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Review

Structure, binding, and antagonists in the IL-4/IL-13 receptor system

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Abstract

Interleukin-4 (IL-4) and IL-13 are the only cytokines known to bind to the receptor chain IL-4Rα. Receptor sharing by these two cytokines is the molecular basis for their overlapping biological functions. Both are key factors in the development of allergic hypersensitivity, and they also play a major role in exacerbating allergic and asthmatic symptoms. Knowledge of structure and function of this system has allowed the development of inhibitors that block the interaction between the cytokines and their shared receptor. Mutational analysis of IL-4 has revealed variants with high-affinity binding to IL-4Rα but no detectable affinity for the second receptor subunit, which is either γc or IL-13Rα1. These IL-4 antagonists fail to induce signal transduction and block IL-4 and IL-13 effects in vitro. IL-4 antagonists prevent the development of allergic disease in vivo and an antagonistic variant of human IL-4 is now in clinical trials for asthma. Detailed knowledge of the site of interaction of IL-4 and IL-4Rα has been gained by structure analysis of the complex of these two proteins and through functional studies employing mutants of IL-4 and its receptor subunits. Based on these new data, the hitherto elusive goal of designing small molecular mimetics may be feasible.

Keywords: IL-4; IL-13; Allergy; Antagonist; Protein structure

1. Introducing IL-4 and IL-13

1.1. Atopic diseases arise due to untimely production of IL-4

Immunologists are hotly debating why atopic diseases like allergies and asthma are increasing in industrialized countries. Social, medical, genetic, and environmental factors all influence the development of allergic sensitivity [1–3]. On a mechanistic level, an explanation could be offered by pointing out that production of interleukin-4 (IL-4) is directing the immune system toward T_H2-dependent allergic reactivity [4–6], and in consequence, the rise in atopic diseases would be explained by an increased tendency of the immune system to produce IL-4 in response to inappropriate stimuli. Rather than debating the causes of this phenomenon, we will focus here on strategies for the inhibition of IL-4. Blocking IL-4 could introduce a completely new range of allergy therapeutics affecting the cytokine network.

Therapeutic application seems feasible since IL-4 is not only critically important for T_H2 cell differentiation at the beginning of an immune response, but is also responsible for downstream events leading to differentiation and activation of effector cells. IL-4 induces class switching to IgE and IgG4 in man, and to IgE and IgG1 in mouse [7,8]. It induces expression of adhesion molecules like VCAM-1 [9–11], T_H2 cytokines like IL-5, IL-6, and IL-9 [5,12], and chemokines like eotaxin-1, -2, and -3 [13–15]. IL-4 can also prime mast cells [16] and basophils [17], leading to enhanced activation of cells during allergic challenge. This wide range of effects suggests that even in ongoing disease, IL-4 inhibition could still be beneficial.

1.2. IL-13 enhances clinical parameters in allergy and asthma

IL-13 shares a receptor with IL-4 (see below) and is, for this reason, able to induce nearly all responses generated by IL-4 [18,19]. An exception is the IL-4-dependent differentiation of uncommitted T_H cells to the T_H2 phenotype, where IL-13 usually cannot replace IL-4 as a differentiation factor [5,12]. However, IL-13 is produced for a longer period

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of time than IL-4, and seems to be particularly important for ongoing atopic and asthmatic diseases [20–24]. Experiments in mice have indeed shown that the selective inhibition of IL-13 is therapeutically more efficient in asthma models than the inhibition of IL-4 [25,26]. The homology (similarity) between both cytokines is as low as about 25% on the amino acid sequence level [27], which makes it unlikely that any single antibody or similar agent could neutralize both of them. Nevertheless, if receptors are shared by both cytokines, it ought to be possible to find a single inhibitor for both.

1.3. The IL-4 and IL-13 receptor system

For signal transduction, both IL-4 and IL-13 require the same receptor subunit, IL-4Rα. This protein is part of the functional heterodimeric receptor complex for either cytokine. It organizes signal transduction through signaling molecules associated with its large intracellular domain [6]. Signalling is initiated by recruiting and binding of the ligand to a second receptor subunit, which can be either γc (type 1 receptor) or IL-13Rα1 (type 2 receptor). Type 1 receptor complexes can be formed only by IL-4, not by IL-13, and appear to be responsible for signalling in T-cells, which do not express functional IL-13Rα1. Type 2 receptor complexes can be formed by either IL-4 or IL-13, and they are activated by both ligands [6]. A difference between the two ligands is that IL-4 contacts first IL-4Rα and then IL-13Rα1, a sequence of events which is reversed for IL-13. Nevertheless, the resulting dimeric receptor subunit assembly is identical.

2. Therapeutic prospects for IL-4/IL-13 inhibitors

2.1. Selecting a target

In this review, we will not deal with strategies that are based on the interference with the production of allergy-associated cytokines, or on the blockage of specific intracellular signalling components which are activated through cytokine receptors. Rather, we will focus on dealing with these cytokines as they are. Once IL-4 and IL-13 have been produced during allergic sensitization or upon challenge with allergen, inhibition of the receptor–ligand interaction may offer the most straightforward approach to interfere with the biological effects of the cytokines. Either the ligands or the receptor subunits could be targets. Inhibition is selective for one cytokine if the ligands are targeted. Such an approach, a humanized antibody against IL-4 (Protein Design Labs and GlaxoSmithKline), is currently undergoing phase II clinical trials. Blocking γc is problematic since this receptor chain is also used by IL-2, IL-7, IL-9, IL-15, and IL-21 [28,29], which implies that severe side effects must be anticipated. Loss of functional γc leads to a severe combined immunodeficiency (X-linked SCID) due to the lack of IL-7 signals during lymphopoiesis [30,31]. IL-13Rα1 appears to be a suitable target since its inhibition would interfere with effector cell activation, but presumably not with T_H1/T_H2 differentiation. An interesting candidate is a high-affinity binding protein for IL-13, IL-13Rα2, described from man [32] and mouse [33], which does not form signal-competent receptor complexes [18,34,35]. A soluble variant of the murine binding protein is a potent inhibitor for IL-13 in mice [25]. Finally, blocking IL-4Rα would lead to a complete inhibition of both IL-4 and IL-13 signalling [36]. Indeed, mice lacking a functional gene for this receptor subunit fail to respond to either cytokine and are severely impaired in T_H2-associated immune responses in models of parasite infections and allergies [37–43].

2.2. How to inhibit cytokines

2.2.1. Cytokine overdose

Some cytokine receptors are inhibited by high doses of their specific ligand, which means that the same molecule can have agonistic and antagonistic effects. An example is the growth hormone, where high levels of ligand lead to saturating formation of 1:1 complexes of ligand and receptor, leaving no free receptor molecules available for receptor dimerization [44,45]. This principle can only be applied to cytokines binding to homodimeric receptors (like the growth hormone or erythropoietin), and is not feasible for heterodimeric receptor complexes as found in the IL-4/IL-13 system. Nevertheless, the observation indicates that prevention of receptor dimerization is a suitable way to disrupt signal generation and inhibit cytokine effects.

2.2.2. Inhibitory antibodies

Protein–protein interaction is prevented by specific antibodies binding at or near the interaction surfaces, where they are precluding access of the natural binding partner. Inhibitory antibodies may be directed against the ligand or against the receptor. Inhibitory antibodies against IL-4 [46–49] and IL-4Rα [50,51] have been developed and were efficient in cellular assays and in mouse models of parasite infection and allergy. It should be noted that antibodies against these two molecules are not functionally equivalent, since only the latter one inhibits IL-13 as well as IL-4. More surprising is the observation that anti-IL-4 antibodies can increase the biological effects of IL-4 [52]. Binding to such an antibody will of course prevent an IL-4 molecule from contacting the receptor, but this inhibition is not irreversible. Cytokine and antibody may dissociate again, with kinetics depending on the off-rate for the specific cytokine–antibody pair. Antibody binding prolongs the serum half-life of IL-4 by slowing down its renal clearance and by protecting the cytokine from proteolytic digestion. In most circumstances, inhibitory anti-IL-4 antibodies are net inhibitors, physiologically, but this cannot be taken for granted and the specific parameters of an experimental setup have to be considered. In contrast, blocking IL-4Rα is always inhibitory unless a conscious effort is made to induce homodimerization of the receptor, which may lead to unphysiologic activation [53–55].

2.2.3. Soluble receptor domains

The extracellular domains of cytokine receptors are produced biologically by shedding or through specific mRNAs generated by differential splicing [56]. It has been suggested that these soluble receptor variants may play a role in the regulation of cytokine activity. The application of soluble receptor-derived binding proteins offers the advantage that these are physiological proteins that should not elicit an immune response. A soluble variant of IL-4Rα binds IL-4 with an affinity that is lower only by a factor of 2–4 than for the heterodimeric receptor. The soluble binding protein is therefore well able to compete with the cell-bound receptor for its ligand and is highly efficient in blocking the effects of IL-4 [57–60]. However, soluble IL-4Rα does not bind IL-13 to a measurable extent and under some circumstances may potentiate IL-4 effects due to the same mechanism as observed for inhibitory anti-IL-4 antibodies [57,58,61]. A soluble variant of IL-4Rα is now undergoing clinical trials for application in the therapy of allergic asthma [62,63].

2.2.4. Cytokine antagonists

Cytokine antagonists are designed by mutating a binding site with the intention to create a variant that is still able to bind one receptor subunit, but not the other one. Antagonistic mutants cannot activate the receptor and are highly specific inhibitors. In the case of IL-4, mutation of amino acids in the binding site for γc leads to the creation of antagonistic variants [64,65]. Cross-linking experiments have confirmed that these antagonists bind to IL-4Rα as predicted, but not to the second receptor subunit [66].

Initially, three amino acids located close to the C-terminus were identified as particularly sensitive toward exchanges: R121, Y124 and S125 [64,65]. Simultaneous mutation of R121 and Y124 to aspartic acid residues (R121D/Y124D) creates a protein that has a high binding affinity for IL-4Rα, no detectable biological activity, and which completely inhibits both IL-4 and IL-13 signalling [50].

The analogous amino acids in mouse IL-4 are Q116 and Y119. The variant Q116D/Y119D corresponding to the human antagonist is a complete inhibitor for IL-4 and IL-13 [39,67]. Inhibitory activity is also created by the single point mutation Y119D [68] or by truncating mouse IL-4 after residue 118 [69].

Cytokine antagonists can be designed by introducing very small changes into the original molecule. For human IL-4, the powerful antagonist R121D/Y124D retains 127 of the 129 amino acids of the natural protein. This does not absolutely prevent immune reactions against the new epitope, but good tolerance can nevertheless be expected.

