 variants [R121D,Y124D]IL-4, [R121D,S125D]IL-4, [Y124D, S125D]IL-4 and [121D,Y124D,S125D]IL-4. The K_d values were determined by means of competitive radioligand binding to IL-4R_{ex} between ^{125}I-labeled IL-4 and unlabeled IL-4 or variants (Kruse et al., 1992). The concentration of ^{125}I-labeled IL-4 was 0.59 nM in experiment 1 and 0.32 nM in experiment 2. The K_d value for IL-4 of 100 pM was determined from the dose-dependence of receptor saturation after correcting for unspecific binding (Kruse et al., 1993). The K_d values represent mean values ± the standard deviation.

Protein	Dissociation constant K_d	
	experiment 1	experiment 2
	pM	
IL-4	100 ± 17	100 ± 18
[R121D,Y124D]IL-4	105 ± 14	90 ± 13
[R121D,S125D]IL-4	50 ± 5	90 ± 3
[Y124D,S125D]IL-4	170 ± 20	95 ± 12
[R121D,Y124D,S125D]IL-4	180 ± 30	200 ± 35

Biological activities of variants as determined by T-cell proliferation

All variants in Table 2 failed to stimulate T-cell proliferation significantly above background levels. Residual activities with mean values of 0–1.3% of wild-type IL-4 maximal response (R_{max}) were obtained with standard deviations in the same range or even larger. Thus, the loss of efficacy for this T-cell response is at least 100-fold. These results might have been anticipated for variants [R121D,Y124D]IL-4, [Y124D, S125D]IL-4, and [R121D,Y124D,S125D]IL-4 all containing the replacement of Tyr124 with aspartic acid, since the [Y124D]IL-4 variant does not stimulate T-cell proliferation. The loss of efficacy in variant [R121D,S125D]IL-4, however, appears to be more pronounced than expected from an additive effect of the single amino acid replacements in variants [R121D]IL-4 and [S125D]IL-4 both of which retained 20–40% partial agonist activity (Kruse et al., 1993).

All variants inhibit IL-4-dependent T-cell proliferation with K_i values of 460–750 pM. The range of these K_i values is 3–6 fold higher than the EC_{50} value of IL-4 (Fig. 1, Table 2).

Partial agonist/antagonist activity of variants during CD23 induction in B-cells

The induction of CD23-positive B-cells requires much lower IL-4 concentrations (EC_{50} values of 1–3 pM) compared to the stimulation of T-cell proliferation. The CD23 content in the positive B-cells increases at somewhat higher IL-4 concentration (EC_{50} values 6–18 pM). As demonstrated in Fig. 2 and Table 3, clear partial agonist activities are observed with some of the signalling site variants in these sensitive B-cell assays. The double variant [R121D,S125D]IL-4 produces similar maximal responses as [Y124D]IL-4, i.e. 28% versus 29% of the maximal number of CD23-positive cells induced by IL-4, or 12% versus 8% of the maximal CD23 content. The R_{max} values produced by variants [Y124D,S125D]IL-4 and [R121D,Y124D,S125D]IL-4 are lower than those produced by [Y124D]IL-4, but are clearly above the background level. No significant response is observed in the presence of variant [R121D,Y124D]IL-4. The loss of agonist activity in [R121D,Y124D]IL-4 is more than

