Molecular Characterization and Functional Analysis of Murine Interleukin 4 Receptor Allotypes

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Summary

The murine interleukin 4 receptor (IL-4R) exists as a transmembrane protein transducing pleiotropic IL-4 functions, or as soluble (s)IL-4–binding molecule with potent immunoregulatory effects. In this study we identified and characterized a murine IL-4R allotype. Sequence analysis of the IL-4R cDNA of BALB/c mice revealed 18 base substitutions leading to three extracellular and five cytoplasmic amino acid changes when compared with the published IL-4R sequence of C57BL/6 mice. Analyses with allotype-specific mAbs revealed that AKR/J and SJL/J mice possess the newly identified BALB/c IL-4R allotype whereas the IL-4Rs of C3H, CBA, DBA-2, and FVB/N mice are identical to that of the C57BL/6 mouse. The extracellular Thr49 to Ile substitution abrogates one *N*-glycosylation site in the naturally occurring BALB/c IL-4R as well as in the experimentally point mutated C57BL/6-T49I sIL-4R, and both molecules display a nearly threefold reduction in IL-4–neutralizing activity compared to the C57BL/6 sIL-4R. In line with this, a significantly enhanced dissociation rate of IL-4 was detected for the BALB/c IL-4R allotype by surface plasmon resonance and in radioligand binding studies with IL-4R–transfected cell lines. These findings suggest that the altered ligand binding behavior of the newly described IL-4R allotype may influence the IL-4 responsiveness, thus contributing to the diverse phenotypes of inbred mouse strains in IL-4–dependent diseases.

Interleukin 4 (IL-4) is a multifunctional cytokine produced by T cells, mast cells, and basophils (for review see reference 1). On B cells IL-4 stimulates proliferation and differentiation, induces the expression of MHC II molecules as well as CD23, and is indispensable for the Ig heavy chain class switching to IgE in mice (for review see reference 2). Cells of the monocytic lineage display enhanced levels of MHC II molecules correlating with an increased antigen-presenting function of macrophages, whereas the production of proinflammatory cytokines is downregulated in the presence of IL-4 (for review see reference 3). In the T cell compartment, IL-4 is required for the development of Th2, which was demonstrated by the use of anti–IL-4 strategies such as neutralizing Abs (4, 5), soluble (s)IL-4[1] receptors (6, 7), or IL-4 gene deficient mice (8) in a variety of infectious disease models, e.g., in susceptible BALB/c mice infected with *Leishmania major* the early IL-4 production is responsible for the severe disease (for review see reference 9). In allergic diseases of humans there is a strong correlation of the frequencies of IL-4–producing Th and serum IgE concentrations (for review see reference 10). This contrasts with helminth infections, e.g., with *Trichuris muris*, where IL-4 protects the host by induction of a Th2 response. A protective role of Th-derived IL-4 was also demonstrated in mouse models of autoimmune diseases such as experimental allergic encephalomyelitis (EAE; reference 11) and lupus-like autoimmune syndrome (12).

The pleiotropic activities of IL-4 are mediated by a high-affinity receptor (K_d = 20–300 pM), which is expressed in low numbers (100–5,000) on a variety of cell types (13). The majority of cells express IL-4R in association with the common γ chain (14), which is needed, at least in T cells, for IL-4 signal transduction. Cloning of the cDNAs coding for the mouse IL-4R (15, 16) and human IL-4R (17, 18) and their expression in COS7 cells resulted in glycosylated transmembrane proteins with a molecular mass of 140 kD. The murine and human IL-4R share ~50% homology on the amino acid level and both molecules share the two cysteine pairs and the WSXWS motif in their extracellular domain, which are characteristic for type I cytokine receptors. In the case of the murine IL-4R, an additional cDNA derived by alternative spliced mRNA coding for a 40 kD sIL-4R has been identified (15).

Allelic variation was first characterized for Igs, HLA/MHC-antigens, TCRs, and complement factors, but,

[1] *Abbreviations used in this paper:* EAE, experimental allergic encephalomyelitis; s, soluble.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/11/1419/11 $2.00
Volume 186, Number 9, November 3, 1997 1419–1429
http://www.jem.org

meanwhile, this important evolutionary mechanism has also been shown for a large number of other molecules. The murine IL-2 is the first example of a cytokine encoded by at least five distinct alleles comprising polymorphic IL-2 molecules differing in their biological activity for the proliferation of human PBL and T cells (19). As shown recently, the expression of a mutant allele of the chemokine receptor 5 in HIV-1–resistant Caucasian patients leads to a nonfunctional receptor that reduces fusion or infection of cells by primary monocytotrophic HIV-1 strains (20). Mice homozygous for a point mutation in the cytoplasmic domain of Fas, which inhibits Fas signal transduction, develop lymphadenopathy and SLE-like autoimmune disease due to a defect in Fas-mediated apoptosis (21). In the case of the leptin receptor the mutant allele encodes a truncated receptor unable to induce activation of STAT (signal transducer and activator of transcription) proteins, causing obesity of mice (22). Typically, these described allelic variations result in the loss of function by major structural alterations of the respective gene products, thereby causing different diseases.

In this work we present the first example for an allelic variation within the type I cytokine receptor family. The naturally occurring allotypes of the murine IL-4R differ not only in their amino acid sequence, but also in their functional properties. These allotypic differences may result in divergent outcomes of IL-4–dependent immune responses in vivo.

Materials and Methods

Mice. Female mice of the inbred strains AKR/J, BALB/c, C3H/HeN, C57BL/6, CBA, C.B-17/SCID, DBA/2, FVB/N, and SJL/J were obtained from Charles River Labs. (Sulzfeld, Germany) and used at 6–12 wk of age.