2.2.5. Small molecular mimetics

Proteins offer the advantage of evolution-selected binding specificity, but are pharmaceutical agents of limited applicability. Mass production of recombinant proteins is expensive and difficult, proteases may quickly dispose of the carefully designed agent, and oral application is still impossible. Small synthetic molecules that mimic the protein binding surface and maintain a high binding affinity would be an attractive alternative. So far, no synthetic substance has been identified which could be used in the IL-4/IL-13 system, but progress in resolving the structures of IL-4 and IL-4Rα combined with functional analysis may facilitate such attempts in the future [70].

3. Structural and functional analysis of the IL-4/IL-13 receptor system

3.1. The structure of IL-4

IL-4 is one of the small four-helix-bundle cytokines [71,72] that are characterized by antiparallel juxtaposed helices A, C, B, D, and two long end-to-end loops, loop AB and CD, which are connected by a short β-sheet packed against helices B and D (Fig. 1a) [73–78]. The conserved exon boundaries present in the IL-4 gene are also indicated in Fig. 2. Helix A, the AB loop, the helix BC hairpin, and the loop CD plus helix D are encoded by four different exons. An invariant disulfide bond (C46/C99) seems to stabilize the helix BC hairpin. IL-4 has two binding epitopes: The binding epitope for the high-affinity receptor chain IL-4Rα is located on the helices A and C [65,79], whereas the binding site for the low-affinity receptor chains, both γc and IL-13Rα1, is located on helices A and D [65,80].

IL-4 is found exclusively in mammals. Nevertheless, the divergence of the amino acid sequence among species is high. A large part of helix C present in the primate protein is absent in rodents and even more is deleted in the sequences of IL-4 from cattle, pig, and cat (Fig. 2). Amino acid differences among human and primate IL-4 proteins are confined mainly to helix A (exon 1) near the binding epitope for the α receptor chain.

Biological activity of IL-4 is species specific in many instances. Murine IL-4 does not interact with the human IL-4Rα chain at physiological concentrations [67]. This is remarkable, since the main binding determinants (E9, R53, Y56, and R88) for the α chain are conserved (Fig. 2). As discussed below, binding specificity between IL-4 and IL-4Rα seems to be modulated by side chains that do not contribute significantly to the binding [81]. Thus, mismatches disturbing the complementarity of the contact epitopes most likely broaden binding specificity. The helix AC face of human IL-4 accumulates a large positive charge due to several basic residues, namely K12, R53, R75, K77, R81, K84, R85, and R88 (Fig. 1b). In contrast, the IL-4Rα binding epitope contains a series of acidic residues (Fig. 3b). This charge complementarity will induce electrostatic steering during the association of IL-4 and IL-4Rα [82] (Fig. 4), and in consequence, the association rate constant k_{ass} is exceedingly high ($>10^7$ M^{-1} s^{-1}). Only two of these basic amino acids are

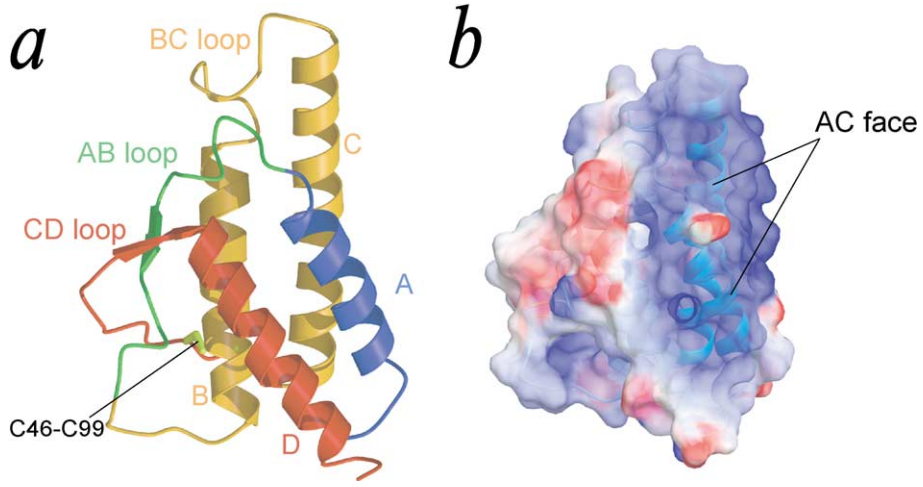

Fig. 1. (a) Ribbon diagram of human IL-4. The four-helix bundle with the up-up-down-down topology is a typical member of the short-chain cytokine family. The exon-intron boundaries found for IL-4 are confined to secondary structure elements. Exon 1 encodes for helix A (blue), exon 2 for the long loop AB (green), exon 3 comprises helices B and C (orange) and exon 4 encodes the second long loop CD and helix D (red). The invariant disulfide bond between Cys46 and Cys99 is shown in yellow. (b) Electrostatic potential of IL-4. The orientation of the molecule is the same as in (a). The surface is color-coded according to its electrostatic potential (intense blue: +2.5 kT/e; intense red: −2.5 kT/e). The electrostatic potential was calculated using the program GRASP (full charges, salt concentration 150 mM). The figure shows the strong positive potential of the binding interface for the IL-4Rα chain, also called AC face.

conserved among the known IL-4 sequences of all species. As a result, the association rates for binding of IL-4 to IL-4Rα should be lower in other species, as seen for mouse IL-4 with a k_{ass} of $<10^7\ M^{-1}\ s^{-1}$ [67].

3.2. The structure of IL-4Rα CHR

The extracellular binding domain of IL-4Rα (IL-4Rα CHR) represents the minimal version of a typical cytokine-

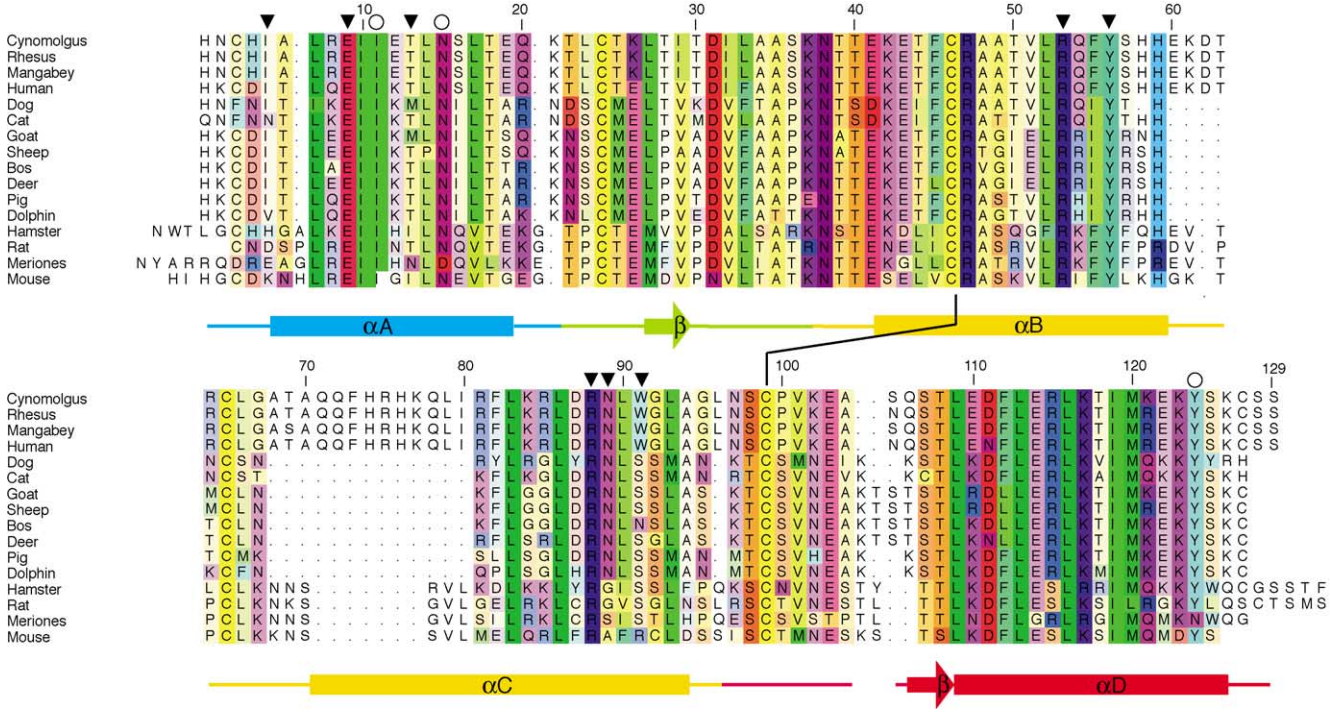

Fig. 2. IL-4 is found only in mammals. The sequences for mature IL-4 were aligned using the programs ClustalW, PILEUP (GCG version 9.1) and Jalview (http://www2.ebi.ac.uk/~michele/jalview/contents.html). The amino acid type is color-coded using the Taylor color scheme. The intensity of the color was manipulated to show the degree of conservation for each residue position; sites showing intense color are exhibiting a higher degree of conservation. Secondary structure elements (helices and sheets as determined from the crystal structure of human IL-4) are indicated together with the color-coding for the intron/exon boundaries (see also Fig. 1a). The main binding determinants for the IL-4Rα chain are marked by black triangles (AC face), for the γc receptor subunit, open circles are used (AD face).

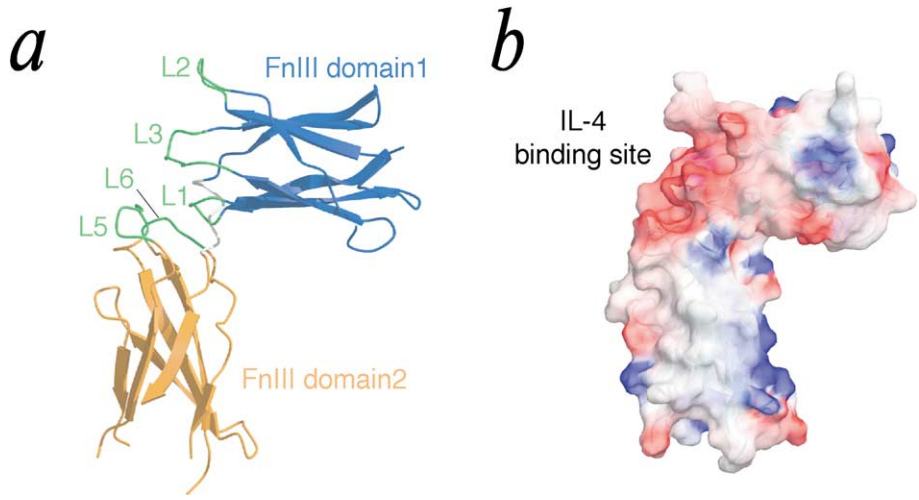

Fig. 3. (a) Ribbon diagram for IL-4Rα CHR. The modular architecture of IL-4Rα CHR is shown. The two fibronectin type III domains are color-coded in blue (FnIII domain 1) and orange (FnIII domain 2), and loop regions in contact with the ligand IL-4 are shown in green. Due to lack of electron density, no coordinates are available for the loop regions from residue 107 to 112 and 163 to 169 in the second FnIII domain. (b) Electrostatic potential map of IL-4Rα CHR. The surface representation of IL-4Rα CHR in its bound form is shown color-coded by the electrostatic potential as calculated in GRASP (red: −2.5 kT/e; blue: +2.5 kT/e). The binding site for the ligand IL-4 exhibits a strong negative potential complementary to the positive potential of the ligand IL-4 itself.

binding homology region (CHR) [83]. Two fibronectin type III (FnIII) domains, each about 100 residues long, are connected by a short linker segment (Fig. 3a). The N-terminal FnIII domain D1 containing the conserved disulfide bonds exhibits the h-type of an immunoglobulin fold [84]. The second FnIII domain D2 does not contain any disulfide bonds and exhibits the FnIII topology of type s [84]. In addition, domain D2 includes the ws × ws motif, typical for the cytokine receptor type I family [83].