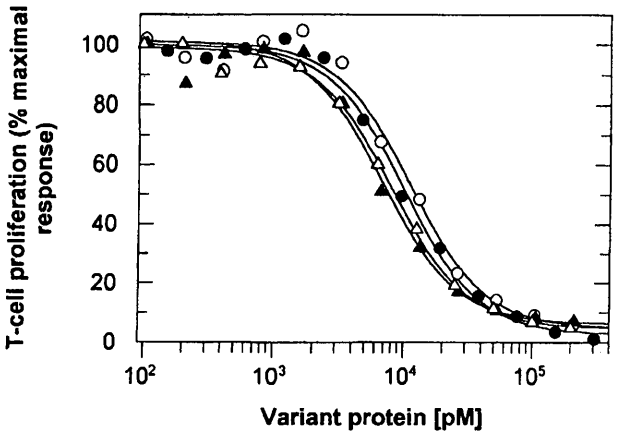

Fig. 1. Inhibition of T-cell proliferation by IL-4 variants [R121D, Y124D]IL-4, [R121D,S125D]IL-4, [Y124D,S125D]IL-4, and [R121D,Y124D,S125D]IL-4. Activated human T-cells (prestimulated with phytohemagglutinin) were incubated with 2 nM IL-4 plus the indicated concentration of IL-4 variant [R121D,Y124D]IL-4 (●), [R121D,S125D]IL-4 (○), [Y124D,S125D]IL-4 (▲), and [R121D,Y124D,S125D]IL-4 (△) for three days before [^{3}H]thymidine incorporation during 4 h was determined.

Table 2. Partial agonist and antagonist activities of variants [R121D,Y124D]IL-4, [R121D,S125D]IL-4, [Y124D,S125D]IL-4 and [R121D,Y124D,S125D]IL-4 on T-cell proliferation. The inhibitory constant K_i was calculated from IC_{50} values as determined by the experiments in Fig. 1; $K_i = IC_{50}/(1 + [IL\text{-}4]/EC_{50})$. [IL-4] was 2 nM and the EC_{50} of IL-4 was 140 pM during this set of experiments. The R_{max} and K_i values represent mean values ± the standard deviation. The background measured without IL-4 was 5.4% ± 1.1 and was substracted from all R_{max} values.

Protein	R_{max} for T-cell proliferation	K_i
	%	pM
IL-4	100 ± 6.5	
[R121D,Y124D]IL-4	0 ± 1	670 ± 15
[R121D,S125D]IL-4	1.3 ± 1.4	750 ± 80
[Y124D,S125D]IL-4	0.4 ± 0.7	460 ± 35
[R121D,Y124D,S125D]IL-4	0.2 ± 1.2	540 ± 20

20-fold compared to variant [Y124D]IL-4. Considering the at least 100-fold loss of agonist activity of [Y124D]IL-4 during the T-cell proliferation response, the efficacy of variant [R121D,Y124D]IL-4 appears to be reduced more than 2000-fold compared to IL-4. It remains unclear why [R121D, Y124D,S125D]IL-4 but not variant [R121D,Y124D]IL-4 retains a small residual partial agonist activity.

Inhibition by antagonistic variants of IL-4-induced CD23 expression

All double and triple variants are efficient inhibitors of IL-4-induced CD23 expression in B-cells (Table 4). The maximal level of inhibition obtainable is determined by the specific partial agonist activity of each variant (Table 3). The K_i values calculated from the concentrations producing 50% inhibition (IC_{50}) and the actual IL-4 concentration and the EC_{50} value of IL-4 for the particular response (see Materials and Methods) are in the range 70−320 pM, and are, hence similar to the K_i value observed for [Y124D]IL-4 (Kruse et al., 1992). Apparently, despite the different partial agonist activities of the variants, the K_i values are similar. This indicates that variant [R121D,Y124D]IL-4, showing the most pronounced activation deficiency, is a complete high-affinity antagonist.