Cell Clones and Lines. The BALB/c mouse–derived Th2 clone L1/1, specific for the protozoan parasite *L. major*, was provided by M. Lohoff (Institute of Clinical Microbiology and Immunology, Erlangen, Germany; reference 23). The mouse Th HT-2 and the human erythroleukemic cell line TF-1 (24) was obtained from the American Type Culture Collection (Rockville, MD). The human kidney cell line 293-EBNA was purchased from Invitrogen (Leek, The Netherlands).

RNA Isolation, Reverse Transcription, and PCR. After RNA extraction from mouse spleen cells with acidic guanidinium thiocyanate (25), cDNAs were synthesized with reverse transcriptase (Pharmacia Biotech, Freiburg, Germany) as previously described (6). For PCR, cDNAs were amplified in a 40-μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 1 mM of each dNTP, 1 U of *Taq* (Pharmacia Biotech) or PFU (Stratagene, Heidelberg, Germany) polymerase, and 100 nM of primers during 35 cycles (1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 72°C). Primers used for PCR were as follows: (*a*) sense primer 1, 5′-GCAGGCACCTTTTGTGTCCCC-3′ (position −97 to −77), and antisense primer 2, 5′-CTCACCACCGCAGCCCCCAAGGTCA-3′ (position 624–648), for the murine sIL-4R (product length 745 bp); and (*b*) primer 1 and antisense primer 3, 5′- CCCTGGCCTCAGCACAGACCTC-3′ (position 2383–2404), for the transmembrane form of the murine IL-4R (product length 2,501 bp). Samples were separated on 1.5% agarose gels containing 0.2 μg/ml ethidium bromide.

Sequence Analysis. Sequence analysis of cloned or PCR-amplified sIL-4R or IL-4R cDNAs was performed using the dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Warrington, UK) as recommended by the manufacturer. Primers used were as follows: in sense orientation, primer 1 and primers 5′-GCCCCAGTGGTAATGTGAAGCCCC-3′ (position 275–298), 5′-TCAACCAAGTACCCGCACTGGA-3′ (position 814–835), 5′-GAGCTGGCTCCAGAGCAGCAGC-3′ (position 1468–1489); and in antisense orientation, primer 2, primer 3, and primers 5′-TCGGGAAGCTCAGCCTGGGTT-3′ (position 470–490), 5′-TTCAGCAAGCAAGGCAGCAGC-3′ (position 852–872), 5′-ATGAGATAGTCAAAGAGGACCTGTAGG-3′ (position 1060–1086), and 5′-CGGGCTTGGAGGCTCCTCTTCT-3′ (position 1506–1527).

Restriction Analysis. The 745-bp PCR fragments obtained from the sIL-4R cDNAs were restricted with the enzymes Eco-47III or HhaI. Resulting fragments were analyzed on 1.5% agarose gels containing 0.2 μg/ml ethidium bromide and compared with the fragment sizes calculated with the Lasergene software package (DNASTAR Inc., Madison, WI).

Analysis of Cell Culture Supernatants. The supernatants of transfected cells were analyzed by sandwich ELISAs. Seven independently derived rat hybridomas (1046-570-2, 1046-957-14, 1046-1273, 1046-1273-2, 1046-1658-13, 1046-1712, and 1046-1910) and nine mouse hybridomas (999-31, 999-258, 999-461, 999-643, 999-707, 999-927, 999-1243, 999-1366, and 999-1378) were raised after immunization of Lewis rats or BALB/c mice with purified recombinant C57BL/6 sIL-4R expressed in BHK-21 cells (6) by applying standard techniques. The anti–IL-4R mAbs produced by these hybridomas were purified by affinity chromatography with protein G–Sepharose (Pharmacia Biotech) and coupled to the bottom of microtiter plate wells in a concentration of 5 μg/ml. Detection was performed with the rat mAb M1 (Genzyme, Cambridge, MA) or with affinity-purified antiserum raised from a rabbit immunized with recombinant murine sIL-4R. Alkaline phosphatase–coupled Abs goat anti–rat or donkey anti–rabbit (Dianova, Hamburg, Germany) were used as secondary Abs. The ELISAs were standardized using purified recombinant murine sIL-4R (6) and had a working range of 40–2,000 pg/ml.

Murine IL-4R Expression Constructs. cDNAs prepared from Con A–stimulated (Sigma Chemical Co., Deisenhofen, Germany) spleen cells of C57BL/6 or BALB/c mice were used as templates for PCR with primers 1 and 2 for the sIL-4R or with primers 1 and 3 for the membrane-bound IL-4R. The products obtained with C57BL/6 and BALB/c cDNA were subcloned and served for the construction of sIL-4R-C57BL/6-BALB/c hybrid cDNAs by use of the internal and plasmid-encoded EcoRI restriction sites. For the C57BL/6-T49I sIL-4R construct, we applied the PCR mismatch technique using the IL-4R primer 1 and the antisense primer 5′-TTCCTCGGGATGCATATGAGGTTTTCAGAG-3′ (position 132–161; underlined nucleotide indicates the G to A exchange) to introduce a point mutation leading to the Thr49 to Ile substitution in the C57BL/6 sIL-4R peptide. The 5′-region of the wild-type sIL 4R cDNA was substituted for the mutated sequence using the NsiI restriction site. Resulting fragments were cloned into the eukaryotic expression vector pCEP4 (Invitrogen). The 2,501-bp cDNAs encoding the transmembrane IL-4Rs were cloned into the eukaryotic expression vector pM5neo, provided by W. Ostertag Heinrich-Pette-Institute (Hamburg, Germany).

