Bioconjugate Chemistry

Article

pubs.acs.org/bc

IL-4 Analogues with Site-Specific Chemical Modification at Position 121 Inhibit IL-4 and IL-13 Biological Activities

Viswanadham Duppatla,*[,†] Maja Gjorgjevikj,[†] Werner Schmitz,[†] Heike M. Hermanns,[§] Carmen M. Schäfer,[§] Mathias Kottmair,[‡] Thomas Müller,[‡] and Walter Sebald[†]

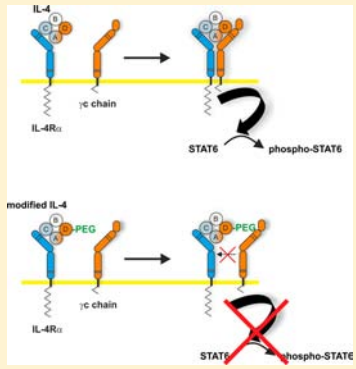
[†]Lehrstuhl für Physiologische Chemie II, Theodor-Boveri-Institut für Biowissenschaften (Biozentrum) der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

[‡]Lehrstuhl für Botanik I-Molekulare Pflanzenphysiologie und Biophysik, Julius-von-Sachs-Institut für Biowissenschaften (Biozentrum) der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

[§]Rudolf-Virchow-Center, Research Center for Experimental Biomedicine, University of Würzburg, Sanderring 2, 97070 Würzburg, Germany

Supporting Information

ABSTRACT: IL-4 signaling into a cell occurs via assembly of a receptor complex that consists of a high-affinity IL-4Rα chain and a low affinity chain, where the low-affinity chain is either γ_c or IL-13Rα1. It has been previously shown that mutational disruption of the low affinity interface in the IL-4DM (double mutein) yields an antagonist that inhibits IL-4 as well as IL-13-dependent responses. The present study reveals that new types of IL-4 antagonists can be generated by site-specific chemical modification. The chemically modified IL-4 analogues consist of (1) mixed disulfides created by refolding IL-4 cysteine muteins in the presence of different thiol compounds or (2) maleimide conjugates created by modifying cysteine muteins with maleimide derivatives. IL-4 analogues chemically modified at position 121 retain marginal binding affinity to γ_c or IL-13Rα1 receptor ectodomains during SPR interaction analysis. The biological activity of the analogues is strongly reduced in HEK-Blue IL-4/IL-13 cells as well as in Jurkat cells. Since the IL-4 analogues modified at position 121 have the ability to inhibit γ_c (IL-4)- and IL13Rα1 (IL-4/IL-13)-dependent responses in Jurkat and HEK-Blue cell lines, they effectively act as IL-4 antagonists. The results of our IL-4 study provide the first example of a cytokine that is transformed into a competitive inhibitor by site-specific chemical modification.



INTRODUCTION

The IL-4-receptor system is ideal for manipulating protein−protein interactions via site-specific chemical modification. IL-4 is not only of great interest as a model system for cytokine receptor structure[1−3] and signaling[4,5] but also of high medical interest since it is one of the major regulatory cytokines that determines immune cell differentiation and growth.[6−8] IL-4 is produced and secreted primarily by Th_2-biased CD4+ T cells, mast cells, basophils, and eosinophils. Together with its close relative IL-13,[9] another Th_2 cytokine, IL-4 plays a dominant role in the development and maintenance of allergic inflammation and asthma.[10−13] Many findings support the view that IL-4 acts mainly as an "immunoregulatory" cytokine, while IL-13 acts largely as an "effector" cytokine.[4] In addition to its crucial role in the immune system, IL-4 functions as a survival factor and inhibitor of apoptosis in cancer cells.[14,15]

IL-4 is a small 4-helix bundle cytokine that features three disulfide bridges and no free cysteine residue.[16] It employs two types of heterodimeric receptors for signaling into a cell.[17,18] Both receptor types contain an IL-4Rα chain that binds IL-4 with high affinity (K_D of about 100 pM). The IL-4Rα chain provides most of the IL-4 affinity within the whole receptor complex.[19] The type I and II receptors that interact with IL-4 differ in their second chain. The type I receptor employs the common γ chain (γ_c) as the second chain, and it is expressed predominantly on immune cells and other hematopoietic cells. The type II receptor contains IL-13Rα1 as the second chain, and it is mainly found on nonhematopoietic cells. The type II receptor is promiscuous in that it binds not only to IL-4 but also to IL-13.

The IL-4 double mutein (IL-4DM, also known as pitrakinra) with substitutions R121D and Y124D retains the ability to bind to the IL-4Rα chain, but the substitutions lead to disrupted binding with the γ_c and IL-13Rα1 chains.[20] IL-4DM has no agonist activity but competes effectively against endogenous wild type IL-4 (IL-4WT) for receptor binding. IL-4DM is being developed as a receptor antagonist (IL-4Rα blocker) and therapeutic for the treatment of eosinophilic asthma and eczema.[21,22] For such applications, there are two relevant points to consider: (1) by antagonizing IL-4Rα, both IL-4- and IL-13-

Received: July 8, 2013
Revised: December 15, 2013
Published: December 16, 2013

dependent responses are effectively inhibited, and (2) IL-4Rα has a very high affinity for IL-4 (K_D of ~100 pM), which is almost identical to IL-4's affinity with the heterodimeric type I or II complex that exists in the cell membrane.[19,23]

Site-specific modification of IL-4 has been used in the past for conjugation with a truncated *Pseudomonas exotoxin* (PE35).[24] To achieve this, a series of IL-4 analogues were prepared where residues at positions 28, 38, 68, 70, 97, or 105 were individually substituted by cysteine. Disulfide-mediated conjugation with PE35 yielded IL-4-toxins with improved binding affinity and biological activity compared to an IL-4-PE35 fusion protein. A major concern in this study was to preserve the receptor-binding affinity of IL-4 as much as possible since it is largely responsible for biological activity. Another study described the site-specific modification of IL-4DM with polyethylene glycol (PEG).[25] Residues 28, 36, 37, 104, 105, or 106 were selected for substitution with cysteine and subsequent conjugation since all of them were considered to be located outside of the binding epitopes of IL-4 for the receptor.

In contrast to existing studies, we used chemical modification to target IL-4 positions within the binding epitopes of the γ_c and IL-13Rα1 receptors. Our chemically modified IL-4 analogues demonstrated abilities to act as receptor antagonists that inhibit IL-4 as well as IL-13 signaling. As a notable advantage compared to the previous double-mutein approach, we found that chemical modification of a single IL-4 site yielded antagonistic analogues. We propose that such PEGylated antagonistic analogues, in particular, hold the most potential for reducing immunogenicity and offer superior pharmacokinetics *in vivo* as an additional benefit.

EXPERIMENTAL PROCEDURES

Chemicals. *E. coli* glutaredoxin (GRX-01) was obtained from IMCO (Stockholm, Sweden), glutathione from Fluka, and NADPH and yeast glutathione reductase (GR) from Sigma (St. Louis, MO, USA). TMM(PEG)12 [(methyl-PEG12)3-PEG4-maleimide, MW 2360,75] was purchased from Thermo Scientific (Dreieich, Germany). MeOH-PEG-SH (M_r 5079) was obtained from Iris Biotech (Markt Redwitz, Germany). *N*-Malenoyl-(*S*)-alanine was purchased from GL Chemtech (Oakville, Canada). *N*-(2-Aminoethyl)maleimide was purchased from Sigma Aldrich.

Preparation of IL-4 Cysteine Muteins as Mixed Disulfides. The cDNA-encoding IL-4 cysteine muteins were generated by recombinant PCR. The IL-4 muteins were expressed in *E. coli*, extracted from inclusion bodies, and refolded as detailed elsewhere.[26] Oxidized and reduced forms of thiol compounds were added during refolding to generate specific mixed disulfides. The mixed disulfide with PEG (121-SS-PEG) was produced by adding 1 mM MeOH-PEG-SH and 0.1 mM dithio-dipyridine to the refolding mixture. IL-4 analogues were purified by SP-Sepharose chromatography and C4 HPLC as described,[26] freeze-dried, and stored in aliquots at −20 °C until further use.