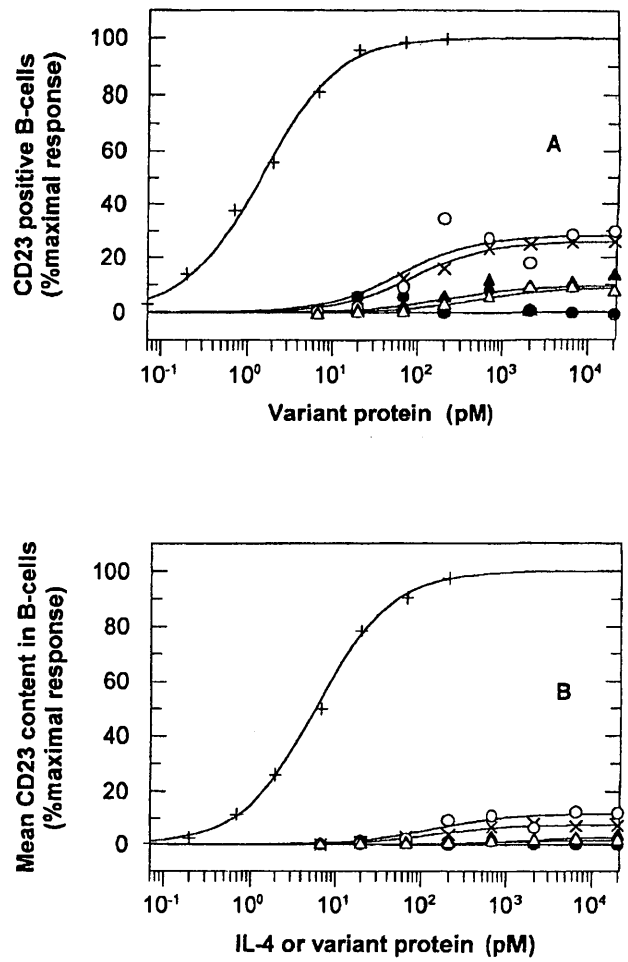

Fig. 2. Partial agonist activities of IL-4 variants during induction of CD23 (Fcε RII) in human B-cells. Human B-cells purified from spleen were incubated with the indicated concentrations of IL-4 (+), or variants [Y124D]IL-4 (×), [R121D,Y124D]IL-4 (●), [R121D, S125D]IL-4 (○), [Y124D,S125D]IL-4 (▲), and [R121D,Y124D, S125D]IL-4 (△) during 18 h and were analysed for the number of CD23-positive cells (A) and the mean CD23 content (B). For evaluation see Table 3.

Inhibition of IL-13-induced CD23 expression

CD23 can also be induced in human B-cells upon stimulation with IL-13 (McKenzie et al., 1993; Minty et al., 1993; Punnonen et al., 1993). The maximal responses are lower, however, than those obtainable with IL-4 (Table 5). At saturation levels of IL-13, the number of CD23-positive cells is only approximately 67% the number of cells inducible by IL-4, and the mean CD23 content is approximately 33% (Table 5). This suggests that only a subpopulation of IL-4-responsive B-cells is also responsive to IL-13, and, furthermore, that even in the IL-13-responsive subpopulation the IL-13-inducible CD23 content is lower (less than 50%). The EC_{50} values of IL-13 are higher than those of IL-4. In the experiment described in Table 5, differences between EC_{50} values were more than tenfold. The variant [R121D,

Table 3. Partial agonist activities of IL-4 variants on CD23 induction in B-cells. A background value of 6.6% (10.1%) CD23-positive cells and of 3.5% (2.9%) mean CD23 content measured without IL-4 was subtracted from all numbers in experiment 1 (experiment 2). The EC_{50} of IL-4 for induction of CD23-positive B-cells was 1 pM (3 pM) and of mean CD23 content 6 pM (18 pM) during experiment 1 (experiment 2). The values represent mean values ± the standard deviation.

Protein	Maximal response R_{max} for induction of			
	CD23-positive B-cells		mean CD23 content	
	experiment 1	experiment 2	experiment 1	experiment 2
	%			
IL-4	100 ± 2.6	100	100 ± 0.1	100
[R121D,Y124D]IL-4	0.6 ± 1.5	− 2.9 ± 3.2	0.7 ± 0.9	− 0.4 ± 0.5
[R121D,S125D]IL-4	28 ± 4.1	25 ± 1.2	12 ± 0.3	9.4 ± 1.1
[Y124D,S125D]IL-4	9.3 ± 2.6	4.3 ± 3.3	2.8 ± 1	1.1 ± 0.6
[R121D,Y124D,S125D]IL-4	8.7 ± 0.9	4.9 ± 3.2	1.7 ± 0.2	1 ± 0.6
[Y124D]IL-4	26 ± 0.0−0.1	19 ± 2.6	7.6 ± 0.4	6.3 ± 0.7

Table 4. Inhibitory constants (K_i) of IL-4 variants during IL-4-induced CD23 expression in B-cells. The K_i values were calculated from the IC_{50} values (Table 2) measured during dose-dependent inhibition of CD23 induction in the presence of 67 pM IL-4. The EC_{50} value of IL-4 for induction of CD23-positive B-cells was 1 pM and the EC_{50} value for the mean CD23 content was 6 pM. The K_i values represent mean values ± the standard deviation.