The elbow region between the two FnIII domains is dominated by the long inter beta-sheet loops L2 and L5 that are flanked by loops L1 and L3 in the first FnIII domain and by loop L6 in the second. The linker L4 connecting the two FnIII domains is buried underneath the contacting loops and is not accessible to the cytokine ligand. Sequence comparison of the loops in the elbow region of the CHR of IL-4Rα, hGHbp, EBP, gp130, and G-CSFbp reveals a high diversity in length as well as amino acid composition. This reflects the specificity of cytokine recognition as well as different binding modes, that is, low- or high-affinity binding and AC- or AD-face interaction.

Since the three-dimensional structure for the free IL-4Rα chain has not been determined so far, we can only speculate about possible changes of the structure of the receptor upon binding to its ligand. However, for two other members of the cytokine receptor superfamily, erythropoietin receptor (EPOR) and gp130, the structures for the free receptor as well as for the receptor in complex with the ligand have been determined. The structure of the ligand-binding domain (CHR) of the receptor gp130, which is shared among IL-6, CNTF, LIF, oncostatin, and IL-11, has been determined in free form and bound to its ligand IL-6 [85,86]. The super-

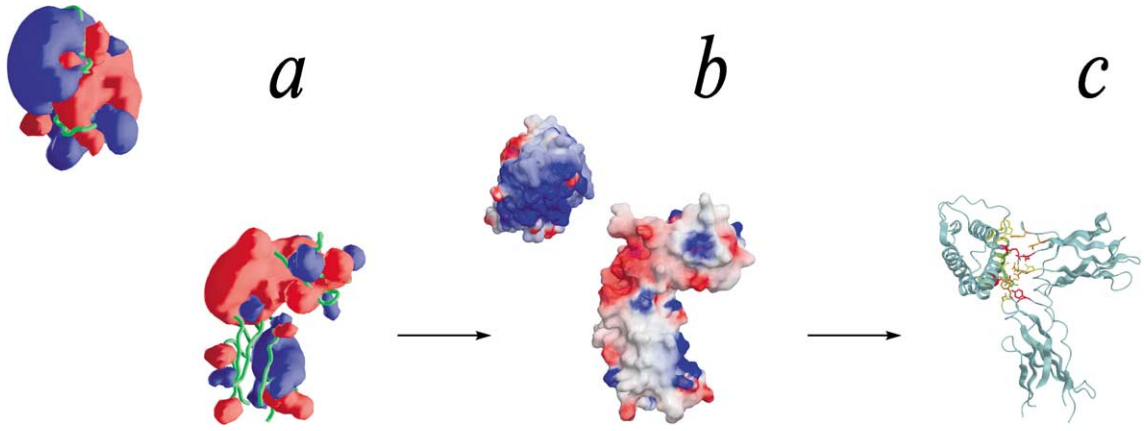

Fig. 4. Electrostatic steering is the cause for an extremely fast association. The strong complementary charge distribution of the ligand IL-4 and the receptor ectodomain IL-4Rα lead to an electrostatic steering effect. At first, the strong dipoles of both molecules cause a "pre-alignment" (a), so that the dipoles of both molecules are aligned parallel (b). The pre-oriented molecules then associate with a very fast on rate (b) to yield the final complex (c).

imposed coordinates for the free and bound form of the gp130 molecule show almost no change in either the side chain conformation or in the hinge angle between the FnIII modules, suggesting that the structural motif of cytokine receptors is rather rigid. However, a detailed analysis of the structure of free gp130 reveals some possible bias; two receptor molecules form a kind of dimer in the crystal lattice with major contacts in the binding region of IL-6. One might therefore speculate that this dimer simulates the binding of IL-6, with the consequence that the similarity of the free and bound form of gp130 could be actually caused by a crystal packing artefact. The structure of the erythropoetin receptor (CHR) was also determined in free and bound (to erythropoietin and also to an EPO mimetic) conformation [87–89]. Similarly to gp130, the structures for the CHR in its free and bound form can be superimposed yielding deviations for the coordinates of the backbone atoms of less than 1 Å. The major differences are in the conformation of a few side chains in the binding region, probably due to plasticity to allow for adaption to the different ligands (native EPO and peptide mimetic). From these two examples, it must be assumed that the CHR is a rather rigid motif in general. The NMR studies, which could give a direct insight into the dynamical behaviour of the modular motif, focus unfortunately on isolated FnIII modules of the receptor ectodomains and thus cannot answer the question of flexibility between the two FnIII modules [90,91]. In the case of the IL-4:IL-4Rα CHR complex, multiple interactions between side chains of the two FnIII domains and the compact packing of the loops in the hinge region also suggest that the CHR of IL-4Rα acts rather like a rigid body [81].

The charge distribution of IL-4Rα CHR presented in Fig. 3b shows a concentration of acidic negatively charged residues in the elbow region which forms the contact with IL-4. Mutational analysis and model calculations indicate that these complementary charge patterns enhance the affinity of the complex of IL-4 and IL-4Rα CHR by about 10-fold due to an increase in the on-rate constant of complex formation [82,92]. The dramatic effect of electrostatic steering becomes obvious when the association rate for hGH and its receptor is compared to the much faster association for the IL-4/IL-4Rα CHR system. The hGH-induced homodimerization of its receptor chains requires a symmetrical charge distribution, which leads to two possible encounter complexes [82]. In addition, with the lower overall charge, the lack of a strong dipole character results in a much lower on-rate of 3.5×10^{-5} M^{-1} s^{-1} [82,93,94].

3.3. High-resolution analysis of the binding epitopes in the IL-4/IL-4Rα CHR complex

Large contact areas of IL-4 and IL-4Rα CHR comprising 17–18 residues and an area of more than 1000 $Å^2$ in each protein become buried upon complex formation. The landscapes of the structural epitopes as established by X-ray analysis of the complex reveal knobs and holes on both sides of the interface, as well as a mixed pattern of charged, polar, and hydrophobic residues [81] (Fig. 5a–d). The hGH/hGHbp paradigm would have predicted that the hydrophobic side chains V69 and Y127 of the IL-4 receptor, which protrude at the center of the contact area, represent a hot spot of hydrophobic binding energy [94–96]. The central position of both residues in the loop L2 and L5 corresponds to the location of the central binding determinants W104 and W168 in hGHbp [95]. Mutational analysis of both IL-4 and IL-4Rα CHR proved, however, that this prediction is wrong [92]. Mutation of the residues V69 and Y127 of IL-4Rα CHR to alanine reduced the binding affinity to IL-4 only about 50-fold. A much larger decrease in affinity ranging from 200- to 1000-fold was found when either residue D72 or Y183 was mutated (Fig. 5d). The receptor mutants D72A and D72N exhibit dissociation constants K_d that are 1500- and 2200-fold larger than that of the wild-type IL-4Rα CHR. This indicates that the negatively charged carboxylate group of D72 is required for binding (Fig. 6a). Substitution of residue Y183 in the mutants Y183A and Y183F results in a 500- and 200-fold increase in K_d, indicating the importance of the tyrosine hydroxyl group for IL-4 binding (Fig. 6b).

Mutational analysis of IL-4 confirmed that two polar interactions provide most of the binding energy in the IL-4/IL-4Rα CHR complex. The side chains of the IL-4 residues E9 and R88 are the main binding determinants [97] (Fig. 5c). Disruptive mutations in the variants E9Q, R88A, or R88Q lead to a 100- to 200-fold reduced binding affinity, respectively [65,97]. X-ray analyses of the free mutant proteins E9A [98] and R88Q [78] prove that there are no structural alterations causing the decrease in binding affinity. The crystal structure of the complex reveals that residue R88 of IL-4 can form an ion pair with residue D72 of IL-4Rα CHR (Fig. 6a), and residue E9 of IL-4 can accept three hydrogen bonds from the receptor residues Y183, Y13 (hydroxyl groups) and S90 (main chain amide group) (Fig. 6b) [81].

The combined mutational and structural analysis also explains the large contribution of these two residues to the interaction of IL-4 and IL-4Rα CHR (hot spots of binding energy). Both interacting side chains of E9 and R88 are surrounded by a shell of minor binding determinants that are mainly hydrophobic (Fig. 5a,b). This kind of binding motif has been termed ‘‘avocado cluster’’ [81], since an avocado fruit has also a core that is surrounded by a hydrophobic layer. The hydrophobic shell of ‘‘avocado cluster’’ I consists of the residues I5, K12, T13, and N89 of IL-4 and residues Y13, A71, Y183, and Y127 of IL-4Rα CHR (Fig. 5a,b). The core is the hydrogen bond network emanating from the carboxylate group of IL-4 E9 (Fig. 6b). In ‘‘avocado cluster’’ II, the ion pair between IL-4 R88 and residue D72 of IL-4Rα CHR is shielded by residues R53, Y56, and W91 of IL-4 and residues L39, F41, and V69 of the IL-4 receptor α-chain (Figs. 5a,b and 6b). It is therefore conceivable that the polar interactions are reinforced by the low dielectricity of the microenvironment. Also, it is interesting to note that the contribution in binding energy does not correlate with the contribution of