Transfection and Cell Cloning. 15 μg of purified plasmid DNA was electroporated into 10^7 293-EBNA or TF-1 cells in 0.8 ml of medium (Click's RPMI 1640 [Life Technologies, Eggenstein, Germany], 2 mM l-glutamine, 10 mM Hepes, 100 μg/ml penicillin,

60 ng/ml streptomycin, 13 mM $NaHCO_3$ and 5×10^{-5} M 2-ME) supplemented with 10% (vol/vol) FCS (Biochrom, Berlin, Germany) at 900 μF and 260 V in an Easyject electroporation unit (Eurogentec, Seraing, Belgium). The selection of sIL-4R–transfected 293-EBNA cells was started after 36 h by addition of hygromycin (Calbiochem, Bad Soden, Germany) to a final concentration of 0.3 mg/ml. Cell supernatants were analyzed for their sIL-4R contents 3 wk after electroporation. Stably transfected TF-1 cells were selected by cultivation in the presence of 1 mg/ml G418 (Calbiochem) for 2 wk and subjected to single cell cloning.

Purification of Soluble Receptors. sIL-4R from supernatants of transfected 293-EBNA cells was affinity-purified using the rat mAb 1046-1712 coupled to an *N*-hydroxysuccinimide–activated HiTrap column (Pharmacia Biotech AB, Uppsala, Sweden). The eluted sIL-4R proteins were dialyzed and concentrated in Centrisart-C30 Microcentrifuge Filters (Sartorius AG, Goettingen, Germany). The purity of the IL-4R preparations was verified by silver-stained SDS-PAGE and the concentration was determined by ELISA and the AminoQuant Series II derivatization method (Hewlett Packard, Waldbronn, Germany), performed by Dr. J. Bernhagen (Fraunhofer Institut, Stuttgart, Germany) according to the manufacturer's recommendations.

Flow cytometry. Transfected TF-1 cells were analyzed by flow cytometric analysis on a FACScan® with Lysis software. IL-4R was detected by indirect immunofluorescence with M1, 1046 (rat) or 999 (mouse) mAbs as primary Abs and FITC-conjugated goat anti–rat Ab or rabbit anti–mouse Ab (Dianova) as secondary Abs.

SDS-PAGE and Western Blotting. TCA-precipitated supernatants or column elutions containing sIL-4R were separated under reducing conditions on a 15% SDS-PAGE gel. Immunodetection of IL-4R on nitrocellulose blots was performed with a rabbit antiserum followed by a swine anti–rabbit Ig conjugated with horseradish peroxidase (Dianova) and the enhanced chemiluminescence Western blotting system (Amersham, Braunschweig, Germany).

Cell Proliferation Assays. The murine Th cells L1/1 and HT-2 were incubated in medium for 48 h with recombinant murine IL-4 (IC Chemikalien, Ismaning, Germany) in the presence or absence of different concentrations of sIL-4R before [^{3}H]thymidine was added for the final 16 h of culture. The cells were harvested and [^{3}H]thymidine incorporation was measured in a β counter (Berthold, Munich, Germany). The ED_{50} values were calculated using Prism software (GraphPAD Software for Science, San Diego, CA).

Surface Plasmon Resonance. Measurements of receptor–ligand interactions were performed using a surface plasmon resonance detector (Pharmacia Biotech AB). Murine IL-4 was immobilized to a nonneutralizing anti–IL-4 mAb, which was coated to a CM5 sensor chip as described by the manufacturer (Pharmacia Biotech AB). The association and dissociation of the C57BL/6 and BALB/c sIL-4R in HBS buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20, pH 7.4) were analyzed at a flow rate of 5 μl/min at 25°C. Five different protein concentrations were analyzed for each sIL-4R preparation. Calculation of the rate constants was performed with the attendant evaluation software.

Iodination of Murine IL-4. 5 μg of recombinant murine IL-4 was iodinated as described by Lowenthal et al. (13) using Iodogen-coated (Pierce Chemical Co., Rockford, IL) coated glass tubes. Iodinated IL-4 (^{125}I–IL-4) was repurified by use of an anti–IL-4 affinity column and the concentrations of the IL-4 preparations were determined by ELISA. The specific activity of the ^{125}I–IL-4 was $\sim 9 \times 10^{14}$ cpm/mmol.

^{125}I–IL-4–binding Assays. Binding of ^{125}I–IL-4 to murine IL-4R expressed on human TF-1 cells was measured according to the methods described by Lowenthal et al. (13). For the equilibrium binding analysis, aliquots of 5×10^5 cells were incubated in the presence of different concentrations of ^{125}I–IL-4 in a final volume of 200 μl in microfuge tubes at 4°C until equilibrium had been achieved (90 min). To separate nonbound ^{125}I–IL-4 from cell-bound ^{125}I–IL-4, the reaction mixture was centrifuged through an oil gradient. Nonspecific binding was determined by addition of a 200-fold excess of unlabeled IL-4 and was subtracted from specific binding. For the dissociation kinetics, the IL-4R–transfected cells were incubated in the presence of 1,200 pM ^{125}I–IL-4 for 90 min at 4°C to obtain receptor saturation. Cells were then washed twice and ligand dissociation was allowed to proceed in medium at 4°C. Aliquots of 5×10^5 cells were taken at the indicated times and bound IL-4 was measured. Obtained data were calculated using Prism software.