Enzymatic Reduction of IL-4 Cysteine Muteins with Glutaredoxin (GRX1). The mixed disulfide between IL-4 mutein and glutathione was reduced enzymatically with *E. coli* glutaredoxin-1.[26] The final reaction conditions were 100 mM potassium phosphate (pH 7), 2 mM EDTA, 3 μM glutathione reductase, 0.2 mM NADPH, 0.5 mM glutathione (reduced form), and 50 μM glutathionylated IL-4 protein. The redox reaction was started by adding 0.01 volumes of a 300 μM glutaredoxin solution prepared according to the manufacturer's recommendation. The progress of the reaction was monitored by the oxidation of NADPH, which was detected by a decrease in the extinction at 340 nm. After 30 min, 0.025 volumes of 4 M ammonium acetate (pH 5) were added. The reaction mixture was immediately loaded onto a 1 mL SOURCE 15S column (GE Healthcare) pre-equilibrated with 25 mM ammonium acetate (pH 5). The IL-4 protein was eluted using a salt gradient of 0.5 to 1.5 M sodium chloride over 60 min at a flow rate of 0.5 mL min^{-1}. The protein-containing fractions from the ion exchange chromatography step (containing about 0.75 M NaCl) were immediately loaded onto a C4 reverse phase HPLC column (Vydac 214TP54, 250 × 4.6 mm) pre-equilibrated with 0.1% TFA for further purification. The IL-4 protein was eluted using a gradient of 20 to 80% acetonitrile over 60 min at a flow rate of 0.8 mL min^{-1}. IL-4 protein-containing fractions were pooled, freeze-dried, and dissolved in water at about 100 μM concentration.

Production of Maleimido-Analogues. Reaction of the reduced IL-4 muteins at preparative scale was performed at a 3-fold molar excess of the maleimido compound in PE7 buffer (0.1 M potassium phosphate (pH 7), 2 mM EDTA) at 20 °C for 30 min. The conjugated proteins were first submitted to SP-Sepharose chromatography at pH 5. The eluted protein was then mixed with 0.1% TFA to a final volume of 5 mL and loaded onto a C4 reverse phase HPLC column (Vydac 214TP54, 250 × 4.6 mm) pre-equilibrated with 0.1% TFA. The modified protein was purified by applying an acetonitrile gradient of 20 to 80% acetonitrile over 60 min at a flow rate of 0.8 mL min^{-1}. Protein-containing fractions were pooled and freeze-dried. The conjugated IL-4 protein was dissolved in water at concentrations of about 100 μM and stored at −20 °C until further use.

Electrospray Ionization Mass Spectrometry Analysis (ESI-MS). ESI-MS was performed using an APEX-II FT-ICR (Bruker Daltonic GmbH, Bremen) equipped with a 7.4 T magnet and an Apollo ESI ion source in positive mode. Desalted proteins were dissolved in methanol/water/acetic acid (49.5/49.5/1) to yield sample concentrations of 1−5 μM. The sample was injected using a Hamilton syringe at a speed of 2 μL min^{-1} with a capillary voltage of 360 V. The detection range was typically set to 300−3000 m/z in initial measurements. The detection range was then optimized to the signal-containing area. An accumulation of 256 scans was combined at a resolution of 256 K.

For evaluation, the mass spectra were deconvolved to the single protonated ion mode using the Bruker Xmas software. The most intense isotope signal was selected for mass determination.

Surface Plasmon Resonance (SPR) Interaction Analysis. Biosensor experiments were carried out on a BIAcore 2000 system (GE Healthcare, Freiburg, Germany) at 25 °C and a flow rate of 10 μL min^{-1} as described.[27] Briefly, the biotinylated ectodomain of the IL-4Rα receptor was immobilized on a CM5 biosensor chip loaded with streptavidin. Using the command COINJECT, IL-4Rα was first saturated with IL-4 protein via a 300-s perfusion with a solution of 10 to 20 nM IL-4 or IL-4 analogue. Thereafter, a solution of the recombinant ectodomain of γ_c (1 μM) or IL-13Rα1 (2 μM) was applied for 120 s.

Cellular Assays for IL-4 and IL-13 Bioactivity. HEK-Blue IL-4/IL-13 cells (Cat. Code hkb-stat6 ; CAYLA-INVIVOGEN EUROPE, Toulouse) were grown in a growth

dx.doi.org/10.1021/bc400307k | *Bioconjugate Chem.* 2014, 25, 52−62

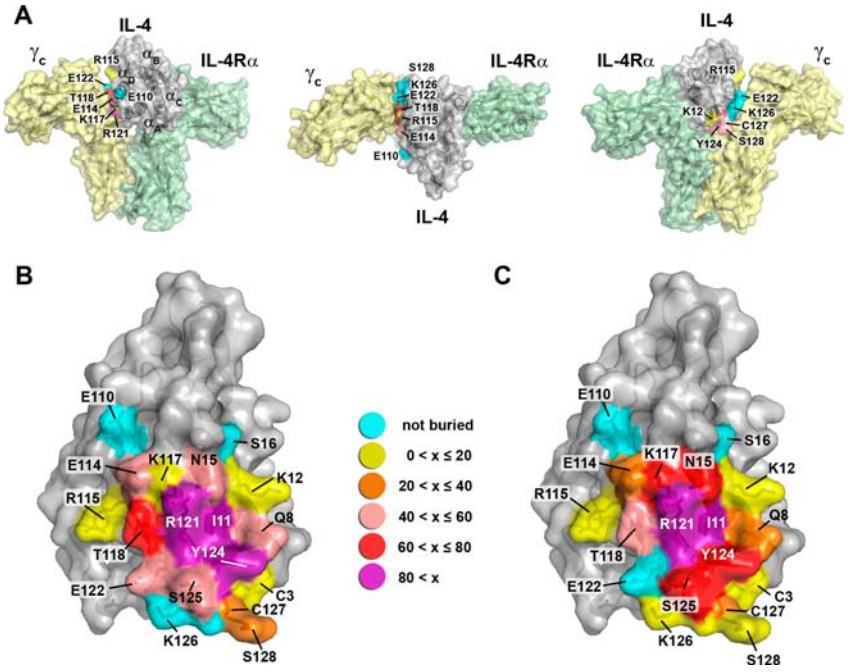


Figure 1. (A) Surface representation of the complex of IL-4 bound to the ectodomains of the IL-4 type I receptor subunits IL-4Rα and γ_c (PDB entry 3BPL).[2] The location of the helices α_A, α_B, α_C, and α_D of IL-4 is indicated in the left panel. The contact residues of IL-4 with γ_c are color-coded according to the accessible surface area buried in the contact (BSA). (B) Surface presentation of only IL-4 with the contact residues to the receptor subunit γc (see also Table S1, Supporting Information). (C) Surface representation of IL-4 with the surface color-coded to highlight the residues buried upon complex formation of IL-4 with the receptor subunit IL-13Rα1. In all panels, the color scheme used to describe the amount of buried surface upon binding is shown in between panels B and C.

medium containing DMEM, 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL normocin, 2 mM L-glutamine at 37 °C, and 5% CO_2. Cells were maintained in growth medium supplemented with 10 μg/mL blasticidin and 100 μg/mL zeocin. Induction of SEAP (soluble alkaline phosphatase) by IL-4WT and IL-4 analogues was measured in the growth medium according to the manufacturer's instructions.

Human acute T-cell leukemia Jurkat cells (DMSZ, ACC 282) were grown at 37 °C and 5% CO_2 in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. One milliliter of cells (2.5 × 10^6/mL) in RPMI containing 1% heat-inactivated fetal bovine serum was plated in a 24-well dish for 4 h. Cells were preincubated for 5 min with the indicated concentration of an IL-4 analogue, before they were incubated for another 15 min with IL-4 (300 pM). Cells were harvested at 4 °C in a 1.5 mL vial by first centrifuging 2× for 1 min at 1000 rpm followed by 10 s at 5000 rpm. The sedimented cells were washed by resuspending in ice cold PBS (phosphate buffered saline) and centrifuging for 2 min at 2500 rpm. Cell lysates were prepared by adding 180 μL of Triton X-100 lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 3 μg/mL pepstatin, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) and thoroughly vortexed as described.[28] After incubation for 15 min on ice, the mixture was centrifuged at 4 °C for 15 min at 14 000 rpm. Supernatants were transferred to new vials. After mixing with 3 volumes of 4× concentrated SDS sample buffer, 20 μL aliquots were submitted to SDS−PAGE on 10% PAA gels before the proteins were transferred to a PVDF (polyvinylidenedifluoride) membrane by Western blotting. Polyclonal antibodies against STAT6 pY641 (Cell Signaling, Cat. No. 9361) were applied for the detection of phosphorylated STAT6. Lower part of the membrane was incubated with monoclonal α-tubulin antibody (Sigma Aldrich, Cat. No. T6074) to verify equal loading. The ECL signals were quantified using the FluorChemQ Imaging System (ProteinSimple, Santa Clara, USA). The STAT6 pY641 signals were normalized to the tubulin signal in each sample.