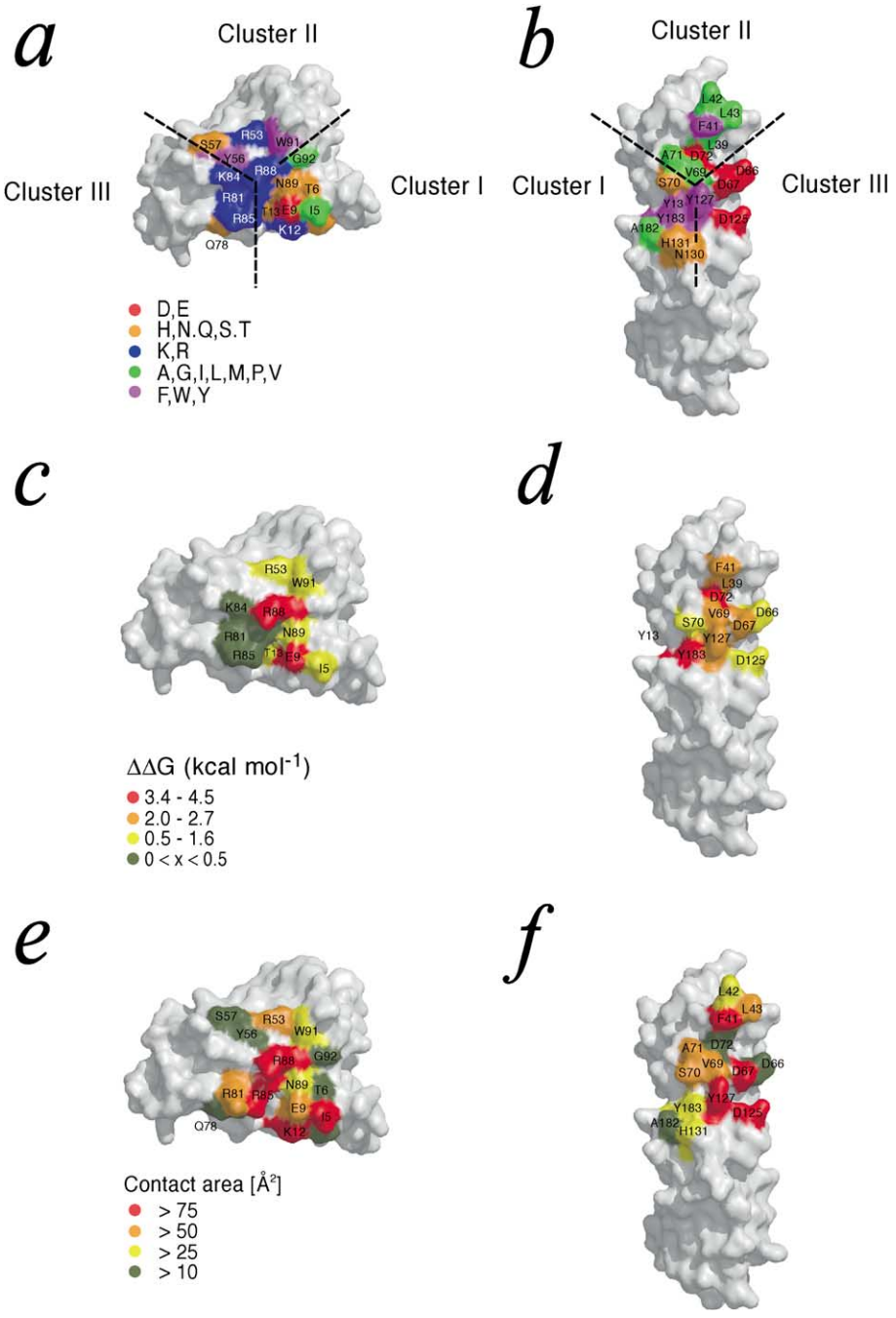

Fig. 5. Comparison of structural and functional epitope. The surface representation for the structural epitopes for IL-4 (a) and IL-4Rα CHR (b) is shown. Negatively charged residues are shown in red (Asp, Glu), positive ones in blue (Arg, Lys), green color represents hydrophobic amino acids (Ala, Gly, Ile, Leu, Val), aromatic residues in magenta (Phe, Tyr, Trp), polar residues are shown in orange (Asn, Gln, His, Ser, Thr). The structural epitope can be divided into three ''avocado clusters''. The IL4:IL-4Rα CHR complex can be obtained by rotating the ligand IL-4 around the *y*-axis by 180°, so that equally numbered clusters are on top of each other. In comparison to the structural epitopes, the functional epitopes are shown in (c) and (d). The energy contribution of each residue was determined by exchange of the respective residue to alanine and measurement of the binding capabilities of the mutant using BIAcore methodology. The color code for the energy contribution is given in (c). (e,f) The residues of IL-4 and IL-4Rα CHR are color-coded according to their contribution to the surface area in the contact interface. As can be seen clearly, contribution of a large area does not correlate with the binding energy supplied by the respective residue. Residues E9 (cluster I) and R88 (cluster II) are clearly the main determinants for the binding energy, despite of the fact that the amount of surface area contributed by residue E9 is smaller than for other less ''important'' residues, for example, I5, K12, or R85.

surface area by a particular residue (Fig 5e,f). Several residues, for example, I5, K12, R81, and R85 that are a major part of the contact interface contribute only little (about 0.2–1.6 kcal mol^{-1}) to the total binding energy of about 13.5 kcal mol^{-1}. The probable cause could be either in the entropy cost of fixing flexible side chains or in the nature of the inter-

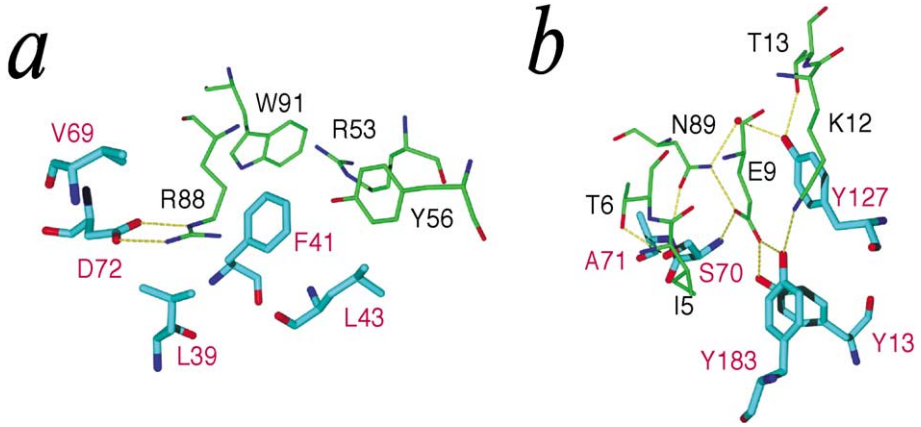

Fig. 6. Hydrogen bonding pattern for the "hot spots of binding". (a) Residue R88 forms a bi-dentate salt bridge with residue D72 of IL-4Rα CHR. The shielding of the salt bridge by mainly hydrophobic residues and thus the reinforcement of these hydrogen bonds explains the high energy contribution of this residue. (b) Similarly, residue E9 of IL-4 is showing a complex hydrogen bond pattern. Intramolecular hydrogen bonds of the carboxylate group of E9 with residue N89 might pre-orient the side chain of E9 for the intermolecular hydrogen bonds. The carboxylate group of E9 is then capable of forming intermolecular hydrogen bonds with the main chain amide of S70 and the hydroxyl groups of the tyrosines Y13 and Y183 of IL-4Rα CHR. In addition, K12 of IL-4 can also form a hydrogen bond with the hydroxyl group of Y13 of IL-4Rα CHR. The large number of possible hydrogen bonds involving residue E9 explains the enormous energy contribution of this single residue.

actions formed, for example, hydrophobic contacts and hydrogen bonds.

In conclusion, high-resolution structural and functional analysis of the IL-4/IL-4Rα CHR interface reveals a sophisticated anatomy and substructure of the binding epitopes. A total of three different types of binding elements can be discriminated. First, charge complementarity contributes a factor of about 10 to the affinity constant K_A (1–1.5 kcal mol^{-1} to the free energy of binding) by increasing the rate of association. Second, "avocado cluster" I contributes a factor of 10^4- to 10^5-fold to the affinity constant K_d (about 6 kcal mol^{-1}). Third, about the same contribution is made by "avocado cluster" II. Assuming that the contributions add up linearly, the sum of all three binding elements would yield a total free energy of binding of 13.5 kcal mol^{-1}, corresponding to a dissociation constant K_d of 100 pM. This value is in remarkably good agreement with the observed K_d of 100–200 pM for IL-4/IL-4Rα binding. Recently, it has been shown that the binding affinity of residues in the two different "avocado clusters" of IL-4 is indeed additive, whereas the binding affinity of residues within one cluster is cooperative [92]. Receptor double mutants with mutations in each cluster exhibit a decrease in binding affinity that corresponds to the sum of the decrease found for the respective single mutants. In contrast, double mutations within one cluster did not show additivity of the contributions found for the single mutants.

3.4. Binding of the second receptor chain

3.4.1. IL-4 utilizes two different low-affinity receptor chains

The high-affinity binding of IL-4 to the receptor IL-4Rα chain has been analyzed in detail. Structural (contact area) and functional (energy contribution of individual residues) epitopes are well characterized. Thermodynamic and kinetic parameters of IL-4 interaction with IL-4Rα CHR are almost identical to results from experiments utilizing the complete IL-4Rα receptor expressed on whole cells. However, binding of the second receptor subunit that is either γc [99,100] or IL-13Rα1 [101–104] is more difficult to analyze. For cells expressing only γc or IL-13Rα, no binding of IL-4 can be observed even at concentrations as high as 100 nM [105]. The receptor subunit IL-4Rα would be fully saturated at this ligand concentration, considering the small dissociation constant of 100 pM for the IL-4/IL-4Rα interaction. The binding affinity of the γc ectodomain to IL-4 was determined using surface plasmon resonance technology to about 150 μM. The affinity is increased by a factor of 50 if γc interacts with the high-affinity complex of IL-4 and IL-4Rα CHR, yielding a dissociation constant K_d of approximately 3.5–5 μM [80].

Although these results prove that IL-4 can bind directly to γc, it has to be considered that subnanomolar concentrations of IL-4 are quite sufficient for biological activity. These concentrations are much too low to allow direct binding of solute IL-4 to γc under physiological conditions. It was therefore proposed that the ligand-induced dimerization of the receptor subunits proceeds in a two-step mechanism. In the first step, the solute IL-4 binds to the membrane-anchored ectodomain of IL-4Rα after a three-dimensional search reaction. In the second step, the low-affinity subunit γc performs a two-dimensional search on the surface of the membrane to find and bind to the high-affinity complex of IL-4:IL-4Rα. The weak interaction of γc with the intermediate complex IL-4:IL-4Rα in the micromolar range, as was measured by BIAcore experiments, seems puzzling at first sight, considering that full biological activity is observed at concentrations as low as 200 pM for the ligand. However, a likely explanation for this contradiction is the sequential nature of the activation mechanism. The very weak affinity of $K_d = 5$ μM as determined by the

BIAcore experiments is likely to be an artificial result of measurement procedure. In reality, the second binding event is not a three-dimensional search as it is in the in vitro measurement but a two-dimensional search on a surface. Subsequently, a smaller number of translational and rotational degrees of freedom shall lead to an increased binding affinity in the in vivo (membrane-anchored) situation. The rate of productive collisions is much larger due to one less degree of freedom of the diffusion. Additionally, the ''local concentration'' of IL-4 on the cell surface is increased upon binding of IL-4 to the IL-4Rα chain as compared to the concentration in solution. A density of about 1000 receptor molecules on a cell surface with a surface area of about 100 μm^2 is comparable to a concentration of about 50 nM in solution. The apparent concentration of IL-4 on the cell surface is therefore much higher than estimated from the serum level. In addition, binding of the second receptor subunit to the 1:1 complex of ligand and high-affinity receptor subunit is limited to a two-dimensional search in the plane of the membrane. These considerations may explain why a relatively low affinity of the second receptor subunit is still sufficient to achieve receptor dimerization.