Results

The Newly Identified BALB/c IL-4R Allotype Contains 18 Base Substitutions Leading to Eight Mainly Nonconservative Amino Acid Changes. Sequence analysis was performed with cloned or PCR-derived cDNAs coding for the transmembrane IL-4R or sIL-4R, respectively. The cDNAs derived from BALB/c mice were not identical in their base sequence, compared to the published data obtained from the T cell line CTLL or mast cell line MC/9 established from C57BL/6 mice (15, 16). We found 18 base substitutions, which were reproducibly detected in cDNAs obtained independently either from five BALB/c mice or the murine Th2 line L1/1, established from a BALB/c mouse (Table 1). Sequencing revealed no differences between C57BL/6, C3H, CBA, DBA/2, and FVB/N mice, whereas the mouse strains AKR/J and SJL/J showed the same base variations found in the extracellular part of the BALB/c IL-4R.

The predicted translated product of the BALB/c IL-4R cDNA varies in eight amino acids, compared to the published C57BL/6 IL-4R amino acid sequence. Three of the substitutions are located in the extracellular and five in the cytoplasmic part of the IL-4R protein (Table 1). The substitutions at positions 49 and 309 have been previously observed in genomic DNA derived from BALB/c mice (26). Of special interest are (*a*) the extracellular substitution of Cys34 to Arg leading to the loss of a potential disulfide bond partner in the BALB/c IL-4R and (*b*) the Thr49 to Ile substitution abrogating a potential *N*-glycosylation site (see below). Due to the charges of the substituted amino acids, the predicted isoelectric point of the sIL-4R is shifted from 5.16 to 5.38 in the BALB/c allotype.

We also sequenced the BALB/c cDNA of the common γ chain, which is also a component of the functional IL-4R complex (14). Compared to the published sequence, no base substitutions in the coding region of the common γ chain were present (data not shown), showing that the allotypic variation in the BALB/c IL-4R complex is confined to one receptor chain.

Since we were interested in whether there are allelic IL-4R variants in other mammalian species, e.g., humans, the

Table 1. *Differences in the Nucleotide and Amino Acid Sequence Comparing the IL-4Rs of C57BL/6 and BALB/c Mice*

	cDNA		Peptide		
Base*	C57BL/6	BALB/c	C57BL/6	BALB/c	aa‡
Extracellular region					
78	T	C			
100	T	C	Cys	Arg	34
105	A	G			
146	C	T	Thr	Ile	49
165	T	C			
174	T	C			
213	A	G			
447	A	T			
503	T	C	Met	Thr	168
Cytoplasmic region					
861	T	C			
926	T	C	Leu	Pro	309
945	A	G			
1046	A	G	Asn	Ser	349
1071	A	G	Ile	Met	357
1248	A	G			
1340	G	A	Gly	Asp	447
1802	A	G	Asp	Gly	601
2157	C	T			

*Numbers indicate the positions of the base exchanges counted from the first nucleotide coding for the mature IL-4R.
‡Numbers indicate the positions of the amino acid substitutions in the mature IL-4R peptide. The sequence data of the BALB/c IL-4R are available from EMBL/GenBank/DDBJ under accession number AF000304.

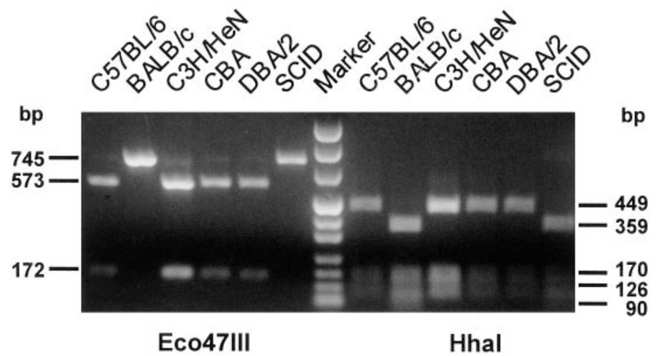

Figure 1. Restriction analysis of sIL-4R cDNAs. The sIL-4R cDNAs of the different mouse strains indicated were PCR-amplified, gel-purified, and digested with the enzymes Eco47III or HhaI. Resulting fragments were analyzed on a 1.5% agarose gel. The pUC Mix Marker (Fermentas AB, Vilnius, Lithuania) was used for the determination of the fragment sizes.

cDNAs coding for the extracellular IL-4R domain from the human cell lines TF-1 (24), HUT-78, HeLa, and Jurkat (all obtained from the American Type Culture Collection) were also sequenced. Only one A to G transition in the HeLa, Jurkat, and TF-1 cDNA was found, compared to the published sequence from T-22 cells (17, 18), leading to the conservative amino acid substitution Ile to Val at position 50 (data not shown).

IL-4R Alleles Are Detectable by DNA Restriction Analysis. The PCR-amplified sIL-4R cDNAs from different mouse strains were digested with the restriction enzymes Eco47III or HhaI, and the resulting fragments were size compared. Except for the PCR products from BALB/c and SCID mice, all tested DNA fragments were digested by Eco47III, yielding two fragments of 573 and 172 bp (Fig. 1). In the case of the BALB/c and SCID sIL-4R cDNAs, the T to C base substitution at position 78 led to the loss of the restriction site and to an undigestable DNA fragment of 745 bp. Digestion of PCR products from C57BL/6-type cDNAs with HhaI resulted in three fragments of 449, 170, and 126 bp, whereas the BALB/c and SCID cDNAs yielded four fragments of 359, 170, 126, and 90 bp due to an additional HhaI restriction site at position 168 (T to C substitution).