Protein Analysis. Protein concentrations of purified IL-4 proteins were determined by measuring UV absorption spectra between 250 and 320 nm. The molar extinction coefficient was calculated from the amino acid sequence using the ProtParam tool and used to determine protein concentration according to the Lambert−Beer law (molar extinction coefficient ε_{278} = 8900 M^{-1} cm^{-1}). SDS−PAGE was performed using a 12% polyacrylamide gel as described previously.[26] Protein samples for SDS−PAGE analysis were diluted 2- to 5-fold with sample buffer, with or without 50 mM DTT for analysis under reducing conditions or analysis under nonreducing conditions to determine disulfide bonding, respectively. The gels were stained with Coomassie Blue R-250 for 1 h and destained overnight. Molecular weight standards phosphorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor soybean (20.1 kDa), and α-lactalbumin (14.4 kDa) were obtained from GE Healthcare (Freiburg, Germany).

RESULTS

Screening of IL-4 Positions by Chemical Modification for Disruption of the γc/IL−13Rα1 Interface. The overall complex of IL-4 and the ectodomains of the receptor subunits can be described as a Y-shaped fork (Figure 1A) where the IL-4 liganding region is located in between the arms of the fork.[2] The "high-affinity" interface between IL-4 and the IL-4Rα ectodomain is termed site I, while the receptor−receptor

dx.doi.org/10.1021/bc400307k | Bioconjugate Chem. 2014, 25, 52−62

interface between the D1 domains of both receptor ectodomains is termed site IIb. The interface site IIa is formed from the loops located between domains D1 and D2 of the classical cytokine receptor interaction motif of both "low-affinity" receptors γ_c and IL-13Rα1. In the ligand IL-4, the epitope for site IIa interactions is centered around residues Arg121 and Tyr124; mutation of these residues to aspartates results in IL-4 antagonists[19,23] that inhibit IL-4 type I and type II receptor responses due to an electrostatic mismatch.[29] Positions 121 and 124 were selected for chemical modification together with more peripheral positions Glu110, Glu114, Lys117, Thr118, and Glu122 (Figure 1B and C). The percentage of buried surface area for IL-4 residues, which upon ternary complex formation come into contact with γ_c or IL-13Rα1, established a first estimate of the location of the residues within and their contribution to the receptor binding epitope (Table S1 (Supporting Information), Figure 1B, and C).

LaPorte and colleagues reported that the IIa site acts as an interface between the ligand IL-4 and the receptors γ_c or IL-13Rα1 by exhibiting excellent shape complementarity.[2] This results in tightly packed interfaces, which might exclude the possibility of adapting to an altered surface geometry. It might therefore be reasonable to assume that modifications at positions at the center or the periphery of the interface (Figure 1B and C) will disrupt the interfacial contacts or yield chemically modified IL-4 analogues that might possibly discriminate between γ_c and IL-13Rα1 binding. To explore these possibilities, IL-4 cysteine mutant proteins S16C, E110C, E114C, K117C, T118C, R121C, E122C, and Y124C were prepared. When expressed in *E. coli*, they were recovered in the insoluble fraction ("inclusion bodies") and could be refolded and purified as previously described.[26] Remarkably, when a glutathione redox couple was present in the refolding buffer, the cysteine mutant proteins were recovered as mixed disulfides with a glutathione residue disulfide-bonded to the engineered cysteine side chain. These glutathionylated IL-4 proteins provided a first collection of analogues to be screened for the effect of site-directed chemical modification.

Table 1 compiles biological activities and the receptor binding of different IL-4 analogues in which a cysteine residue introduced in positions within or close to the receptor contacts was modified with glutathione. As for the accessibilities of residues of the IL-4 ligand when bound to either γ_c or IL-13Rα1, several categories of sites could be discriminated. IL-4 residues Ser16 and Glu110 represent the first category of sites, which are located at the border of the "low affinity"-receptor epitopes and are thus still fully accessible in the ternary ligand–receptor complexes (percentage of buried surface area (%BSA) = 0%). The respective IL-4 cysteine analogues modified with a glutathione moiety (-SG) at these positions exhibited wild type-like biological activities and showed the same (110-SS-G; for abbreviations of analogues see Figure S1, Supporting Information) or only slightly reduced (16-SS-G) levels of receptor binding as that of IL-4WT. IL-4 residues Glu114 and Thr118 represent the second category of sites, which are partially buried in the IL-4 type I and type II receptor complexes. The glutathione-modified IL-4 cysteine analogues 114-SS-G and 118-SS-G exhibited a biological activity comparable to that of IL-4WT; their binding to the two receptor ectodomains γ_c and IL-13Rα1 was between 35 and 72% of IL-4WT (Table 1). IL-4 residues Lys117 and Glu122 represent a third category of sites, which show different ligand accessibilities in both IL-4 receptor complexes. Lys117 of IL-4 is buried to a large degree (%BSA = 72%) when IL-4 is bound to IL-13Rα1, but the same residue shares little contact with the receptor ectodomain γ_c (%BSA = 3%). The opposite holds for IL-4 Glu122, which is part of the structural binding epitope for γ_c but does not share any contact with IL-13Rα1. The glutathione-modified IL-4 mutein E122C (abbreviated as 122-SS-G) showed reduced affinities *in vitro* to both receptors, but binding to IL-13Rα1 was higher than that to γ_c. Despite the significant changes in receptor binding, biological activities remained unaltered. The IL-4 analogue 117-SS-G retained only 4% (γ_c) and 7% (IL-13Rα1) binding to the low-affinity receptors, as revealed in an SPR-based *in vitro* interaction analysis. However, the biological activities of this IL-4 analogue were only slightly reduced in the HEK-Blue and in the Jurkat cell assays (Table 1). Finally, residues Arg121 and Tyr124 represent a fourth category of sites. Tyr124 is buried completely in the interface with γ_c. When in contact with IL-13Rα1, very little of the Y124 residue is accessible to the solvent. Residue Arg121 is hardly accessible in the type I and type II receptor complexes. Conforming to the idea of its central position in the "low-affinity" receptor interface, the IL-4 analogue 124-SS-G exhibited strongly reduced receptor binding compared to that of IL-4WT, at 3% of the IL-4WT level for binding to γ_c and 6% for binding to IL-13Rα1. These residual affinities for γ_c and IL-13Rα1, however, were clearly higher compared to the receptor-binding capabilities of the IL-4 analogue 121-SS-G (IL-4 variant R121C modified with glutathione), which were below the limit of detection. Surprisingly, despite these low receptor-binding efficiencies the biological activities of the IL-4 analogue 124-SS-G were rather high in the HEK-Blue cell assay (61% of IL-4WT), which measured the IL-4 type II receptor-dependent response, and only somewhat lower (25% of IL-4WT) in the Jurkat cell assay, which specifically measured the IL-4 type I receptor activation. Even for the IL-4 analogue 121-SS-G, which showed seemingly no receptor binding to γ_c and IL-13Rα, a small residual activity in both the HEK-Blue and the Jurkat STAT6 phosphorylation assays could be measured (Table 1).