Protein	Inhibitory constants K_i for IL-4-dependent induction of	
	CD23-positive B-cells	mean CD23 content
	pM	
[R121D,Y124D)IL-4	180 ± 10	280 ± 25
[R121D,S125D]IL-4	98 ± 13	190 ± 11
[Y124D,S125D]IL-4	210 ± 22	320 ± 44
[R121D,Y124D,S125D]IL-4	120 ± 13	180 ± 12
[Y124D]IL-4	71 ± 11	110 ± 24

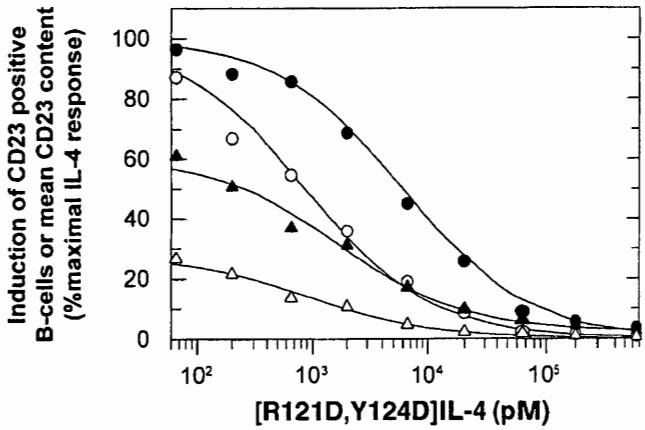

Fig. 3. Inhibition of IL-13-dependent or IL-4-dependent CD23 expression in B-cells by variant [R121D,Y124D]IL-4. Human B-cells purified from spleen were incubated with 67 pM IL-4 (●, ○) or 800 pM IL-13 (▲, △) plus the indicated concentration of variant [R121D,Y124D]IL-4. After 18 h, the number of CD23-positive B-cells (●, ▲) and the mean CD23 content of B-cells (○, △) were measured by flow cytometry. For evaluation see Table 5.

Y124D]IL-4 inhibits IL-13-dependent CD23 induction to background levels (Fig. 3). The K_i values were calculated to be 130−160 pM. This is in the range of K_i values measured for the IL-4-dependent response although the values are somewhat lower.

Monoclonal antibody X2/45 directed against IL-4R_{ex} inhibits both IL-4-induced and IL-13-induced CD23 expression in B-cells

The monoclonal antibody (mAb) X2/45 was prepared by immunizing mice with a recombinant extracellular domain

Table 5. Inhibition by IL-4 variant [R121D,Y124D]IL-4 of IL-13-dependent or IL-4-dependent expression of CD23 in human B-cells. In the inhibition assays, the concentration of IL-13 was 800 pM and the concentration of IL-4 was 67 pM. The K_i values were calculated from the concentration of [R121D,Y124D]IL-4 causing half-maximal inhibition (IC_{50}) of the B-cell responses; $K_i = IC_{50}/(1+[IL]/EC_{50})$. The EC_{50} values represent mean values ± the standard deviations. MFI designates the mean fluorescence intensity during CD23 FACS analysis.