Allotype-dependent Binding and Epitope Mapping of mAbs Specific for the Extracellular Part of the IL-4R. A panel of mAbs obtained either by immunization of BALB/c mice with the recombinant C57BL/6 IL-4R or from similarly immunized rats was analyzed with regard to their IL-4R binding characteristics. mAbs were coupled to microtiter plates and the IL-4R ELISAs were developed with polyclonal rabbit Abs. While all of the murine mAbs were allotype specific, recognizing the C57BL/6-type IL-4R, but not the IL-4R of BALB/c, AKR/J, and SJL/J mice, the rat mAbs did not distinguish between IL-4Rs obtained from the different inbred strains of mice (Fig. 2). Thus, these binding data obtained with cell culture supernatants as well as sera (data not shown) confirmed the allotypic differences deduced from the cDNA sequences. To map the allotype-specific binding epitopes of the different murine mAbs, we cloned C57BL/6, BALB/c, and C57BL/6-BALB/c-hybrid sIL-4Rs and expressed them in 293-EBNA cells. The two hybrid receptors, C57BL/6-C34R-T49I and C57BL/6-M168T, were constructed using the internal restriction site EcoRI as indicated in Fig. 3. The PCR mismatch technique was used to introduce a single point mutation in the C57BL/6 sIL-4R cDNA leading to the C57BL/6-T49I sIL-4R.

Summarizing the mAb binding patterns, one-third of the allotype-specific mAbs (three out of nine) was dependent on the presence of Cys34, while the other mAbs were dependent on Met168. Interestingly, none of the allotype-specific mAbs appeared to be influenced by Thr49 and none of them displayed IL-4–neutralizing capacity (Table 2).

We also analyzed seven rat mAbs, which showed no detectable differences in their binding activity for all sIL-4R constructs (Table 2 and data not shown). Importantly, the binding behavior of the commercially available mAb M1 was quantitatively different for the two tested IL-4R allotypes: this mAb displayed threefold reduced OD_{410} values in the ELISAs over a broad concentration range when BALB/c or

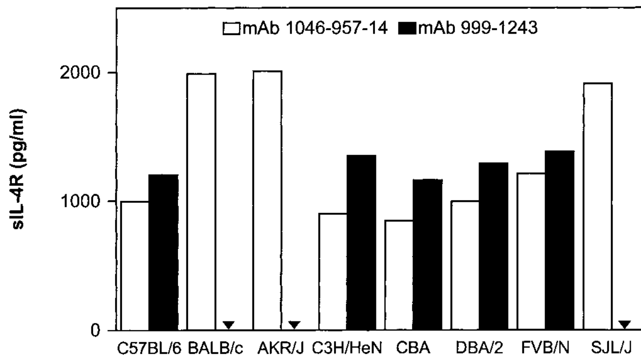

Figure 2. Comparison of sIL-4R ELISAs applying rat or mouse mAbs. Sandwich ELISAs were performed with either the rat mAb 1046-957-14 (*white bars*) or the mouse mAb 999-1243 (*black bars*) coupled to microtiter plates. Spleen cells of the indicated mouse strains were cultured for 48 h in the presence of 7.5 μg/ml Con A and the supernatants were analyzed in the ELISAs. Bound sIL-4R was detected with affinity-purified polyclonal IL-4R–specific rabbit Abs followed by donkey anti–rabbit Ig coupled to alkaline phosphatase.

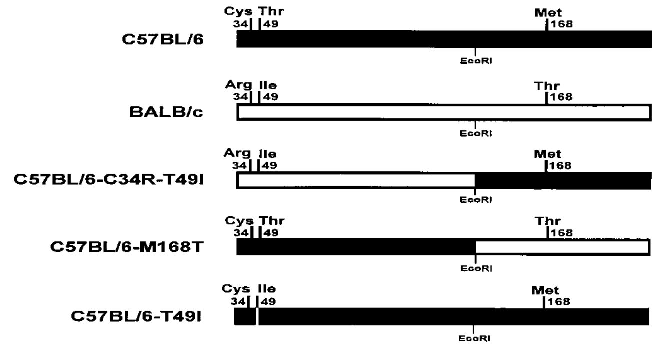

Figure 3. Murine sIL-4R constructs designated for the expression in 293-EBNA cells. Boxes represent the coding regions of the sIL-4Rs derived from C57BL/6 (*black*) or from BALB/c (*white*) mice. The amino acids differing between the C57BL/6 and BALB/c sequences are indicated. The names of the constructs include the substituted amino acids in the C57BL/6 sIL-4R and their position in the mature peptide. See Materials and Methods for details of the cloning strategies.

C57BL/6-T49I sIL-4Rs were compared with equivalent concentrations of the C57BL/6 IL-4R type in contrast to measurements with the polyclonal antiserum (data not shown). Thus, the sIL-4R quantification by this mAb is critically dependent on the presence of Thr49. The fact that M1 is the only neutralizing mAb of all tested antibodies leads to the hypothesis that Thr49 is either directly or indirectly involved in IL-4–IL-4R interactions. According to the model of human IL-4R (27), the substitutions at position 34 and 49 are located near the postulated IL-4–binding region of the IL-4R, whereas the Met168 is located more proximal to the cell surface.