The results obtained from this collection of glutathionylated IL-4 analogues might be summarized as follows: First, compared to the modified IL-4 121-SS-G, all other analogues

Table 1. Biological Activities and Receptor Binding of Glutathionylated IL-4 Analogues

	biological activity[b]		receptor binding[b]	
	% IL-4		% IL-4	
analogues[a]	HEK-Blue cells	Jurkat cells	γ_c	IL-13Rα1
16-SS-G	102		86	71
110-SS-G	111		125	105
114-SS-G	110		66	72
117-SS-G	64	71	4	7
118-SS-G	115		38	35
121-SS-G	3.4	9	<1	<1
122-SS-G	113		29	52
124-SS-G	61	25	3	6

[a]The glutathione-modified analogues were obtained from IL-4 cysteine muteins after refolding involving a glutathione redox couple as detailed under Experimental Procedures. For abbreviation see Figure S1, Supporting Information. [b]For experimental details see Legends to Figures 3, 4, and 5.

dx.doi.org/10.1021/bc400307k | *Bioconjugate Chem.* 2014, 25, 52–62

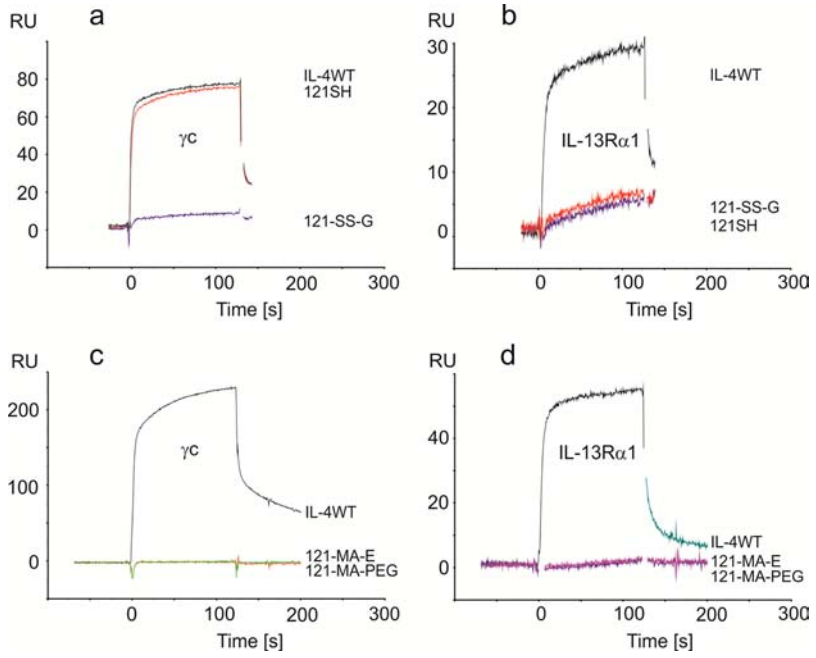


Figure 2. Surface plasmon resonance (SPR) analysis of the interaction of IL-4 analogues with the γ_c and IL-13Rα1 receptor ectodomains. The IL-4 analogues were bound to the IL-4Rα ectodomain, which was immobilized on a CM5 biosensor chip (see Figure S5, Supporting Information). At time point 0 s, perfusion with 1 μM γ_c (a,c) or 2 μM IL-13Rα1 (b,d) was started for a duration of 120 s after which time the biosensor was again perfused with buffer to measure dissociation. The sensorgrams recorded during perfusion with buffer-only were subtracted from the sensorgrams obtained with the receptor proteins. For abbreviations of analogues, see Figures S1 and S2, Supporting Information.

showed substantial biological activities. This was not anticipated since at least for some IL-4 analogues, chemical modification with the tripeptide glutathione occurred within the core of the receptor-binding epitopes for γ_c and IL-13Rα1. Considering the size of the glutathione tripeptide, which measures about 9 Å in length, the conjugated glutathione moiety was expected to exert a large steric hindrance when bound to either γ_c or IL-13Rα1. Second, none of the modified IL-4 analogues exhibited a pronounced selectivity with respect to γ_c- or IL-13Rα1-mediated signaling, not even 117-SS-G and 122-SS-G which showed clear differences in participation with the two IL-4-receptor interfaces (see Figure 1B and C and Table S1, Supporting Information). This suggests that the glutathione moiety (possibly also applicable to other ligands), which is bound to the introduced cysteine residue in IL-4 via a disulfide bridge, can "freely" rotate around its rotatable bonds, thereby shielding or occupying a larger area around the modified site. This possibly explains the unexpected high agonistic activity and low receptor specificity of some IL-4 analogues, in which modification sites were defined by *in silico* predictions. Finally, for all IL-4 analogues except 121-SS-G, the decrease in biological activity was much less than what was expected from the loss in receptor binding. This difference might be explained by known receptor biology in whole cells (see the Discussion section).

The above results of the glutathionylation screening of the IL-4 site IIA interface suggested that position 121 is the most promising target for disrupting interaction with γ_c and IL-13Rα1. Therefore, chemical modifications of this site were studied in more detail.

Mixed Disulfides of IL-4 Cysteine Mutein R121C. Two types of experiments were performed for the site-specific chemical modification of the cysteine mutein R121C. First, the mutein was prepared as mixed disulfides with glutathione by employing a glutathione redox couple during refolding as mentioned in the previous section (2 mM glutathione reduced and 5 mM glutathione oxidized). Mass spectrometry analyses (Figure S3, Supporting Information) identified species with molecular weights confirming that the mutein has been recovered with all three native disulfide bonds present and that the seventh cysteine in the mutein was completely conjugated with the thiol compound. The yield of conjugated R121C (abbreviated 121-SS-G; see also Figure S1, Supporting Information) was similar to that of IL-4WT.

The mixed disulfides of IL-4 R121C with cysteamine or thioglycolate were generated in a similar way. The IL-4 analogue conjugated with thio-PEG (121-SS-PEG; for abbreviation see Figure S1, Supporting Information) was obtained by adding the reduced thiol compound plus an appropriate concentration of dithiodipyridine (see Experimental Procedures). SDS−PAGE analysis (Figure S4D, Supporting Information) of the PEGylated R121C analogue (121-SS-PEG) showed that only a high-molecular weight analogue was present in the nonreduced sample, which was converted to a protein with IL-4-like mobility under reducing conditions. In conclusion, these results show that the formation of mixed IL-4 disulfides during protein refolding represents an efficient method to effect site-specific modifications.

Preparation of IL-4 Cysteine Mutein R121C with a Free Thiol Group and of Analogues Modified by Maleimido Compounds. Maleimidation was employed as a second highly specific thiol-modifying reaction. A variety of maleimide compounds, including maleimide-PEGs, are commercially available. However, it is a prerequisite that the cysteine mutein contains the engineered cysteine in a nonconjugated form. Previous experiments have shown[26] that even when specific refolding conditions are employed, an IL-4 cysteine mutein with a fully reduced, engineered cysteine could

dx.doi.org/10.1021/bc400307k | *Bioconjugate Chem.* 2014, 25, 52−62

only be obtained in low yield. To further compound the problem, the desired IL-4 cysteine mutein was heavily contaminated by partially refolded and/or conjugated IL-4 proteins. Likewise, a selective chemical reduction of IL-4 mutein mixed disulfides (via the use of DTT, for instance) was not achievable since the structure-stabilizing native disulfide bonds were reduced simultaneously (see also ref 30).

An enzymatic reduction of glutathionylated mutein employing glutaredoxin 1 from *E. coli* (GRX1) proved to be more specific and enabled preparation of the nonconjugated mutein in good yield. One example presented in Figure S3A (Supporting Information) showed that the IL-4 analogue 121-SS-G became rapidly reduced enzymatically to a species with one free thiol group within 5−10 min. Thereafter, additional free thiol groups were generated in the R121C mutein, as confirmed with SDS−PAGE analysis of MA-PEGylated species (Figure S3B, Supporting Information).

The purified nonconjugated mutein IL-4 R121C (121SH) was successfully reacted with various maleimide compounds. The selectivity of the reaction was documented with purified analogues modified with *N*-ethyl-maleimide (NEM; 121-MA-E), *N*-maleonyl-(*S*)-alanine (121-MA-ALA), or *N*-(2-amino-ethyl) maleimide (121-MA-EA). Mass spectrometry analyses showed that from each reaction, only one major protein species with the expected mass was present (Figure S4B,C, Supporting Information). Minor species represented oxidized (+16 Mr) or deaminated (−16 Mr) analogues. Mass spectrometry of PEGylated analogues was not possible since the molecular weight of the PEG reagents was not homogeneous. However, SDS−PAGE analysis revealed that any nonconjugated protein was present in only minor amounts and that the attached PEG was not cleaved off by reduction (see Figure S3B, Supporting Information; data not shown).

Disruption of γ_c and/or IL-13Rα1 Receptor Binding after Chemical Modification of IL-4 at Position 121. SPR-based interaction analyses were performed to assess whether the binding affinity of IL-4 conjugates for γ_c, IL-13Rα1, or IL-4Rα are affected by chemical modification. As detailed under Experimental Procedures, the ectodomain of IL-4Rα was immobilized on a sensor chip using the streptavidin/biotin method. The receptor was first saturated by perfusion with the IL-4 analogue, followed by the ectodomain of γ_c or IL-13Rα1. The sensorgrams shown in Figure S5 (Supporting Information) were generated by subtracting the signal obtained from a control surface without the immobilized receptor. IL-4WT and the analogues at 20 nM concentration bound rapidly to the immobilized IL-4Rα ectodomain within the first 100 to 200 s. Equilibrium binding occurred when the receptor was virtually saturated with the ligand. A buffer-only solution after 300 s of perfusion, which led to a slow dissociation of IL-4 proteins from the IL-4Rα receptor (half-life ($t_{1/2}$) ~10 min) was observed. When buffer containing γ_c or IL-13Rα1 ectodomains were perfused, instantaneous and rapid binding commenced during the association phase, while rapid dissociation occurred after 120 s when the buffer-alone solution was applied again. The differential sensorgrams presented in Figure 2 show only the specific signals generated during the period of γ_c and IL-13α1 association and dissociation.