3.4.2. Selective antagonists of IL-4

So far, no experimental structural data are available for the low-affinity complexes of IL-4/IL-4Rα CHR with either the γc or the IL-13Rα1 receptor chain. However, extensive mutagenesis studies of IL-4 and also of the γc receptor chain give insights into the mechanism of recognition and binding of IL-4 to its low-affinity receptor subunits [106] (T. Hom and

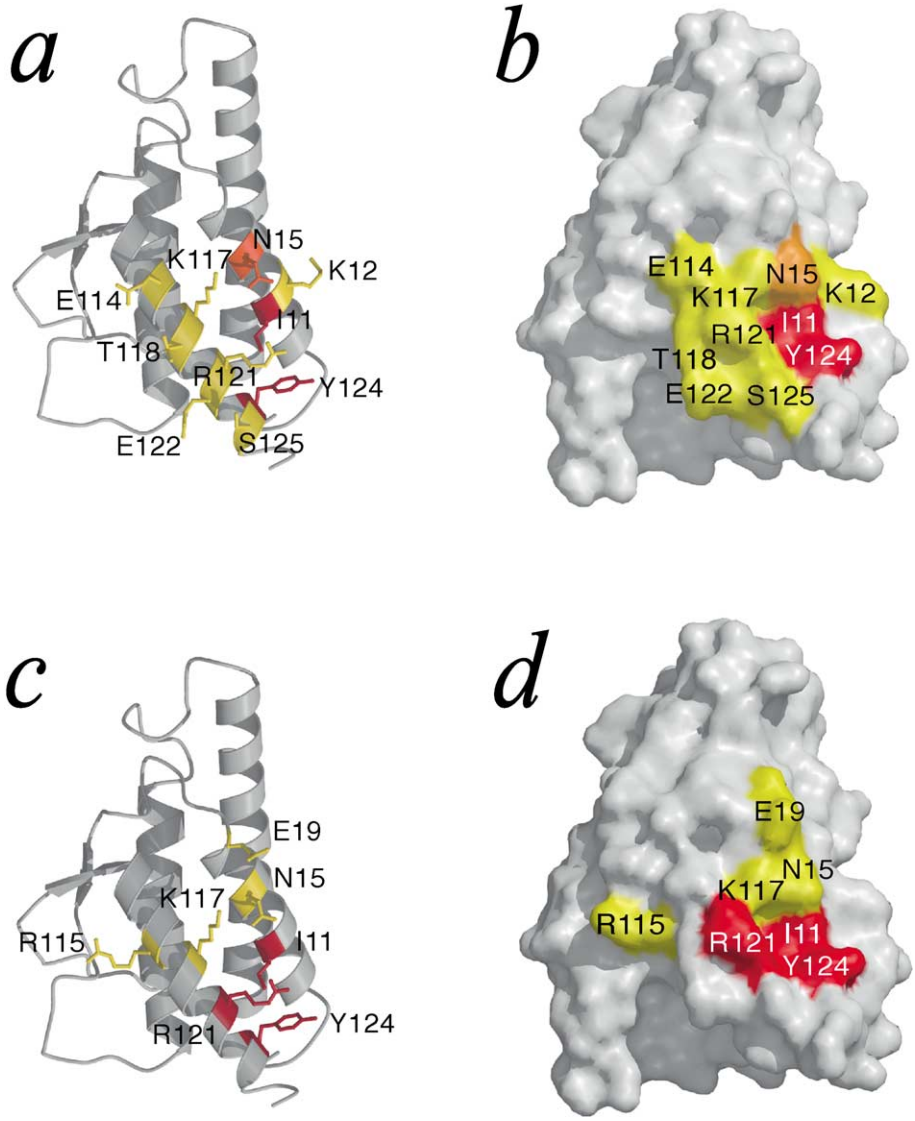

Fig. 7. Selective antagonists for γc and IL-13Rα. Ribbon (a) and surface (b) representation of IL-4. The color-coding represents the loss of binding affinity to the γc receptor upon mutation to alanine. Red color is used for residues for which mutation to alanine results in almost total loss of binding, whereas orange and yellow indicate a significant and small change in binding affinity, respectively. (c,d) Same as in (a,b) but for the binding of IL-4 to IL-13Rα. Although the epitopes are similar in size and location, there are significant differences in utilizing respective residues for the recognition. The main binding determinants of IL-4 in binding to γc are the residues I11 and Y124, whereas for binding of IL-4 to IL-13Rα, residue R121 is also absolutely required.

W. Sebald, unpublished). For the ligand IL-4, antagonists have been discovered which can selectively inhibit either signal transduction through the γc (Fig. 7a,b) [65,80] or the IL-13Rα1 chain (Fig. 7c,d) [107,108] (T. Hom and W. Sebald, unpublished). The first partial antagonistic mutant of IL-4 was described in 1991 [109]. Residue Y124 of IL-4 at the C-terminal end of the fourth helix was replaced with aspartate, resulting in a mutant protein capable of competing for binding to IL-4Rα with wild-type IL-4, but reduced to about 5% of wild-type activity [64]. Mutating further residues around Y124 also led to partially antagonistic behaviour, for example, residues R121 and S125 [65]. The double mutant R121D/Y124D has been shown to be the most effective antagonist for the interaction with γc, while retaining almost complete binding affinity for IL-4Rα [50]. Structure analyses for the partial antagonists Y124D and Y124G proved that the antagonistic effect is not mediated through a significant change in structure or loss in structural integrity but rather through an exchange of the side chain functionality [110]. The observation that negatively charged residues are most effective in creating an antagonistic mutant supports the theory of introduction of an electrostatic mismatch in the binding interface. A more detailed study using alanine replacement revealed that several residues are used to discriminate for the binding to either γc or IL-13Rα1 (T. Hom and W. Sebald, unpublished).

The epitope of IL-4 used for binding to γc comprises residues I11, K12, N15 on helix A and residues E114, K117, T118, R121, E122, Y124, and S125 on helix D (Fig. 7a,b). Only three residues are showing a larger decrease in binding affinity upon mutation to alanine, namely, I11, N15, and Y124. However, if the interaction with IL-13Rα1 is analyzed, differences in the contribution of individual residues are observed, although the size of the functional epitope is very similar, and in both cases, the dissociation constant is roughly 5 μM. While mutation of residue R121 to alanine does not alter the affinity to the receptor chain γc to any large extent, this residue is of significant importance for the interaction with IL-13Rα (Fig. 7c,d). The interaction of N15 with either receptor subunit is also of different strengths, although not to the extent as seen for R121. The observation that several residues contribute differently to the binding affinity of the two low-affinity receptor subunits suggests that recognition and binding of both receptors subunit is different in detail [107,108] (T. Hom and W. Sebald, unpublished).

3.4.3. The binding epitope of a low-affinity receptor subunit

The binding site of IL-4 on γc was determined by mutagenesis [106]. Comparison of the IL-4 epitope responsible for the high-affinity interaction with IL-4Rα with the epitope determined for the interaction with either γc or IL-13Rα1 suggests that the mechanism of recognition is probably very different. For the IL-4/IL-4Rα interaction, two charged residues, E9 and R88, are contributing almost all of the binding energy, whereas for the much weaker interaction of IL-4 with either γc or IL-13Rα two hydrophobic residues, I11

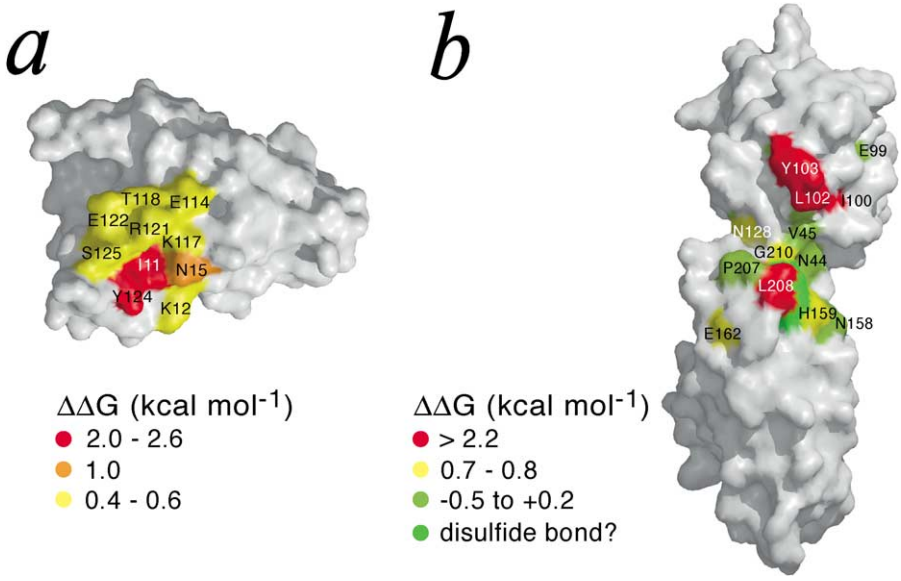

Fig. 8. Binding epitope for γc. Surface representation of IL-4 (a) and a theoretical model of γc (b) color-coded by the loss of binding affinity upon mutation to alanine. (a) is identical to Fig. 7b, but shown additionally on that panel to facilitate a comparison of the ligand and receptor epitopes. Residues I11 and Y124 contribute 2–2.6 kcal mol^{-1} each for the interaction with the receptor subunit γc. (b) Receptor variants I100A, L102A and Y103A in loop L3 as well as L208A in loop L6 show the largest decrease in binding affinity upon substitution by alanine. An even larger loss in affinity is observed when either cysteine residues C160 or C209 are mutated, but model building suggests a disulfide bridge between these two cysteine residues which would stabilize the loop conformation of loops L5 and L6. Therefore, mutation of either one cysteine is likely to cause structural perturbations, resulting in an indirect loss of binding affinity. Figs. 1–8 were generated using the programs Molscript [128], Jalview (http://www2.ebi.ac.uk/~michele/jalview/contents.html), Raster3D [129], DINO (http://www.dino3d.org) and Grasp [130].

and Y124, are essential (Figs. 7a,b and 8a). Using alanine replacement, four residues, I100, L102, Y103 (loop L2), and L208 (loop L6), were identified as major binding determinants (Fig. 8b). Double mutation cycle analysis allowed to determine possible interaction pairs, indicating that residues in the loop L2 (I100, L102, Y103) probably interact with residues Y124 and S125 of IL-4 and residues in the loops L6 (L208, G210) and L5 (L161) probably interact with residues I11, N15, and R121 of IL-4 (Fig. 8a,b).