To investigate whether the binding epitopes on the transmembrane IL-4R are accessible for the mouse mAbs,

Table 2. *Binding Epitopes of IL-4R–specific mAbs*

	ELISA*					Flow cytometry‡			
	C57BL/6	BALB/c	C57BL/6-C34R-T49I	C57BL/6-M168T	C57BL/6-T49I	C57BL/6	BALB/c	Epitope dependent on§	Neutralization of IL-4 function¶
Mouse mAb									
999-31	Yes	No	No	Yes	Yes	Yes	No	Cys34	No
999-258	Yes	No	Yes	No	Yes	No	No	Met168	No
999-461	Yes	No	No	Yes	Yes	Yes	No	Cys34	No
999-643	Yes	No	Yes	No	Yes	No	No	Met168	No
999-707	Yes	No	Yes	No	Yes	No	No	Met168	No
999-927	Yes	No	Yes	No	Yes	Yes	No	Met168	No
999-1243	Yes	No	Yes	No	Yes	No	No	Met168	No
999-1366	Yes	No	No	Yes	Yes	Yes	No	Cys34	No
999-1378	Yes	No	Yes	No	Yes	No	No	Met168	No
Rat mAb									
1046-957-14	Yes	Yes	Yes	Yes	Yes	Yes	Yes	—	No
1046-1658-13	Yes	Yes	Yes	Yes	Yes	No	No	—	No
M1	Yes	Yes[‖]	Yes[‖]	Yes	Yes[‖]	Yes	Yes	Thr49[‖]	Yes

*ELISA data obtained with the different sIL-4R constructs as designated in Fig. 3. Supernatants of transfected 293-EBNA cells were tested.
‡Flow cytometry was performed with transfected TF-1 cells expressing IL-4R allotypes.
§Amino acids of the C57BL/6 IL-4R located in the binding epitopes of the mouse mAbs as judged by the ELISA measurements.
¶The neutralizing function of the mAbs was detected by their capability to inhibit the IL-4–induced proliferation of HT-2 cells.
‖Binding of mAb M1 was present but the OD values in the ELISA were three-fold reduced as compared to the detection with a polyclonal antiserum raised against sIL-4R.

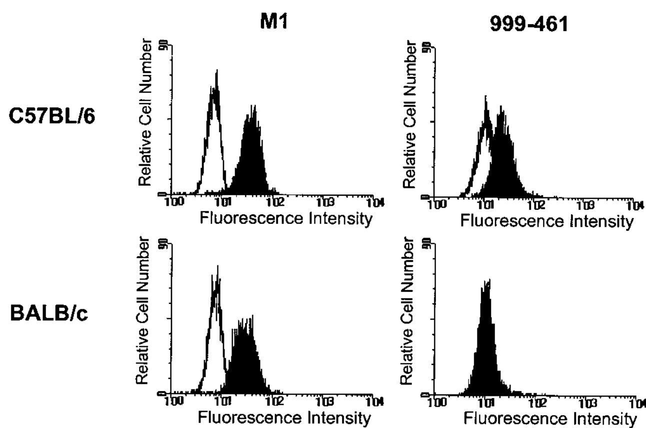

Figure 4. Flow cytometric analysis of IL-4R allotypes expressed on human TF-1 cells. Transfected TF-1 cells stably expressing the transmembrane IL-4R of C57BL/6 or BALB/c mice were stained with the rat mAb M1 or the mouse mAb 999-461, respectively. Fluorescence intensity of the background staining with the FITC-conjugated secondary Abs alone (*white graphs*) or the specific binding in the presence of the primary mAbs (*black graphs*) was determined by use of a FACScan®.

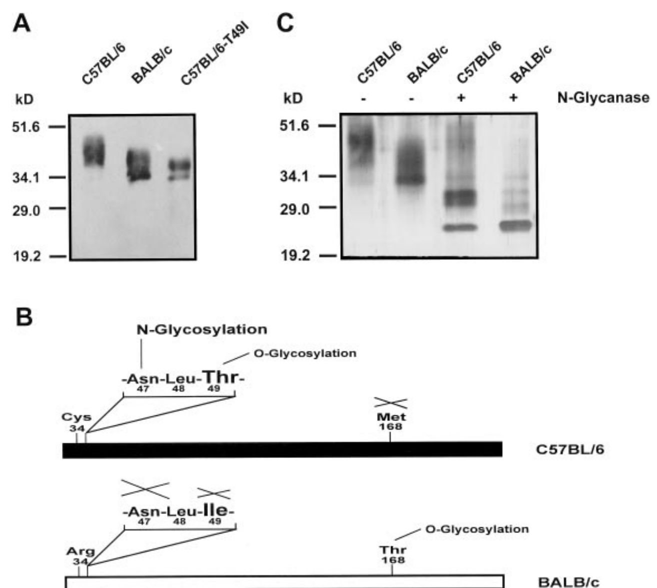

Figure 5. Analysis of the glycosylation of different sIL-4R variants. (*A*) Western blot of sIL-4R variants. Supernatants of transfected 293-EBNA cells expressing the C57BL/6, BALB/c, or C57BL/6-T49I sIL-4Rs were TCA-precipitated and separated on a 15% SDS-PAGE gel. Proteins were blotted onto nitrocellulose and IL-4R bands were detected with an IL-4R–specific polyclonal antiserum and visualized by enhanced chemiluminescence. (*B*) The potential *N*-glycosylation site of the C57BL/6 sIL-4R was absent in the BALB/c sIL-4R. The amino acids Asn47-Leu48-Thr49 found in the C57BL/6 sIL-4R (*black box*) represent an *N*-glycosylation site, which is abrogated in the BALB/c sIL-4R by the Thr49 to Ile substitution (*white box*). Threonins, which can be potentially *O*-glycosylated, are indicated. (*C*) SDS-PAGE of affinity-purified C57BL/6 and BALB/c sIL-4R allotypes. Purified sIL-4Rs were treated with (+) or without (−) *N*-glycanase for 16 h at 37°C. Samples were separated on a 15% SDS-PAGE gel and silver stained. (*A* and *C*) Molecular mass markers are indicated on the left.