The mixed disulfides of IL-4 and the maleimide analogues retained high-affinity binding to the IL-4Rα ectodomain. The initial velocities measuring the diffusion-limited phase of IL-4 binding to IL-4Rα were comparable between IL-4WT and the IL-4 analogues (Figure S5 and Table S2, Supporting Information). The saturation levels obtained with IL-4 or IL-4 analogues were also similar with the exception of the PEGylated analogues where a slightly higher signal at saturation level was obtained. For the measurement of the kinetic and equilibrium constants of the interaction between IL-4 and IL-4Rα, low levels (<50 RU) of the IL-4Rα ectodomain had to be immobilized.[27] Some IL-4 analogues were analyzed under this condition, and the constants were found to be comparable (Table S2, Supporting Information). This suggested that the conformation of the IL-4 protein and its binding affinity for the IL-4Rα receptor was not altered by the R121C mutation itself or by the site-specific modification at position 121.

The IL-4 mutein R121C (121SH) demonstrated binding abilities with the γ_c ectodomain at levels comparable to those of IL-4WT (Figure 2A and Table 2). However, binding of the R121C mutein to the IL-13Rα1 receptor was strongly reduced (Figure 2B). All analogues of R121C mutein exhibited a strongly reduced affinity for the γ_c and IL-13Rα1 receptors. Remarkably, the wild-type-like binding of R121C to γ_c was also severely disrupted by the chemical modification (Table 1 and Figure 2). The faint residual binding of 121-SS-G and 121-SS-PEG to the γ_c receptor was reproducibly observed and therefore appeared to be significant. However, the binding of all other R121C analogues was below the limit of detection (<1% of the RU equilibrium value observed for IL-4WT). The calculated residual binding of the analogues to IL-13Rα1 showed a larger variability than binding to γ_c since the equilibrium binding of IL-13Rα1 (2 μM) to IL-4WT was only 30 to 50 RU.

Table 2. Interaction of IL-4 R121C Analogues with γ_c and IL-13Rα1 Receptor Ectodomains[a]

	γ_c binding	IL-13Rα1 binding
analogues[b]	% IL-4WT	% IL-4WT
IL-4WT	=100	=100
121-SS-G	1.5	0.6
121-SS-CA	−0.2	2.0
121-SS-TG	0.2	1.2
121-SS-PEG	1.2	3.8
121SH	98	0
121-MA-E	0.6	3.9
121-MA- PEG	0.7	4.2
121-MA-ALA	0.6	−0.1
121-MA-EA	0.2	−0.4

[a]Sensorgrams as shown in Figure 2 have been evaluated for equilibrium binding of 1 μM γ_c or the 2 μM IL-13Rα1 ectodomain. Equilibrium binding with IL-4WT was normalized to account for 100%. [b]For abbreviations of analogues, see Figures S1 and S2, Supporting Information.

In conclusion, biosensor interaction analyses demonstrated that chemical modification at position 121 of IL-4 reduces its binding affinity to receptor γ_c by more than 99% and to receptor IL-13Rα1 by at least 95%.

Antagonist Activities of IL-4 Analogues Modified at Position 121. HEK-Blue IL-4/IL-13 cells have been engineered to respond to IL-4 or IL-13 by expressing a secreted alkaline phosphatase (SEAP). These cells also express the IL-4Rα and IL-13Rα1 receptors. As shown in Figure 3, the response was very sensitive with EC_{50} values (concentration effecting half-maximal response) of 3 pM for IL-4 and 8 pM for IL-13.

dx.doi.org/10.1021/bc400307k | *Bioconjugate Chem.* 2014, 25, 52−62

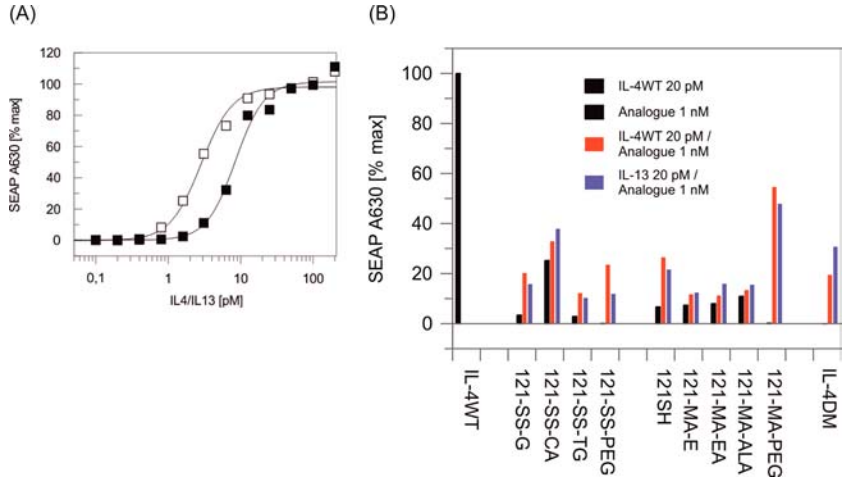


Figure 3. Biological activities of IL-4 R121C analogues in HEK-Blue cells. (A) Dose-dependent stimulation of SEAP activity by IL-4 and IL-13. HEK-Blue cells were incubated with the indicated concentrations of IL-4 (open boxes) or IL-13 (filled boxes) for 30 min before SEAP activity was measured in the supernatant. (B) Agonist activity of IL-4 analogues and inhibition of IL-4 or IL-13 dependent activity in HEK-Blue cells. HEK-Blue cells were incubated with 1 nM IL-4 analogues or with 1 nM IL-4 analogues containing 20 pM IL-4WT or 20 pM IL-13 as detailed under Experimental Procedures. The induced SEAP activity measured in all samples was normalized against the SEAP activity induced by 20 pM IL-4WT.

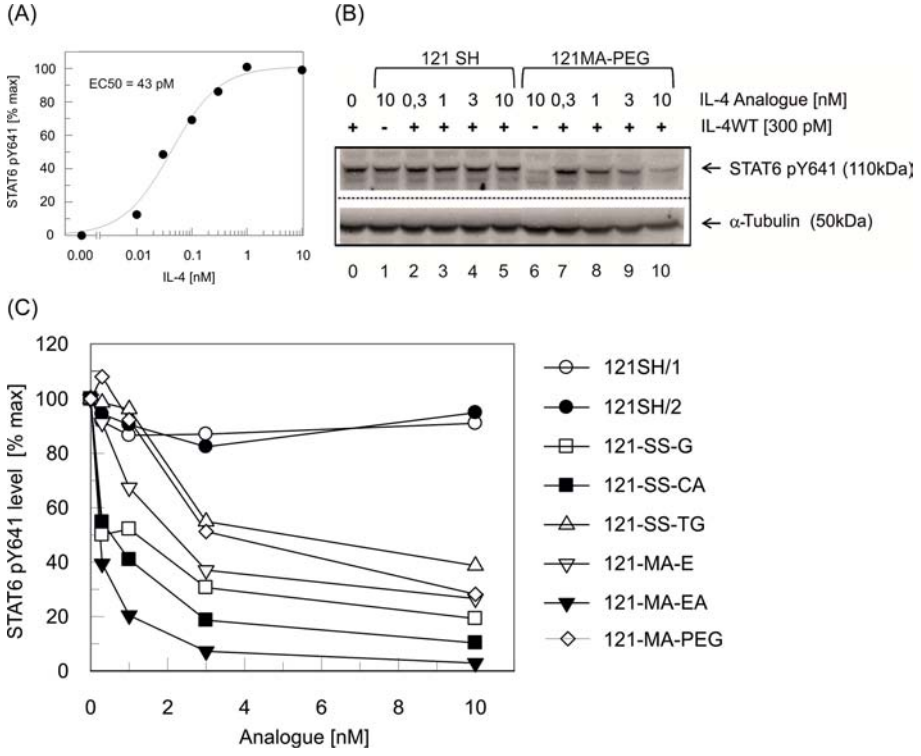


Figure 4. Biological activities of IL-4 R121C analogues in Jurkat cells. (A) Dose-dependent stimulation of STAT6 phosphorylation by IL-4. (B) Inhibition of 300 pM IL-4WT in the presence of mutein 121SH and IL-4 analogue 121-MA-PEG at the indicated concentrations. Cell lysates were submitted to SDS−PAGE and Western blot analysis as detailed under Experimental Procedures. For a quantitative evaluation, the intensity of the band corresponding to STAT6 P641 was normalized to α-tubulin analyzed in the same lysate. (C) Dose-dependent inhibition of IL-4 activity by IL-4 R121C analogues. The level of STAT6 pY641 was evaluated at 300 pM IL-4WT in the presence of IL-4 analogues at the indicated concentrations. The activity of the IL-4SH mutein was included as a reference.