The binding determinants of γc for IL-4 appear to follow the rules for the hydrophobic hot spot of binding as seen for hGHR [95]. The key residues in hGHR required for binding of the ligand are W104 and W169 in the loops L3 and L5. Similar clusters of hydrophobic determinants have been found in several other type I cytokine receptors, for example, hEPOR [88], gp130 [111], and hβc (human common β chain) [112,113]. Two mutant proteins of γc, C160A and C209A, also showed a dramatic loss in binding affinity (K_d>500 μM). However, model building suggests that C160 and C209 form a disulfide bridge that would stabilise the loop conformation of the binding loops L6 and L5 (T. Müller, PhD thesis, 1995) [106,114]. The observed decrease in binding affinity for the two cysteine mutants is therefore likely to be a result of structural perturbations, although a participation in direct binding cannot be ruled out.

Since the γc receptor subunit is shared by the cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [28,29], it is interesting to know whether the residues identified for the IL-4/γc interaction are also playing a similar role in binding the other cytokines. Residue Y103 was shown to participate in binding of IL-2 and IL-7, although mutation of the tyrosine to either alanine or arginine did not exhibit a similarly large effect as found for IL-4 [115]. Further residues of γc, I100, L102, and L208, which are all important for the interaction with IL-4, did not show any significant contribution to the IL-2 and IL-7 interaction in this particular study. However, using the genetic information reported for X-SCID patients [116], missense mutations of γc are reported for the residues Y103, L161, L208, and G210, indicating that residues other than Y103 are also playing an important role in binding of IL-2, IL-7, and IL-15.

4. IL-4 receptor antagonists in vitro, in vivo and in the clinic

4.1. Cellular effects of antagonistic IL-4 mutants

Antagonistic IL-4 mutants were initially identified by their loss of proliferative activity despite seemingly intact receptor binding. It was subsequently found that mutants with such a behaviour actually prevented the induction of cell proliferation by wild-type IL-4, and their antagonistic potential was recognized [64,65]. Other cellular responses were later shown to be blocked as well, including phosphorylation of IL-4Rα [117] and Stat6 [107], expression of CD23 [50,59], and class switching to IgE [59,118,119]. Inhibition of IL-4/IL-13-dependent responses by IL-4 antagonists was shown for a wide variety of cell types, since functional IL-4 receptors are essentially ubiquitously expressed. The available data indicate that IL-4 antagonists block all IL-4- and IL-13-dependent responses in all cell types. This corresponds well with results from transgenic mice lacking IL-4Rα, which also lacked responsiveness toward these two cytokines [37–43].

4.2. Animal studies and clinical trials

The value of IL-4 antagonists has been demonstrated in mice, where the application of antagonists created by mutating murine IL-4 prevented the development of allergic reactivity [39]. Typical allergic responses like serum titers for antigen-specific IgE and IgG1, local cutaneous anaphylaxis upon intradermal delivery of antigen, and systemic anaphylactic shock upon intravenous application of the antigen were absent if mice had been treated with the antagonistic mouse IL-4 variant Q116D/Y119D along with antigen during the sensitization protocol [39].

Another murine IL-4 antagonist was created by deleting the C-terminal amino acids following Q119 [69]. This antagonist was assayed in an asthma model, where it inhibited IL-4/IL-13-induced Stat6 phosphorylation and IL-4/IL-13-induced IgE production in a splenocyte culture in vitro. Administration during antigen challenge inhibited the development of airway eosinophilia and acute hyperresponsiveness. Levels of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid were reduced, as were serum levels of specific IgE [69]. These data confirm findings of decreased bronchial hyperresponsiveness and reduced infiltration of eosinophils following the application of a human IL-4 antagonist in a monkey (*Macaca fasciculata*) model of allergic asthma (R. Gundel, W. Sebald et al., unpublished).

The antagonistic IL-4 mutant R121D/Y124D has now entered a phase II clinical trial where it is evaluated for the treatment of allergic asthma. The positive results from the animal studies suggest that there is hope for therapeutic benefits in humans as well.

5. What do we learn from IL-4/IL-13 antagonists for other cytokines?

Activation of cytokine receptors is achieved by homo- or heterodimerisation of receptor chains in the cell membrane. In some cases, for example, for IL-2 and IL-6, a third receptor chain is used to enhance the affinity for the ligand. So far for all cytokine systems studied, the mechanism of activation has been shown to be sequential. The first step involves the binding of the ligand to its high-affinity receptor, and in the second step, this intermediate complex interacts with the low-affinity receptor chain, leading subsequently to signal transduction into the cell.

Does this mechanism allow us to generate antagonistic variants in a general manner by blocking the second interaction, as demonstrated with the IL-4 receptor antagonists? One major obstacle is in the binding affinity of the ligand and its high-affinity receptor chain compared to the affinity for the whole receptor complex. Although most cytokine receptors bind their respective ligands with an affinity of $K_d = 100-500$ pM, the total affinity of binding is rarely dominated by one receptor subunit as it is for IL-4. Almost all of the binding affinity ($K_d = 100$ pM) is generated by the interaction between IL-4 and its high-affinity receptor chain IL-4Rα. Super-antagonistic mutants that do not bind to γc at all still exhibit a binding affinity of $K_d = 200-300$ pM for IL-4Rα, which is only lower by a factor 2–3 than for the wild-type protein. This allows an effective competition of wild-type activity with the antagonistic mutant. However, in most cases, the ligand binds to the high-affinity chain with an affinity 100 times lower than was measured for the whole receptor complex on the cell. Only the additional association with the low-affinity receptor chain then yields the full-binding strength.

For example, IL-3 binds to its receptor α-chain with a $K_d = 20-100$ nM, while upon association with the common beta chain βc shared between GM-CSF, IL-3, and IL-5, the affinity is increased to $K_d = 100-500$ pM [120]. Similar differences also occur in the γc family. Since the binding affinity of IL-2 to the IL-2Rβ chain is low, effective antagonistic variants of IL-2 have not been developed so far, although structure and sequence comparison of IL-2 and IL-4 suggest a similar binding interface to γc. A possible solution is the development of so-called super-agonists, which bind to their first receptor chain with a much higher affinity. Such mutants have been found in mutagenesis studies for hGH [121], IL-6 [122,123], and IL-4 [92]. The difficulty in finding such super-agonists is that even with knowledge of the structure of the ligand–receptor complex, a rational design or prediction of such mutants has so far not been achieved. Nevertheless, it should be possible to transfer the principle of antagonistic inhibition from IL-4 to other cytokines by using a combination of super-agonistic and antagonistic mutations. Shanafelt et al. [124] have shown another nice example of "molecular medicine" by designing a cell-selective IL-2 variant. IL-2 has a great potential as antitumor therapeutic, but systemic toxicity is a limiting factor in its application. However, the toxicity effect is mainly confined to the IL-2 activity on NK cells, which express the intermediate affinity complex comprising IL-2Rβ and γc, whereas the tumor-suppressing activity is mediated by T-cells, which express the high-affinity receptor consisting of IL-2Rα, IL-2Rβ, and γc. By elegantly modulating the affinity of IL-2 to its IL-2Rβ chain, a mutant IL-2 was designed that preferentially binds to the high-affinity complex [124]. Since this receptor complex resides almost exclusively on the desired target T cells, the application-limiting toxicity could be reduced.

6. Structure/function analysis and the prospect for small molecular mimetics

The development of antagonistic variants of IL-4 that are now in clinical phase II trials is a nice example for the power of structure/function analysis and its value in molecular medicine. The knowledge of the structure of the ligand IL-4 in its free form as well as the structure of the ligand–receptor complex IL-4:IL-4Rα CHR, together with the information about the energy contribution of each individual residue, enabled us to rationally design mini-proteins that mimic the binding of IL-4 to its IL-4Rα chain. The epitope of IL-4 for the high-affinity interaction with its α-chain is located on two antiparallel helices and occupies an area of about 800 $Å^2$. By model building, this motif was placed onto the parallel α-helices of the GAL4 leucine zipper [125] and the newly designed peptide was synthesized. The synthetic peptides exhibited binding to IL-4Rα with a K_d of about 5 μM. Although the affinity would be far too low for therapeutic purposes, the design of such synthetic peptides clearly shows that it is in principle possible to design inhibiting analogs that are highly different from the wild-type protein.

Using peptide libraries and in vitro evolution, a dimeric peptide-mimetic for erythropoietin was developed which exhibits agonistic activity in the nanomolar concentration range [88,126,127]. These results are encouraging, since the large and relatively flat binding interfaces are very challenging for the classical computational drug design. However, minimizing the protein size is not sufficient, as peptides will still exhibit similar pharmacological problems, for example, concerning oral application and development of immune responses. Small mimetics of IL-4 that are capable to bind with high affinity to IL-4Rα and compete effectively for IL-4 and IL-13 would be ideal drugs. The modular architecture of the IL-4/IL-4Rα CHR complex with the epitope consisting of three independently binding clusters suggests an approach where separate ligands for each cluster are being developed. Combining these low-affinity ligands in one molecule might then generate a high-affinity ligand due to adding up of their binding energies as seen in the clusters of the native protein ligand IL-4.

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References

[1] D. Diaz-Sanchez, Immunology 101 (2000) 11–18.
[2] G. Folkerts, G. Walzl, P. Openshaw, Immunol. Today 21 (2000) 249–250.
[3] O. Strannegard, I.L. Strannegard, Allergy 56 (2001) 91–102.
[4] D.Y. Leung, Mol. Genet. Metab. 63 (1998) 157–167.
[5] E. Maggi, Immunotechnology 3 (1998) 233–244.