FACS® analyses of transfected TF-1 cells were performed. The transfectants expressed functionally active C57BL/6 or BALB/c transmembrane IL-4Rs, since these factor-dependent human cells were grown with species-specific murine IL-4 for several months (data not shown). All three Cys34-dependent mAbs, but only one of six Met168-dependent mAbs, bound to the C57BL/6 IL-4R at the cell surface, whereas the BALB/c IL-4R was not recognized (Table 2 and Fig. 4). The mAb M1 was capable of staining both IL-4R allotypes expressed on the cell surface (Fig. 4). These results show that the Cys34-containing epitope is easily accessible whereas the region around Met168 may be more hidden through steric interactions with the cell membrane, with the IL-4R itself, or with other cell surface molecules. Two of the seven rat mAbs were able to bind to the cell surface IL-4R, but in an allotype-independent manner as expected (Table 2 and data not shown).

The BALB/c sIL-4R Lacks One N-glycosylation Site. The properties of the sIL-4R allotypes were compared in Western blot analyses with supernatants of transfected 293-EBNA cells. Compared to the C57BL/6 sIL-4R, the BALB/c sIL-4R and the mutated sIL-4R construct C57BL/6-T49I show an increased mobility on SDS-PAGE (Fig. 5 *A*) and share a Thr49 to Ile substitution. This exchange leads to the loss of a potential *N*-glycosylation site by inactivation of the recognition sequence (Asn^{47}-X^{48}-Thr^{49}) commonly used by oligosaccharyl transferases to fix the glycan to Asn as illustrated in Fig. 5 *B*. When we used *N*-glycanase to deglycosylate affinity-purified C57BL/6 and BALB/c sIL-4R, the differences between the migration behavior of these molecules vanished (Fig. 5 *C*). These findings and the migration behavior of the C57BL/6-T49I sIL-4R strongly argue for *N*-glycosylation of Asn47, which occurs only in the C57BL/6 but not in the BALB/c IL-4R allotype.

The Substitution of Thr49 to Ile Results in a Reduced IL-4–neutralizing Capacity of the BALB/c sIL-4R. Since the C57BL/6 and BALB/c sIL-4R display different biochemical properties, we addressed the question of whether the allotypic differences also result in functional changes. Therefore, the sIL-4R proteins were analyzed for their capacity to inhibit IL-4–induced proliferation of the Th2 clone L1/1 and HT-2 cells. The dose–response curves clearly revealed a diminished IL-4–neutralizing effect of the BALB/c sIL-4R when compared to the C57BL/6 sIL-4R, irrespective of the cell type used (Fig. 6 *A* and *B*). The calculated ED_{50} values obtained from seven experiments performed with different sIL-4R preparations averaged 640 ± 158 pM for the C57BL/6 and 1,650 ± 193 pM for the BALB/c sIL-4R. Interestingly, the C57BL/6-T49I sIL-4R construct displayed a dose–response curve (Fig. 6 *A* and *B*) and an ED_{50} value (1,370 ± 243 pM) very similar to that of the BALB/c sIL-4R. This indicates that the substitution of Thr49 and the resulting loss of N-glycosylation at this part of the IL-4R causes the allotypic differences of IL-4 neutralization.

Purified Recombinant C57BL/6 and BALB/c sIL-4R Allotypes Differ in their Association and Dissociation Constants for IL-4. To directly analyze the IL-4/IL-4R interactions, biomolecular interaction analysis was performed using the technique of surface plasmon resonance. The kinetics re-

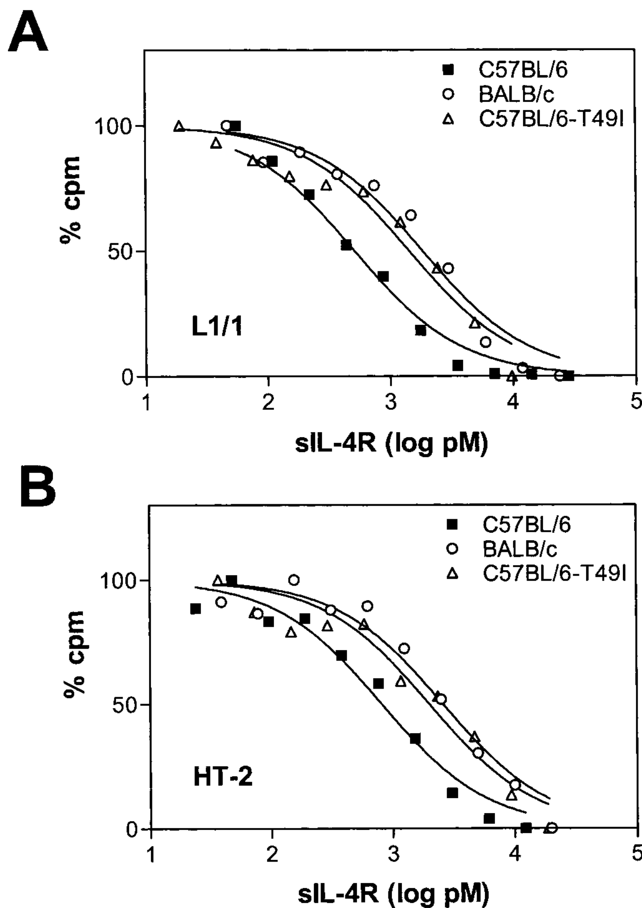

Figure 6. Inhibition of IL-4–induced proliferation by different sIL-4R variants. The Th L1/1 (*A*) or HT-2 (*B*) were incubated with 2 ng/ml recombinant murine IL-4 and serial diluted concentrations of C57BL/6, BALB/c, or C57BL/6-T49I sIL-4Rs, respectively. After 48 h [^{3}H]-thymidine was added and 16 h later the cells were harvested and radioactivity was measured in a β counter. Sigmoidal dose–response curves were calculated for each set of data.