Even at 1 nM concentration, the IL-4 R121C analogues exhibited low activity in HEK-Blue cells. Compared to 20 pM of IL-4WT (Figure 3 and Table S3, Supporting Information), the activity of the 121-SS-CA analogue amounted to 25%. The PEGylated analogues were virtually inactive and similar to the IL-4DM, which was analyzed in parallel. The mixed disulfides 121-SS-G and 121-SSTG showed about 3% residual activity, and the maleimide analogues between 7.4−11%. At 1 nM concentration of the analogue, the cellular receptor was nearly saturated with IL-4 (K_D ~100 pM[31,32]). The measured activities thus approached the maximally attainable responses.

Remarkably, the IL-4 R121C analogues at 1 nM concentration inhibited the IL-4- or IL-13-dependent responses to levels of 11% and 55% of the 20 pM IL-4WT control, respectively (Table S3, Supporting Information). Most of the analogues were more potent inhibitors than IL-4DM. Notable

dx.doi.org/10.1021/bc400307k | Bioconjugate Chem. 2014, 25, 52−62

exceptions were the PEGylated analogues and 121-SS-CA which inhibited to a lesser extent than IL-4DM. The observed lower antagonist activity of IL-4DM compared to most of the analogues may be related to the previous finding that the affinity of IL-4DM to IL-4Rα is lower than that of IL-4WT.[32]

During a second biological assay, T-cell leukemia Jurkat cells were employed, which express IL-4Rα and the γ_c receptor.[33] The cells responded to IL-4 in the form of tyrosine phosphorylation of the STAT6 protein with an EC_{50} of about 50 pM (Figure 4A and B). The cytokine IL-13 was not active in Jurkat cells since no IL-13Rα1 receptor is present.[33]

The 121SH mutein at 10 nM concentration showed almost the same agonist activity as 300 pM IL-4WT and almost no inhibitory activity (Figure 4B and C). This finding is in agreement with the receptor-binding assay where a wild-type-like affinity for the γ_c receptor was observed (see Table 2). For the various analogues at 10 nM concentration, the agonist activity was found to be 3 to 50 times lower than that of 0.3 nM IL-4WT (Table 2). No significant activity was found with IL-4DM mutein (2 nM) and IL-13 (1 nM).

The residual activity measured in the presence of 0.3 nM IL-4WT containing the 10 nM analogue was similar to the agonist activity of the analogues (Table 2). The 121-MA-EA analogue demonstrated behavior similar to that of IL-4DM, which only showed minimal agonist activity and attenuated 300 pM IL-4WT activity to very low levels. Half-maximal inhibition of 300 pM IL-4WT occurred at 300 pM concentration of analogues 121-SS-G, 121-SS-CA, and 121-MA-EA (Figure 4C). This suggests that these analogues bind as effectively to the receptor as IL-4WT.

In conclusion, the agonist activities as well as the inhibitory (antagonist) activities of the IL-4 R121C analogues varied more than expected from their equally low binding affinity to the γ_c chain (Table 3). As discussed below, the same disparity was previously observed when the activities of IL-4 muteins were analyzed.[34] Nonetheless, the results indicate that chemical modification at IL-4 position 121 can generate analogues with low agonist activity and strong antagonist activity comparable to that of IL-4DM.

Table 3. Agonist and Antagonist Activities of IL-4 R121C Analogues in the Jurkat Cell Assay

	STAT6 pY641 [% max]	
IL-4 analogues[a]	analogue [10 nM]	analogue [10 nM] plus IL-4WT [300 pM]
121-SS-G	9	19
121-SS-CA	18	10
121-SS-TG	32	39
121-SS-PEG	n.m.	n.m.
121SH	82	91
121-MA-E	20	27
121 MA-EA	2	3
121-MA-ALA	n.m.	n.m.
121-MA-PEG	24	28
300 pM IL-4WT	=100	
2 nM IL-4DM	1	2
1 nM IL-13	2	

[a]For experimental details, see Figure 5. The agonist/antagonist activities of 2 nM IL-4DM and the agonist activity of 1 nM IL-13 are shown for comparison in the lower part of the table.

DISCUSSION

The results we have obtained with human IL-4 protein demonstrate that site-specific chemical modification can be used to disrupt protein−protein interactions and thereby transform a cytokine into a cytokine inhibitor. Unexpectedly, however, the generation of IL-4 antagonists by introducing modifications in the contact regions between IL-4 and its low affinity receptor chains γ_c or IL-13Rα1 was not straightforward. The structural epitopes at the interface were reported to be quite rigid and of high surface complementarity.[2] Thus, it was initially surmised that any bulky chemical group bound at the IL-4 epitope would disrupt binding and inhibit biological activity to a large extent, thereby creating potent inhibitors. This turned out to be not completely true. Only modifications at position 121 yielded a series of IL-4 analogues that exhibit minimal residual biological activity and potent antagonist properties in different cell lines. Modification at other sites of the epitope only reduced biological activity to a small extent (117-SS-G and 124-SS-G) or not at all.

Such resilience of the IL-4 binding epitope to chemical modification might have several explanations. At the more peripheral positions (114, 118, and 122), the moiety attached to the introduced cysteine residue could move out of the contact area during assembly of the ternary complex. The rotational mobility of the attached residue around the disulfide bond (or the *S*-maleimide bond in case of the maleimide analogues) will facilitate such a displacement and clearance of the contact area. However, at the more central positions (117 and 124) the interface will be necessarily disturbed by the attached bulky moiety. The residual activities of the analogues modified at these positions suggests that the γ_c and IL-13Rα1 chains can dock to IL-4 in different orientations that utilize only some of the original binding determinants and/or alternative binding sites. Such a rigid body reorientation might be facilitated by factors such as the small area of the contact epitopes, the largely hydrophobic side chains of the "elbow (receptor)/canyon (cytokine)" binding motif, and the two-point attachment of γ_c to IL-4.[2] Structural features of the γ_c epitope that allow for the promiscuous interaction of γ_c with other members of the γ_c-binding interleukin family (see below) could also facilitate different cytokine-receptor orientations.

The most efficient disruption of IL-4 receptor binding and biological activities was observed after modification of position 121 suggesting that this site represents an anchor point of critical importance for the interaction. All types of IL-4 R121C analogues retained marginal affinities at the detection limit for γ_c and IL-13Rα1 binding. The equally large deficiencies in affinity of all analogues modified at position 121 suggest that geometrical constraints in the binding epitope as depicted in the models presented in Figure 5 are responsible for impairing γ_c binding by about 100-fold. Charge and size of the attached residue appear to be of secondary importance for this drop in affinity since the *N*-ethyl maleimide (MA-E) analogues suffered the same high loss of affinity as the charged or bulky maleimide analogues. The large decrease in binding affinity for IL-13Rα1 likely resulted from the loss of the side chain of Arg121 since the R121C mutein (121SH) already exhibited an immeasurably low affinity for the IL-13Rα1 chain. The disruptive effect of the chemical modification at position 121 could therefore only be recognized for the interaction with γ_c.