Protein	CD23-positive B-cells			Mean CD23 content		
	EC_{50}	R_{max}	K_i	EC_{50}	R_{max}	K_i
	pM	%	pM	pM	MFI	pM
IL-4	3 ± 0.1	54 (= 100)		13 ± 0.8	361 (= 100)	
IL-13	60 ± 5	41 (64)		152 ± 9	110 (30.5)	
[R121D,Y124D]IL-4 plus IL-4			260			140
[R121D,Y124D]IL-4 plus IL-13			130			150

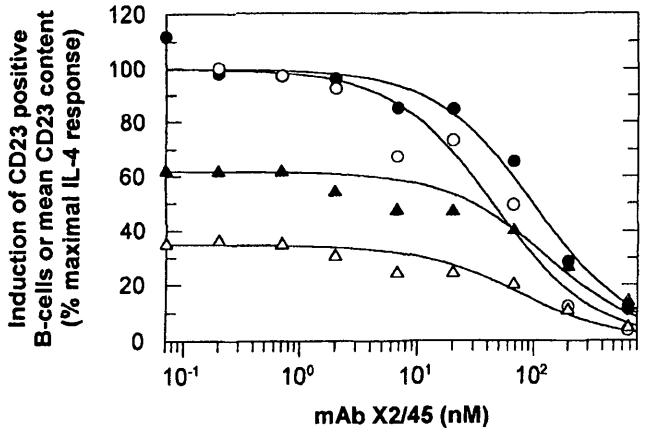

Fig. 4. Inhibition by mAb X2/45 of IL-13-dependent or IL-4-dependent CD23 expression in human B-cells. Purified B-cells were incubated with 67 pM IL-4 (●, ○) or 800 pM IL-13 (▲, △) plus increasing concentrations of mAb X2/45. After 18 h, CD23-positive cells (●, ▲) and mean CD23 content (○, △) were determined by fluorescence-activated cell sorting. A quantitative evaluation of the data is provided in Table 6 (experiment 2).

Table 6. Inhibition by mAb X2/45 of IL-4-dependent or IL-13 dependent expression of CD23 in B-cells. The K_i values were calculated from the IC_{50} value in the presence of 67 pM IL-4 or 800 pM IL-13. The respective EC_{50} values were taken from Table 5.

Protein	mAb X2/45 inhibition of induction of			
	CD23-positive B-cells		mean CD 23 content	
	K_i			
	experiment 1	experiment 2	experiment 1	experiment 2
	nM			
IL-4	4.3	3.1	7.7	6.4
IL-13	8.8	3.6	11.4	3.1

$IL\text{-}4R_{ex}$ of the IL-4 receptor α subunit (Kruse et al., 1993). X2/45 interferes with IL-4 binding and inhibits IL-4-induced T-cell proliferation with a K_i value of 20 nM. X2/45 specifically recognizes the IL-4 receptor α chain on the cell surface as was shown by flow cytometry (Reusch, P., unpublished results).

As demonstrated in Fig. 4 and Table 6, mAb X2/45 inhibits IL-13-dependent CD23 induction in B-cells as efficiently as the IL-4-dependent response. The activity of both interleukins can be inhibited to background levels and the K_i values are in the same range (3−12 nM). An isotype matched control mAb (anti-CD4) had no effect at comparable concentrations. This indicates that $IL\text{-}4R_{ex}$ is necessary for IL-4 and for IL-13 activity.

Inhibition of IL-4-induced and IL-13-induced proliferation of the TF1 cell line by mAb X2/45 and IL-4 variant [R121D,Y124D]IL-4

The premyeloid erythroleukemia cell line TF-1 proliferates in response to both human IL-4 and human IL-13. Both responses are inhibited by signalling site variant [Y124D]IL-4 (Zurawski et al., 1993). The experiments described in Table 7 extend these findings employing variant [R121D, Y124D]IL-4. In addition, the inhibitory action of anti $IL\text{-}4R_{ex}$ mAb X2/45 on both IL-4 and IL-13 activity is demonstrated. In TF-1 cells, the maximal response for IL-13 (R_{max}) was only 20−25% of the R_{max} produced by IL-4. The EC_{50} value of IL-13 was 4−8-fold higher than the EC_{50} value of IL-4. The activity of both cytokines could be inhibited by variant [R121D,Y124D]IL-4 with a K_i value of 40−80 pM. The very high-affinity binding site on TF-1 cells for human IL-4 (K_d less than 10 pM, see Zurawski et al., 1993) could not be detected during competitive radioligand binding. A K_d of 100 to 150 pM was determined for IL-4 binding in the presence of 125 pM ^{125}I-labeled IL-4 as well as in the presence of 2.5 pM ^{125}I-labeled IL-4 (data not shown).