[6] K. Nelms, A.D. Keegan, J. Zamorano, J.J. Ryan, W.E. Paul, Annu. Rev. Immunol. 17 (1999) 701–738.
[7] M. Worm, B.M. Henz, J. Mol. Med. 75 (1997) 440–447.
[8] L.B. Bacharier, H. Jabara, R.S. Geha, Int. Arch. Allergy Immunol. 115 (1998) 257–269.
[9] R.P. Schleimer, S.A. Sterbinsky, J. Kaiser, C.A. Bickel, D.A. Klunk, K. Tomioka, W. Newman, F.W. Luscinskas, M.A. Gimbrone Jr., B.W. McIntyre, et al., J. Immunol. 148 (1992) 1086–1092.
[10] B. Schnyder, S. Lugli, N. Feng, H. Etter, R.A. Lutz, B. Ryffel, K. Sugamura, H. Wunderli-Allenspach, R. Moser, Blood 87 (1996) 4286–4295.
[11] C. Doucet, D. Brouty-Boye, C. Pottin-Clemenceau, G.W. Canonica, C. Jasmin, B. Azzarone, J. Clin. Invest. 101 (1998) 2129–2139.
[12] S. Romagnani, Ann. Allergy, Asthma, & Immun. 85 (2000) 9–18 (quiz 18, 21).
[13] M. Mochizuki, J. Bartels, A.I. Mallet, E. Christophers, J.M. Schroder, J. Immunol. 160 (1998) 60–68.
[14] N. Zimmermann, S.P. Hogan, A. Mishra, E.B. Brandt, T.R. Bodette, S.M. Pope, F.D. Finkelman, M.E. Rothenberg, J. Immunol. 165 (2000) 5839–5846.
[15] J. Hoeck, M. Woisetschlager, J. Immunol. 166 (2001) 4507–4515.
[16] S.C. Bischoff, G. Sellge, A. Lorentz, W. Sebald, R. Raab, M.P. Manns, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8080–8085.
[17] T. Hara, K. Yamada, H. Tachibana, Biochem. Biophys. Res. Commun. 247 (1998) 542–548.
[18] J.E. de Vries, J. Allergy Clin. Immunol. 102 (1998) 165–169.
[19] D.B. Corry, Curr. Opin. Immunol. 11 (1999) 610–614.
[20] M. Wills-Karp, J. Luyimbazi, X. Xu, B. Schofield, T.Y. Neben, C.L. Karp, D.D. Donaldson, Science 282 (1998) 2258–2261.
[21] L. Li, Y. Xia, A. Nguyen, Y.H. Lai, L. Feng, T.R. Mosmann, D. Lo, J. Immunol. 162 (1999) 2477–2487.
[22] Z. Zhu, R.J. Homer, Z. Wang, Q. Chen, G.P. Geba, J. Wang, Y. Zhang, J.A. Elias, J. Clin. Invest. 103 (1999) 779–788.
[23] A. Heinzmann, X.Q. Mao, M. Akaiwa, R.T. Kreomer, P.S. Gao, K. Ohshima, R. Umeshita, Y. Abe, S. Braun, T. Yamashita, M.H. Roberts, R. Sugimoto, K. Arima, Y. Arinobu, B. Yu, S. Kruse, T. Enomoto, Y. Dake, M. Kawai, S. Shimazu, S. Sasaki, C.N. Adra, M. Kitaichi, H. Inoue, K. Yamauchi, N. Tomichi, F. Kurimoto, N. Hamasaki, J.M. Hopkin, K. Izuhara, T. Shirakawa, K.A. Deichmann, Hum. Mol. Genet. 9 (2000) 549–559.
[24] P.G. Fallon, C.L. Emson, P. Smith, A.N. McKenzie, J. Immunol. 166 (2001) 2712–2716.
[25] G. Grunig, M. Warnock, A.E. Wakil, R. Venkayya, F. Rennick, D.M. Rennick, D. Sheppard, M. Mohrs, D.D. Donaldson, R.M. Locksley, D.B. Corry, Science 282 (1998) 2261–2263.
[26] K. Blease, C. Jakubzick, J. Westwick, N. Lukacs, S.L. Kunkel, C.M. Hogaboam, J. Immunol. 166 (2001) 5219–5224.
[27] A. Minty, P. Chalon, J.M. Derocq, X. Dumont, J.C. Guillemot, M. Kaghad, C. Labit, P. Leplatois, P. Liauzun, B. Miloux, et al., Nature 362 (1993) 248–250.
[28] K. Sugamura, H. Asao, M. Kondo, N. Tanaka, N. Ishii, K. Ohbo, M. Nakamura, T. Takeshita, Annu. Rev. Immunol. 14 (1996) 179–205.
[29] H. Asao, C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, K. Sugamura, J. Immunol. 167 (2001) 1–5.
[30] S.M. Russell, A.D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M.C. Friedmann, A. Miyajima, R.K. Puri, W.E. Paul, et al., Science 262 (1993) 1880–1883.
[31] M. Noguchi, H. Yi, H.M. Rosenblatt, A.H. Filipovich, S. Adelstein, W.S. Modi, O.W. McBride, W.J. Leonard, Cell 73 (1993) 147–157.
[32] D. Caput, P. Laurent, M. Kaghad, J.M. Lelias, S. Lefort, N. Vita, P. Ferrara, J. Biol. Chem. 271 (1996) 16921–16926.
[33] D.D. Donaldson, M.J. Whitters, L.J. Fitz, T.Y. Neben, H. Finnerty, S.L. Henderson, R.M. O'Hara Jr., D.R. Beier, K.J. Turner, C.R. Wood, M. Collins, J. Immunol. 161 (1998) 2317–2324.
[34] N. Feng, S.M. Lugli, B. Schnyder, J.F. Gauchat, P. Graber, E. Schlagenhauf, B. Schnarr, M. Wiederkehr-Adam, A. Duschl, M.H. Heim, R.A. Lutz, R. Moser, Lab. Invest. 78 (1998) 591–602.
[35] T. Murata, J. Taguchi, R.K. Puri, Blood 91 (1998) 3884–3891.
[36] A. Duschl, Eur. Cytokine Netw. 11 (2000) 521–522.
[37] N. Noben-Trauth, L.D. Shultz, F. Brombacher, J.F. Urban Jr., H. Gu, W.E. Paul, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10838–10843.
[38] M. Barner, M. Mohrs, F. Brombacher, M. Kopf, Curr. Biol. 8 (1998) 669–672.
[39] S.M. Grunewald, A. Werthmann, B. Schnarr, C.E. Klein, E.B. Brocker, M. Mohrs, F. Brombacher, W. Sebald, A. Duschl, J. Immunol. 160 (1998) 4004–4009.
[40] J.F. Urban Jr., N. Noben-Trauth, D.D. Donaldson, K.B. Madden, S.C. Morris, M. Collins, F.D. Finkelman, Immunity 8 (1998) 255–264.
[41] M. Mohrs, B. Ledermann, G. Kohler, A. Dorfmuller, A. Gessner, F. Brombacher, J. Immunol. 162 (1999) 7302–7308.
[42] L. Cohn, R.J. Homer, H. MacLeod, M. Mohrs, F. Brombacher, K. Bottomly, J. Immunol. 162 (1999) 6178–6183.
[43] N. Noben-Trauth, W.E. Paul, D.L. Sacks, J. Immunol. 162 (1999) 6132–6140.
[44] J. Beattie, Biochim. Biophys. Acta 1203 (1993) 1–10.
[45] J.A. Wells, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 1–6.
[46] F.D. Finkelman, I.M. Katona , J.F. Urban Jr., J. Holmes, J. Ohara, A.S. Tung, J.V. Sample, W.E. Paul, J. Immunol. 141 (1988) 2335–2341.
[47] R. Moser, P. Groscurth, J.M. Carballido, P.L. Bruijnzeel, K. Blaser, C.H. Heusser, J. Fehr, J. Lab. Clin. Med. 122 (1993) 567–575.
[48] J.C. Guery, F. Galbiati, S. Smiroldo, L. Adorini, J. Exp. Med. 183 (1996) 485–497.
[49] H. Renz, K. Bradley, K. Enssle, J.E. Loader, G.L. Larsen, E.W. Gelfand, Int. Arch. Allergy Immunol. 109 (1996) 167–176.
[50] H.P. Tony, B.J. Shen, P. Reusch, W. Sebald, Eur. J. Biochem. 225 (1994) 659–665.
[51] A.C. Fluckiger, F. Briere, G. Zurawski, J.M. Bridon, J. Banchereau, Immunology 83 (1994) 397–403.
[52] F.D. Finkelman, K.B. Madden, S.C. Morris, J.M. Holmes, N. Boiani, I.M. Katona, C.R. Maliszewski, J. Immunol. 151 (1993) 1235–1244.
[53] W. Kammer, A. Lischke, R. Moriggl, B. Groner, A. Ziemiecki, C.B. Gurniak, L.J. Berg, K. Friedrich, J. Biol. Chem. 271 (1996) 23634–23637.
[54] S.Y. Lai, J. Molden, K.D. Liu, J.M. Puck, M.D. White, M.A. Goldsmith, EMBO J. 15 (1996) 4506–4514.
[55] M. Reichel, B.H. Nelson, P.D. Greenberg, P.B. Rothman, J. Immunol. 158 (1997) 5860–5867.
[56] M.L. Heaney, D.W. Golde, Blood 87 (1996) 847–857.
[57] T.A. Sato, M.B. Widmer, F.D. Finkelman, H. Madani, C.A. Jacobs, K.H. Grabstein, C.R. Maliszewski, J. Immunol. 150 (1993) 2717–2723.
[58] C.R. Maliszewski, T.A. Sato, B. Davison, C.A. Jacobs, F.D. Finkelman, W.C. Fanslow, Proc. Soc. Exp. Biol. Med. 206 (1994) 233–237.
[59] B. Konig, A. Fischer, W. Konig, Immunology 85 (1995) 604–610.
[60] H. Renz, Inflamm. Res. 48 (1999) 425–431.
[61] T. Jung, K. Wagner, C. Neumann, C.H. Heusser, Eur. J. Immunol. 29 (1999) 864–871.
[62] L.C. Borish, H.S. Nelson, M.J. Lanz, L. Claussen, J.B. Whitmore, J.M. Agosti, L. Garrison, Am. J. Respir. Crit. Care Med. 160 (1999) 1816–1823.
[63] L.C. Borish, H.S. Nelson, J. Corren, G. Bensch, W.W. Busse, J.B. Whitmore, J.M. Agosti, J. Allergy Clin. Immunol. 107 (2001) 963–970.
[64] N. Kruse, H.P. Tony, W. Sebald, EMBO J. 11 (1992) 3237–3244.
[65] N. Kruse, B.J. Shen, S. Arnold, H.P. Tony, T. Muller, W. Sebald, EMBO J. 12 (1993) 5121–5129.
[66] A. Duschl, Eur. J. Biochem. 228 (1995) 305–310.
[67] S.M. Grunewald, S. Kunzmann, B. Schnarr, J. Ezernieks, W. Sebald, A. Duschl, J. Biol. Chem. 272 (1997) 1480–1483.
[68] M. Schnare, H. Blum, S. Juttner, M. Rollinghoff, A. Gessner, J. Immunol. 161 (1998) 3484–3492.
[69] A. Tomkinson, C. Duez, G. Cieslewicz, J.C. Pratt, A. Joetham, M.C. Shanafelt, R. Gundel, E.W. Gelfand, J. Immunol. 166 (2001) 5792–5800.