Notwithstanding the effective disruption of receptor binding, cell assays showed that several IL-4 analogues modified at

dx.doi.org/10.1021/bc400307k | *Bioconjugate Chem.* 2014, 25, 52−62

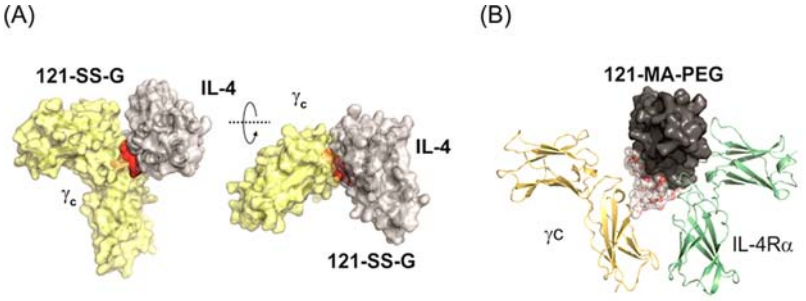


Figure 5. Structural model high-lighting the steric constraints imposed in IL-4 analogues 121-SS-G and 121-MA-PEG for interaction with γ_c. (A) The glutathionyl residue (colored red) in the mixed disulfide with IL-4 (121-SS-G) disturbs the contact with γ_c. (B) The maleimide-PEG moiety in the 121-MA-PEG analogue creates a pronounced steric hindrance for the interaction with γ_c.

position 121 retained significant biological activities that varied among the analogues. Analysis of the IL-13Rα1-dependent type II receptor in the HEKBlue cell assay indicates that the high residual activity (25%) of the 121-SS-CA analogue may have resulted from a resemblance of the disulfide bonded cysteamine moiety to the IL-4 Arg121 side chain that is normally present at this position. Similarly, the low residual activity (3%) of 121-SS-TG may be caused by the negative charge of the thioglycolate moiety, as IL-4 R121E and R121D mutations also lead to a strong disruption of activity.[32,34] The immeasurably low activities of the PEGylated analogues 121-SS-PEG and 121-MA-PEG possibly resulted from the large size of the attached compounds.

All analogues inhibited IL-4- and IL-13-dependent activity to a similar extent. Under the experimental conditions employed for determining antagonist activity (1 nM analogue in the presence of 20 pM IL-4WT or 20 pM IL-13WT), both the analogues and IL-4DM demonstrated a higher residual activity than expected from their observed agonist activity. It is likely that the cellular IL-4 receptor was not completely saturated with the analogue at 1 nM concentration, and as such, some free receptor could still respond to IL-4 or IL-13 in the very sensitive HEK-Blue cell assay (EC_{50} IL-4 = 3 pM; EC_{50} IL-13 = 8 pM).

In measuring the γ_c-dependent type I receptor in the Jurkat cell assay, 121-MA-EA showed the lowest residual activity among all analogues. Similar to IL-4DM, the activity was nearly at background level. Importantly, 121-MA-EA at 10 nM concentration completely inhibited IL-4WT (300 pM) activity. Thus, the 121-MA-EA analogue represents a promising lead compound for the development of useful IL-4 antagonists. The IL-4 Arg121Cys mutein with a free thiol group (121SH) demonstrated almost IL-4WT-levels of activity, clearly indicating that the chemical modification and not the cysteine substitution is responsible for the IL-4 type I receptor inhibition of the analogues.

Remarkably, the above results indicate that the loss in receptor affinity is not linearly related to the loss in biological activity. The same disproportionality has been previously observed in the analysis of IL-4 muteins. For instance, an IL-4 mutein with a Y124A substitution showed a > 50-fold reduction in γ_c binding affinity compared to that of IL-4WT. However, in a T-cell proliferation assay the residual maximal response was still 50% of IL-4WT.[34] Likewise, an IL-4 superkine with 3700-fold higher affinity for γ_c compared to IL-4WT was only 3- to 10-fold more potent than IL-4WT.[35]

There are several mechanisms that could lead to the lack of correlation between receptor binding affinity and receptor signaling as observed in this study. First, the binding affinities were determined by SPR measurements that utilize soluble receptor ectodomain proteins for the second "low-affinity" ligand−receptor interaction. In a cellular environment, however, the same interaction occurs within the two-dimensional plane of the plasma membrane (for a recent review, see ref 36). According to the reduction-of-dimensionality principle, the interaction might generate larger binding affinities as artificially measured by SPR. Furthermore, preassembled and/or clustered receptor complexes might exist, thereby altering association rates and lifetimes of ternary complexes. Second, our equilibrium-based view of receptor activation by IL-4 might be inadequate for interpreting dose-dependency. Very short-term encounters with γ_c or IL-13Rα1 might be sufficient to trigger a full activation response. It is also possible that signaling ternary-complexes are stabilized by cytoplasmic factors and removed by endocytosis from the cell surface.[36]

Site-specific chemical modification of human IL-4 at position 121 has proved to be a robust and adaptable technique to disrupt and modify the interface between IL-4 and the low affinity γ_c and IL-13Rα1 receptor chains. Thus, with the reservations discussed above, site-specific chemical modification has the potential to become a general technological platform for disrupting interactions between other extracellular proteins of medical interest. Promising candidates are interleukins IL-2, IL-15, IL-7, IL-9, and IL-21, each of which uses the γ_c chain for transactivation of their specific receptor chain. X-ray[2,37] and mutational analyses,[38] and molecular modeling[39] of receptor complexes suggest that all interleukins of the γ_c family expose a "γ_c recognition motif"[2,39] similar to IL-4. Although amino acid residues within interleukins of the γ_c family show low sequence similarities, equivalent side chains exert similar structural roles in the cytokine/γ_c complexes. In all receptor complexes, side chains at positions corresponding to IL-4 Arg121 are located at the center of the binding epitope. It will be interesting to see if site-specific chemical modification at this site will disrupt γ_c binding and create antagonists in other cytokines of the γ_c family.

It is noteworthy that chemical modification of proteins offers opportunities that are not possible with genetic modification. For instance, modification with PEG may generate analogues which are less immunogenic than a mutein with a substituted amino acid side chain.[40,41] Chemical modification might also allow for targeting of interfaces, which are otherwise difficult or impossible to disrupt via mutations of amino acid residues. While it is not usually possible to inactivate overlapping binding epitopes with single mutations,[20] there is potential for more than one epitope to be disrupted via a single chemical

dx.doi.org/10.1021/bc400307k | Bioconjugate Chem. 2014, 25, 52−62

modification. Where a mutation only partially disrupts binding affinity, a chemical modification might prove more efficient. Moreover, the ability to generate an antagonist while performing PEG modification of a single site is certainly an attractive technical advantage. In conclusion, disrupting a protein−protein interface using site-directed chemical modification offers the potential to complement and even extend the possibilities presently available via genetic modification.

■ ASSOCIATED CONTENT

Supporting Information

Compilation of surface areas of IL-4WT residues buried upon complex formation with γ_c or IL-13Rα1; the interaction of IL-4 analogues with the IL-4Rα complex; agonist and antagonist activity of IL-4 R121C analogues in the HEK-Blue cell assay; compilation of types of synthesis, names, structural formulas, and abbreviations of the IL-4 analogues used in this study; the kinetics of enzymatic reduction of the glutathionylated IL-4 analogue 121-SS-G; mass spectrometry and SDS−PAGE analysis of IL-4 R121C analogues; surface plasmon resonance (SPR) analyses of the interaction of selected IL-4 analogues with γc and IL-13Rα1 receptor ectodomains. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Physiological Chemistry II, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany. Fax: +49-931-3184113. E-mail: viswanadham@daad-alumni.de,

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank N. Seher for excellent technical assistance and Dr. K. Sugama and Dr. T. Schulze for valuable discussions.

■ ABBREVIATIONS

IL-4 analogue nomenclature (see also Figures S1 and S2, Supporting Information), the number specifies the position of the engineered cysteine in the IL-4 mutein; -SS- or −MA-, indicates the presence of a disulfide or maleimide linker, respectively; the ending code indicates the presence of a conjugated PEG (polyethylenglycol); E, ethyl; EA, ethylamine; ALA, -(*S*)-alanine; TG, thioglycolate; CA, cysteamine; G, glutathione; 121SH, designates the IL-4 R121C mutein with a free thiol group

■ REFERENCES

(1) Hage, T., Sebald, W., and Reinemer, P. (1999) Crystal structure of the interleukin-4/receptor alpha chain complex reveals a mosaic binding interface. *Cell 97*, 271−281.

(2) LaPorte, S. L., Juo, Z. S., Vaclavikova, J., Colf, L. A., Qi, X., Heller, N. M., Keegan, A. D., and Garcia, K. C. (2008) Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell 132*, 259−272.

(3) Sebald, W., Nickel, J., Zhang, J. L., and Mueller, T. D. (2010) Molecular basis of cytokine signalling–theme and variations. *FEBS J. 277*, 106−118.