DISCUSSION

Extending our previous studies on IL-4 variants, the present results demonstrate that it is feasible to rationally design a second generation of human IL-4 antagonists/partial agonists displaying extremely low efficacy. Furthermore, these data together with the data on the inhibitory activity of anti $IL\text{-}4R_{ex}$ monoclonal antibodies yields information on the functional interaction of IL-4 with its receptor subunits and on the essential role of the established IL-4 receptor α subunit during both IL-4-dependent and IL-13-dependent responses in T-cells and B-cells.

The loss of signalling activity (efficacy) in multiple IL-4 variants appears to be caused by the defective signalling site comprising the juxtaposed amino acid side chains at positions 121, 124 and 125 of helix D. [Allosteric effects due to the amino acid replacement are not detected by high-resolution three-dimensional NMR spectroscopy (Müller et al., 1994; Müller, unpublished results) and the binding affinity to $IL\text{-}4R_{ex}$ remains largely unaltered]. The signalling activity of this site depends on a hydrophobic patch provided by Tyr124 (or another large hydrophobic side chain) and is weakened specifically if the negatively charged side chain of aspartic acid is present (Demchuk et al., 1994). The occurrence at positions 121 and 125 of small, large hydrophobic, polar, or glutamyl residues introduced at this position has no or only small effects (Kruse N. and Sebald, W., unpublished results). A positional effect of the aspartic acid side chain is suggested by the observation that IL-4 variants [Y124N]IL-4 and [Y124D]IL-4 show an at least 20-fold difference in efficacy, whereas the difference between wild-type IL-4 and [R121D]IL-4 or [S125D]IL-4 is only 2−5-fold. The partial agonist activities for B-cell CD23 induction indicates that variants [Y124D, S125D]IL-4 and [R121D,Y124D,S125D]IL-4 are slightly more active than [R121D,Y124D]IL-4. Accordingly, an aspartic acid residue at position 121 is more inhibitory than an aspartic acid residue at position 125. But these small differences should not be overemphasized.

In the double variants, the effect of the individual amino acid replacements may be more than additive. The loss of potency in variant [R121D,S125D]IL-4 is more than 100-fold. The additive loss would have been approximately ten-fold. In variant [R121D,Y124D]IL-4, no agonist activity could be measured. This indicates a more than 2000-fold reduced potency compared to IL-4 (considering the background and the sensitivity of the B-cell assay). This is again slightly above the value expected from the added effects in [R121D]IL-4 (approximately three-fold) and [Y124D]IL-4 (greater than 100 fold).

Table 7. Inhibition of IL-4-dependent or IL-13-dependent TF1 cell proliferation by variant [R121D,Y124D]IL-4 or mAb X2/45. R_{max} values are given relative to the maximal IL-4 response (30800 cpm incorporated ^{3}H-thymidine radioactivity). The inhibitory constants K_i were calculated from the concentration of [R121D,Y124D]IL-4 or mAb X2/45 causing half-maximal inhibition (IC_{50}); $K_i = IC_{50}/(1+[IL]/EC_{50})$. The concentration in the inhibition assays was 200 pM for IL-4 and 800 pM for IL-13.

Protein	TF-1 cell proliferation					
	experiment 1			experiment 2		
	EC_{50}	R_{max}	K_i	EC_{50}	R_{max}	K_i
	pM	%	nM	pM	%	pM
IL-4	14	100		25	100	
IL-13	250	20		170	21	
IL-4 plus [R121D,Y124D]IL-4						76
IL-4 plus X2/45			2.6			
IL-13 plus [R121D,Y124D]IL-4						36
IL-13 plus X2/45			1.6			

It has been discussed (Rigley et al., 1991) that B-cells contain two different IL-4 receptors as one possibility to explain the different sensitivities to IL-4 and anti-CD19 mAb and the different intracellular signalling requirement for induction of surface IgM and mean CD23 content in human tonsillar B-cells. The present study did not analyse IL-4-dependent surface IgM expression in B-cells. The inhibition by anti IL-4R_{ex} mAb X2/45 of both the very sensitive induction of CD23-positive B-cells (EC_{50} 1−3 pM in these experiments) and the mean CD23 content in B-cells (EC_{50} 3−13 pM) indicate however, that the IL-4 receptor α subunit is involved in both of these B-cell responses. Thus, it is a distinct possibility that these graded sensitivities of IL-4 responses result from different post-receptor signalling steps.