[70] P. Reinemer, W. Sebald, A. Duschl, Angew. Chem., Int. Ed. Engl. 39 (2000) 2834–2846.
[71] S.R. Sprang, J.F. Bazan, Curr. Opin. Struct. Biol. 3 (1993) 815–827.
[72] D.A. Rozwarski, A.M. Gronenborn, G.M. Clore, J.F. Bazan, A. Bohm, A. Wlodawer, M. Hatada, P.A. Karplus, Structure 2 (1994) 159–173.
[73] R. Powers, D.S. Garrett, C.J. March, E.A. Frieden, A.M. Gronenborn, G.M. Clore, Science 256 (1992) 1673–1677.
[74] L.J. Smith, C. Redfield, J. Boyd, G.M. Lawrence, R.G. Edwards, R.A. Smith, C.M. Dobson, J. Mol. Biol. 224 (1992) 899–904.
[75] M.R. Walter, W.J. Cook, B.G. Zhao, R.P. Cameron Jr., S.E. Ealick, R.L. Walter Jr., P. Reichert, T.L. Nagabhushan, P.P. Trotta, C.E. Bugg, J. Biol. Chem. 267 (1992) 20371–20376.
[76] A. Wlodaver, A. Pavlovsky, A. Gustchina, FEBS Lett. 309 (1992) 59–64.
[77] T. Muller, T. Dieckmann, W. Sebald, H. Oschkinat, J. Mol. Biol. 237 (1994) 423–436.
[78] T. Muller, F. Oehlenschlager, M. Buehner, J. Mol. Biol. 247 (1995) 360–372.
[79] B.J. Shen, T. Hage, W. Sebald, Eur. J. Biochem. 240 (1996) 252–261.
[80] F. Letzelter, Y. Wang, W. Sebald, Eur. J. Biochem. 257 (1998) 11–20.
[81] T. Hage, W. Sebald, P. Reinemer, Cell 97 (1999) 271–281.
[82] E. Demchuk, T. Mueller, H. Oschkinat, W. Sebald, R.C. Wade, Protein Sci. 3 (1994) 920–935.
[83] J.F. Bazan, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6934–6938.
[84] P. Bork, L. Holm, C. Sander, J. Mol. Biol. 242 (1994) 309–320.
[85] D. Chow, X. He, A.L. Snow, S. Rose-John, K.C. Garcia, Science 291 (2001) 2150–2155.
[86] J. Bravo, D. Staunton, J.K. Heath, E.Y. Jones, EMBO J. 17 (1998) 1665–1674.
[87] R.S. Syed, S.W. Reid, C. Li, J.C. Cheetham, K.H. Aoki, B. Liu, H. Zhan, T.D. Osslund, A.J. Chirino, J. Zhang, J. Finer-Moore, S. Elliott, K. Sitney, B.A. Katz, D.J. Matthews, J.J. Wendoloski, J. Egrie, R.M. Stroud, Nature 395 (1998) 511–516.
[88] O. Livnah, E.A. Stura, D.L. Johnson, S.A. Middleton, L.S. Mulcahy, N.C. Wrighton, W.J. Dower, L.K. Jolliffe, I.A. Wilson, Science 273 (1996) 464–471.
[89] O. Livnah, E.A. Stura, S.A. Middleton, D.L. Johnson, L.K. Jolliffe, I.A. Wilson, Science 283 (1999) 987–990.
[90] T. Kernebeck, S. Pflanz, G. Muller-Newen, G. Kurapkat, R.M. Scheek, K. Dijkstra, P.C. Heinrich, A. Wollmer, S. Grzesiek, J. Grotzinger, Protein Sci. 8 (1999) 5–12.
[91] H. Anaguchi, O. Hiraoka, K. Yamasaki, S. Naito, Y. Ota, J. Biol. Chem. 270 (1995) 27845–27851.
[92] J.L. Zhang, I. Simeonowa, Y. Wang, W. Sebald, J. Mol. Biol. 315 (2002) 399–407.
[93] B.C. Cunningham, J.A. Wells, Science 244 (1989) 1081–1085.
[94] B.C. Cunningham, J.A. Wells, J. Mol. Biol. 234 (1993) 554–563.
[95] A.M. de Vos, M. Ultsch, A.A. Kossiakoff, Science 255 (1992) 306–312.
[96] J.A. Wells, Biotechnology (N. Y.) 13 (1995) 647–651.
[97] Y. Wang, B.J. Shen, W. Sebald, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1657–1662.
[98] M. Hulsmeyer, C. Scheufler, M.K. Dreyer, Acta Crystallogr., D Biol. Crystallogr. 57 (2001) 1334–1336.
[99] M. Kondo, T. Takeshita, N. Ishii, M. Nakamura, S. Watanabe, K. Arai, K. Sugamura, Science 262 (1993) 1874–1877.
[100] M. Noguchi, Y. Nakamura, S.M. Russell, S.F. Ziegler, M. Tsang, X. Cao, W.J. Leonard, Science 262 (1993) 1877–1880.
[101] M.J. Aman, N. Tayebi, N.I. Obiri, R.K. Puri, W.S. Modi, W.J. Leonard, J. Biol. Chem. 271 (1996) 29265–29270.
[102] D.J. Hilton, J.G. Zhang, D. Metcalf, W.S. Alexander, N.A. Nicola, T.A. Willson, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 497–501.
[103] J.F. Gauchat, E. Schlagenhauf, N.P. Feng, R. Moser, M. Yamage, P. Jeannin, S. Alouani, G. Elson, L.D. Notarangelo, T. Wells, H.P. Eugster, J.Y. Bonnefoy, Eur. J. Immunol. 27 (1997) 971–978.
[104] B. Miloux, P. Laurent, O. Bonnin, J. Lupker, D. Caput, N. Vita, P. Ferrara, FEBS Lett. 401 (1997) 163–166.
[105] K. Sugamura, H. Asao, M. Kondo, N. Tanaka, N. Ishii, M. Nakamura, T. Takeshita, Adv. Immunol. 59 (1995) 225–277.
[106] J.L. Zhang, M. Buehner, W. Sebald, Eur. J. Biochem. 269 (2002) 1490–1499.
[107] B. Schnarr, J. Ezernieks, W. Sebald, A. Duschl, Int. Immunol. 9 (1997) 861–868.
[108] A.B. Shanafelt, C.P. Forte, J.J. Kasper, L. Sanchez-Pescador, M. Wetzel, R. Gundel, J.M. Greve, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9454–9458.
[109] N. Kruse, T. Lehrnbecher, W. Sebald, FEBS Lett. 286 (1991) 58–60.
[110] T. Muller, W. Sebald, H. Oschkinat, Nat. Struct. Biol. 1 (1994) 674–676.
[111] U. Horsten, G. Muller-Newen, C. Gerhartz, A. Wollmer, J. Wijdenes, P.C. Heinrich, J. Grotzinger, J. Biol. Chem. 272 (1997) 23748–23757.
[112] J.M. Woodcock, B. Zacharakis, G. Plaetinck, C.J. Bagley, S. Qiyu, T.R. Hercus, J. Tavernier, A.F. Lopez, EMBO J. 13 (1994) 5176–5185.
[113] A. Haman, C. Cadieux, B. Wilkes, T. Hercus, A. Lopez, S. Clark, T. Hoang, J. Biol. Chem. 274 (1999) 34155–34163.
[114] R.T. Kroemer, W.G. Richards, Protein Eng. 9 (1996) 1135–1142.
[115] F. Olosz, T.R. Malek, J. Biol. Chem. 275 (2000) 30100–30105.
[116] J.M. Puck, Immunol. Today 17 (1996) 507–511.
[117] C. Smerz-Bertling, A. Duschl, J. Biol. Chem. 270 (1995) 966–970.
[118] G. Aversa, J. Punnonen, B.G. Cocks, R. de Waal Malefyt, F. Vega Jr., S.M. Zurawski, G. Zurawski, J.E. de Vries, J. Exp. Med. 178 (1993) 2213–2218.
[119] J.M. Carballido, G. Aversa, D. Schols, J. Punnonen, J.E. de Vries, Int. Arch. Allergy Immunol. 107 (1995) 304–307.
[120] J.M. Woodcock, C.J. Bagley, B. Zacharakis, A.F. Lopez, J. Biol. Chem. 271 (1996) 25999–26006.
[121] H.B. Lowman, J.A. Wells, J. Mol. Biol. 234 (1993) 564–578.
[122] A. Cabibbo, E. Sporeno, C. Toniatti, S. Altamura, R. Savino, G. Paonessa, G. Ciliberto, Gene 167 (1995) 41–47.
[123] C. Toniatti, A. Cabibbo, E. Sporena, A.L. Salvati, M. Cerretani, S. Serafini, A. Lahm, R. Cortese, G. Ciliberto, EMBO J. 15 (1996) 2726–2737.
[124] A.B. Shanafelt, Y. Lin, M.C. Shanafelt, C.P. Forte, N. Dubois-Stringfellow, C. Carter, J.A. Gibbons, S.L. Cheng, K.A. Delaria, R. Fleischer, J.M. Greve, R. Gundel, K. Harris, R. Kelly, B. Koh, Y. Li, L. Lantz, P. Mak, L. Neyer, M.J. Plym, S. Roczniak, D. Serban, J. Thrift, L. Tsuchiyama, M. Wetzel, M. Wong, A. Zolotorev, Nat. Biotechnol. 18 (2000) 1197–1202.
[125] H. Domingues, D. Cregut, W. Sebald, H. Oschkinat, L. Serrano, Nat. Struct. Biol. 6 (1999) 652–656.
[126] O. Livnah, D.L. Johnson, E.A. Stura, F.X. Farrell, F.P. Barbone, Y. You, K.D. Liu, M.A. Goldsmith, W. He, C.D. Krause, S. Pestka, L.K. Jolliffe, I.A. Wilson, Nat. Struct. Biol. 5 (1998) 993–1004.
[127] S.A. Middleton, F.P. Barbone, D.L. Johnson, R.L. Thurmond, Y. You, F.J. McMahon, R. Jin, O. Livnah, J. Tullai, F.X. Farrell, M.A. Goldsmith, I.A. Wilson, L.K. Jolliffe, J. Biol. Chem. 274 (1999) 14163–14169.
[128] P.J. Kraulis, J. Appl. Cryst. 24 (1991) 946–950.
[129] E.A. Merritt, D.J. Bacon, Methods Enzymol. 277 (1997) 505–524.
[130] A. Nicholls, K.A. Sharp, B. Honig, Proteins 11 (1991) 281–296.