(4) Junttila, I. S., Mizukami, K., Dickensheets, H., Meier-Schellersheim, M., Yamane, H., Donnelly, R. P., and Paul, W. E. (2008) Tuning sensitivity to IL-4 and IL-13: differential expression of IL-4Ralpha, IL-13Ralpha1, and gammac regulates relative cytokine sensitivity. *J. Exp. Med. 205*, 2595−2608.

(5) Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. (1999) The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol. 17*, 701−738.

(6) Coffman, R. L. (2013) Converging discoveries: the first reports of IL-4. *J. Immunol. 190*, 847−848.

(7) Liang, H. E., Reinhardt, R. L., Bando, J. K., Sullivan, B. M., Ho, I. C., and Locksley, R. M. (2012) Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat. Immunol. 13*, 58−66.

(8) Paul, W. E., and Zhu, J. (2010) How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol. 10*, 225−235.

(9) Ramalingam, T. R., Pesce, J. T., Sheikh, F., Cheever, A. W., Mentink-Kane, M. M., Wilson, M. S., Stevens, S., Valenzuela, D. M., Murphy, A. J., Yancopoulos, G. D., Urban, J. F., Jr., Donnelly, R. P., and Wynn, T. A. (2008) Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat. Immunol. 9*, 25−33.

(10) Brandt, E. B., and Sivaprasad, U. (2011) Th2 cytokines and atopic dermatitis. *J. Clin. Cell. Immunol. 2*, 110−120.

(11) Ingram, J. L., and Kraft, M. (2012) IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J. Allergy Clin. Immunol. 130*, 829−842 quiz 843−844..

(12) Kuperman, D. A., and Schleimer, R. P. (2008) Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. *Curr. Mol. Med. 8*, 384−392.

(13) Arron, J. R., Scheerens, H., and Matthews, J. G. (2013) Redefining approaches to asthma: developing targeted biologic therapies. *Adv. Pharmacol. 66*, 1−49.

(14) Li, Z., Jiang, J., Wang, Z., Zhang, J., Xiao, M., Wang, C., Lu, Y., and Qin, Z. (2008) Endogenous interleukin-4 promotes tumor development by increasing tumor cell resistance to apoptosis. *Cancer Res. 68*, 8687−8694.

(15) Di Stefano, A. B., Iovino, F., Lombardo, Y., Eterno, V., Hoger, T., Dieli, F., Stassi, G., and Todaro, M. (2010) Survivin is regulated by interleukin-4 in colon cancer stem cells. *J. Cell. Physiol. 225*, 555−561.

(16) Smith, L. J., Redfield, C., Smith, R. A., Dobson, C. M., Clore, G. M., Gronenborn, A. M., Walter, M. R., Naganbushan, T. L., and Wlodawer, A. (1994) Comparison of four independently determined structures of human recombinant interleukin-4. *Nat. Struct. Biol. 1*, 301−310.

(17) Kelly-Welch, A. E., Hanson, E. M., Boothby, M. R., and Keegan, A. D. (2003) Interleukin-4 and interleukin-13 signaling connections maps. *Science 300*, 1527−1528.

(18) Mueller, T. D., Zhang, J. L., Sebald, W., and Duschl, A. (2002) Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim. Biophys. Acta 1592*, 237−250.

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

(20) Tony, H. P., Shen, B. J., Reusch, P., and Sebald, W. (1994) Design of human interleukin-4 antagonists inhibiting interleukin-4-dependent and interleukin-13-dependent responses in T-cells and B-cells with high efficiency. *Eur. J. Biochem. 225*, 659−665.

(21) Tomkinson, A., Tepper, J., Morton, M., Bowden, A., Stevens, L., Harris, P., Lindell, D., Fitch, N., Gundel, R., and Getz, E. B. (2010) Inhaled vs subcutaneous effects of a dual IL-4/IL-13 antagonist in a monkey model of asthma. *Allergy 65*, 69−77.

(22) Wenzel, S., Wilbraham, D., Fuller, R., Getz, E. B., and Longphre, M. (2007) Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet 370*, 1422−1431.

(23) Kruse, N., Shen, B. J., Arnold, S., Tony, H. P., Muller, T., and 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.

(24) Kreitman, R. J., Puri, R. K., Leland, P., Lee, B., and Pastan, I. (1994) Site-specific conjugation to interleukin 4 containing mutated cysteine residues produces interleukin 4-toxin conjugates with improved binding and activity. *Biochemistry 33*, 11637−11644.

(25) Pan, C., Roczniak, S., Greve, J. M., Yung, S. L., Longphre, M., Wong, T. M. and Tomkinson, A. (2008) Modified IL-4 Mutein Receptor Antagonists, United States Patent, US 7,404,957 B2.
(26) Duppatla, V., Gjorgjevikj, M., Schmitz, W., Kottmair, M., Mueller, T. D., and Sebald, W. (2012) Enzymatic deglutathionylation to generate interleukin-4 cysteine muteins with free thiol. *Bioconjugate Chem. 23*, 1396−1405.
(27) Shen, B. J., Hage, T., and Sebald, W. (1996) Global and local determinants for the kinetics of interleukin-4/interleukin-4 receptor alpha chain interaction. A biosensor study employing recombinant interleukin-4-binding protein. *Eur. J. Biochem. 240*, 252−261.
(28) Drechsler, J., Grotzinger, J., and Hermanns, H. M. (2012) Characterization of the rat oncostatin M receptor complex which resembles the human, but differs from the murine cytokine receptor. *PLoS One 7*, e43155.
(29) Letzelter, F., Wang, Y., and Sebald, W. (1998) The interleukin-4 site-2 epitope determining binding of the common receptor gamma chain. *Eur. J. Biochem. 257*, 11−20.
(30) Kioi, M., Kawakami, K., and Puri, R. K. (2004) Mechanism of action of interleukin-13 antagonist (IL-13E13K) in cells expressing various types of IL-4R. *Cell. Immunol. 229*, 41−51.
(31) Park, L. S., Friend, D., Sassenfeld, H. M., and Urdal, D. L. (1987) Characterization of the human B cell stimulatory factor 1 receptor. *J. Exp. Med. 166*, 476−488.
(32) Shanafelt, A. B., Forte, C. P., Kasper, J. J., Sanchez-Pescador, L., Wetzel, M., Gundel, R., and Greve, J. M. (1998) An immune cell-selective interleukin 4 agonist. *Proc. Natl. Acad. Sci. U.S.A. 95*, 9454−9458.
(33) Kioi, M., Kawakami, K., and Puri, R. K. (2004) Mechanism of action of interleukin-13 antagonist (IL-13E13K) in cells expressing various types of IL-4R. *Cell. Immunol. 229*, 41−51.
(34) Letzelter, F., Wang, Y., and Sebald, W. (1998) The interleukin-4 site-2 epitope determining binding of the common receptor gamma chain. *Eur. J. Biochem. 257*, 11−20.
(35) Junttila, I. S., Creusot, R. J., Moraga, I., Bates, D. L., Wong, M. T., Alonso, M. N., Suhoski, M. M., Lupardus, P., Meier-Schellersheim, M., Engleman, E. G., Utz, P. J., Fathman, C. G., Paul, W. E., and Garcia, K. C. (2012) Redirecting cell-type specific cytokine responses with engineered interleukin-4 superkines. *Nat. Chem. Biol. 8*, 990−998.
(36) Piehler, J., Thomas, C., Garcia, K. C., and Schreiber, G. (2012) Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation. *Immunol. Rev. 250*, 317−334.
(37) Ring, A. M., Lin, J. X., Feng, D., Mitra, S., Rickert, M., Bowman, G. R., Pande, V. S., Li, P., Moraga, I., Spolski, R., Ozkan, E., Leonard, W. J., and Garcia, K. C. (2012) Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat. Immunol. 13*, 1187−1195.
(38) Kang, L., Bondensgaard, K., Li, T., Hartmann, R., and Hjorth, S. A. (2010) Rational design of interleukin-21 antagonist through selective elimination of the gammaC binding epitope. *J. Biol. Chem. 285*, 12223−12231.
(39) Walsh, S. T. (2012) Structural insights into the common gamma-chain family of cytokines and receptors from the interleukin-7 pathway. *Immunol. Rev. 250*, 303−316.
(40) Bailon, P., and Won, C. Y. (2009) PEG-modified biopharmaceuticals. *Expert Opin. Drug Delivery 6*, 1−16.
(41) Gefen, T., Vaya, J., Khatib, S., Harkevich, N., Artoul, F., Heller, E. D., Pitcovski, J., and Aizenshtein, E. (2013) The impact of PEGylation on protein immunogenicity. *Int. Immunopharmacol. 15*, 254−259.