In previous studies it has been demonstrated (Aversa et al., 1993; Zurawski et al., 1993) that IL-13 partially inhibits IL-4 binding to TF-1 cells and that IL-13-dependent responses are inhibited by variant [Y124D]IL-4. These results showed that the IL-4 and IL-13 receptor systems are complex and share a common component. The present data indicate that this common component comprises at least the IL-4 receptor α subunit, since both anti IL-4R_{ex} mAb X2/45 and the new IL-4 antagonist [R121D,Y124D]IL-4 (similar but more efficient than [Y124D]IL-4) inhibit B-cell CD23 induction by both IL-4 and IL-13.

It is a reasonable working hypothesis that the signalling site of human IL-4, i.e. the amino acid side chains at positions 121, 124 and 125 interact with the common γ chain, which has been identified first in the IL-2 receptor system and which has been later established to be also a constitutent of the IL-4 and IL-7 receptor systems (Kondo et al., 1993; Russell et al., 1993; Kondo et al., 1994). Actually, conspicuous similarities can be detected if the corresponding surface areas of human IL-4 and IL-2 are compared (Müller, T., unpublished results). It is unknown at this time whether or not the common γ chain also functions in the IL-13 receptor system. The C-terminus of human and murine IL-13 exhibits some similarities to the signalling site of IL-4, although one published alignment (Zurawski et al., 1993) postulates the insertion of a glycine residue at the corresponding IL-13 sequence. It is tempting to speculate that the IL-13 receptor system uses both the IL-4 receptor α subunit and the common γ chain and that IL-13 specificity is determined by an additional third chain similar to the ciliary-neutrophic-factor receptor system (Davis et al., 1993; Kishimoto et al., 1994). Accordingly, the nonproductive complex postulated to be formed by the human IL-4 antagonists (Zurawski et al., 1993) might be a 1:1 complex between the IL-4 receptor α subunit and the IL-4 antagonist, which cannot associate further due to the defective signalling site with the common γ chain to form the functional IL-4 receptor or with the common γ chain plus the IL-13-binding protein to form the functional IL-13 receptor.

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REFERENCES

Aversa, G., Punnonen, J., Cocks, B. G., de Waal Malefyt, R., Vega, F. Jr, Zurawski, S. M., Zurawski, G. & de Vries, J. E. (1993) 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, *J. Exp. Med. 178*, 2213−2218.

Beckmann, M. P., Cosman, D., Fanslow, W., Maliszewski, C. R. & Lyman, S. D. (1992) The IL-4 receptor: structure, function, and signal transduction, in *Interleukins: molecular biology and immunology. Chem. Immunol.* (Kishimoto, T., ed.) vol. 51, pp. 107−134, Karger, Basel.

Cunningham, B. C., Ultsch, M., De Vos, A. M., Mulkerrin, M. G., Clauser, K. R. & Wells, J. A. (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule, *Science 254*, 821−825.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. & Yancopoulos, G. D. (1993) LIFR beta and gp130 as heterodimerizing signals of the tripartite CNTF receptor, *Science 260*, 1805−1808.

Demchuk, E., Müller, T., Oschkinat, H., Sebald, W. & Wade, R. C. (1994) Receptor binding properties of four-helix-bundle growth factors deduced from electrostatic analysis, *Protein Sci. 3*, 920−935.

De Vos, A. M., Ultsch, M. & Kossiakoff, A. A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex, *Science 255*, 306−312.

Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V. & Wells, J. A. (1992) Rational design of potent antagonists to the human growth hormone receptor, *Science 256*, 1677−1680.

Galizzi, J.-P., Zuber, C. E., Harada, N., Gorman, D. M., Djossou, O., Kastelein, R., Banchereau, J., Howard, M. & Miyajima, A. (1990) Molecular cloning of a cDNA encoding the human interleukin 4 receptor, *Int. Immunology 2*, 669−675.

Idzerda, R. L., March, C. J., Mosley, B., Lyman, S. D., Vanden-Bos, T., Gimpel, S. D., Din, W. S., Grabstein, K. H., Widmer, M. B. & Park, L. S. (1990) Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily, *J. Exp. Med. 171*, 861–873.

Kishimoto, T., Taga, T. & Akira, S. (1994) Cytokine signal transduction, *Cell 76*, 253–262.

Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K. & Sugamura, K. (1993) Sharing of the interleukin-2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4, *Science 262*, 1874–1883.

Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S. I. & Sugamura, K. (1994) Functional participation of the IL-2 receptor γ chain in IL-7 receptor complexes, *Science 263*, 1453–1454.

Kruse, N., Lehrnbecher, T. & Sebald, W. (1991) Site-directed mutagenesis reveals the importance of disulfide bridges and aromatic residues for structure and proliferative activity of human interleukin-4, *FEBS Lett. 286*, 58–60.

Kruse, N., Tony, H.-P. & Sebald, W. (1992) Conversion of human IL-4 into a high-affinity antagonist by a single amino acid replacement, *EMBO J. 11*, 3237–3244.

Kruse, N., Shen, B.-J., Arnold, S., Tony, H.-P., Müller, T. & Sebald, W. (1993) Two distinct functional sites of human interleukin 4 are identified by variants impaired in either receptor binding or receptor activation, *EMBO J. 12*, 5121–5129.

McKenzie, A. N. J., Culpepper, J. A., de Waal Malefyt, R., Brière, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S., de Vries, J. E., Banchereau, J. & Zurawski, G. (1993) Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function, *Proc. Natl Acad. Sci. USA 90*, 3735–3739.

Minty, A., Chalon, P., Derocq, J.-M., Dumont, X., Guillemot, J.-C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P. & Caput, D. (1993) Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses, *Nature 362*, 248–250.

Müller, T., Dieckmann, T., Sebald, W. & Oschkinat, H. (1994) Aspects of receptor binding and signalling of IL-4 investigated by site-directed mutagenesis and NMR spectroscopy, *J. Mol. Biol. 237*, 423–436.

Punnonen, J., Aversa, G., Cocks, B. G., McKenzhie, A. N. J., Menon, S., Zurawski, G., de Waal Malefyt, R. & de Vries, J. E. (1993) Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells, *Proc. Natl Acad. Sci. USA 90*, 3730–3734.

Ramanathan, L., Ingram, R., Sullivan, L., Greenberg, R., Reim, R., Trotta, P. P. & Le, H. V. (1993) Immunochemical mapping of domains in human interleukin 4 recognized by neutralizing monoclonal antibodies, *Biochemistry 32*, 3549–3556.

Reusch, P., Arnold, S., Heusser, C., Wagner, K., Weston, B. & Sebald, W. (1994) Neutralizing monoclonal antibodies define two different functional sites in human interleukin 4, *Eur. J. Biochem, 222*, 491–499.

Rigley, K. P., Thurstan, S. M. & Callard, R. E. (1991) Independent regulation of interleukin 4 (IL-4)-induced expression of human B cell surface CD23 and IgM: functional evidence for two IL-4 receptors, *Int. Immunol. 3*, 197–203.

Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E. & Leonard, W. J. (1993) Interleukin-2 receptor γ chain: a functional component of the IL-4 receptor, *Science 262*, 1880–1883.

Zurawski, S. M., Vega, F. Jr, Huyghe, B. & Zurawski, G. (1993) Receptors for IL-13 and IL-4 are complex and share a novel component that functions in signal transduction, *EMBO J. 12*, 2663–2670.