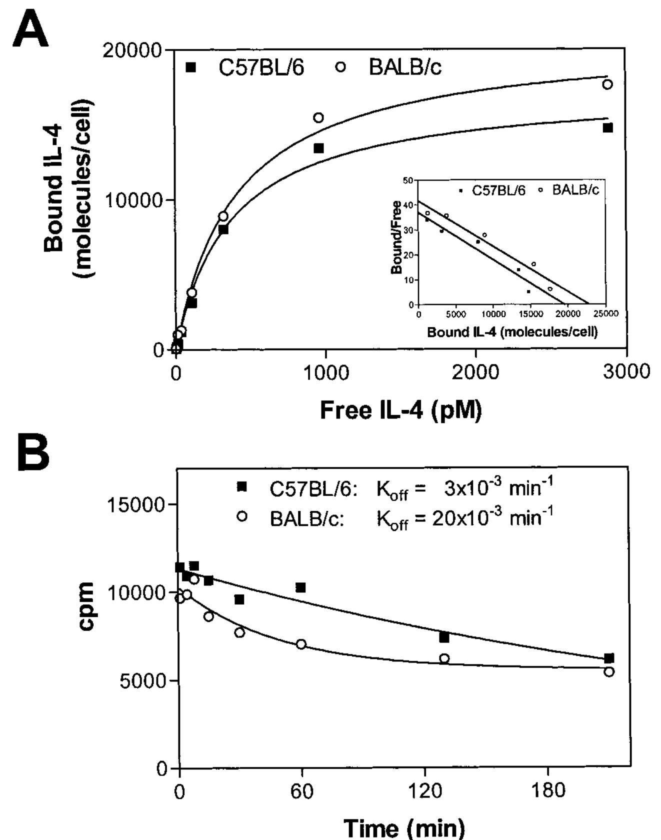

Figure 7. ^{125}I–IL-4–binding to the IL-4R allotypes expressed as cell surface molecules. Ligand binding analysis was performed with human TF-1 cells expressing C57BL/6 or BALB/c IL-4R. (*A*) Equilibrium binding studies. Cells were incubated with various concentrations of ^{125}I–IL-4 for 90 min at 4°C and assayed for binding as described in Materials and Methods. Data are corrected for nonspecific binding and are presented as saturation binding curve or Scatchard plot (*inset*). (*B*) Dissociation of IL-4 from the transmembrane receptors. Transfected TF-1 cells were incubated in the presence of 1.2 nM ^{125}I–IL-4 for 90 min at 4°C, washed twice, resuspended in medium, and incubated at 4°C. Aliquots of 5 × 10^5 cells were taken at the indicated time points and the amount of IL-4 still bound to the cell surface was determined. The calculated K_{off} values for this experiment are indicated.

vealed a twofold increased association rate (K_{on}) and a slightly increased dissociation rate (K_{off}) for the BALB/c sIL-4R in comparison to the C57BL/6 sIL-4R. Table 3 summarizes the data obtained with independently purified sIL-4R preparations. The affinity constant (K_d), which is defined as the quotient of K_{off}/K_{on}, revealed a relatively low affinity of both sIL-4R allotypes for IL-4 with this type of measurement.

Table 3. *Association (K_{on}) and Dissociation (K_{off}) Rates of sIL-4R Allotypes Evaluated with Surface Plasmon Resonance*

sIL-4R		K_{on} ($M^{-1} s^{-1}$)	K_{off} (s^{-1})	K_d (nM)
C57BL/6	Exp. 1	5.06×10^4	1.48×10^{-3}	29.25
	Exp. 2	4.33×10^4	1.30×10^{-3}	30.02
BALB/c	Exp. 1	8.73×10^4	2.07×10^{-3}	23.71
	Exp. 2	8.54×10^4	2.08×10^{-3}	24.36

Binding of sIL-4R allotypes to immobilized IL-4 and resulting K_{on} and K_{off} rates obtained with two independent protein preparations (Exp. 1 and 2). The affinity constant (K_d) is calculated by the equation K_{off}/K_{on}.

Cell Surface BALB/c IL-4R Complexes Show a Markedly Increased K_{off}. Since the experiments described thus far were performed exclusively with soluble and most probably monomeric IL-4R molecules, we set out to analyze the ligand-binding characteristics of IL-4R complexes expressed at the cell surface. Equilibrium binding analysis was performed with transfected human TF-1 cells expressing functionally active C57BL/6 or BALB/c allotypic IL-4Rs. The resulting saturation binding curves and Scatchard plots revealed K_d values of 370 and 410 pM for the C57BL/6 and BALB/c IL-4Rs, respectively, which were expressed in comparable numbers (17,000–22,000 molecules/cell) on the transfected TF-1 cells (Fig. 7 *A*). Importantly, the kinetic measurements revealed a markedly enhanced dissoci-

ation rate for the BALB/c IL-4R (Fig. 7 *B*), while the association rates were similar (200–260 $\times$ 10^6 M^{-1} min^{-1}). In several experiments, the calculated K_{off} values were in the range of 7–16-fold faster for the BALB/c IL-4R than for the C57BL/6 IL-4R.

Discussion

We have shown that the murine IL-4R exists in different allotypes in inbred strains of mice. To our knowledge, this represents the first example of a type I cytokine receptor encoded by two different alleles. These IL-4R allotypes differ in eight amino acids which result in differences in their functional properties concerning IL-4–binding by the transmembrane IL-4R as well as IL-4–neutralizing activity by the sIL-4R. This report also provides evidence that *N*-glycosylation at Asn47 of the IL-4R influences IL-4 binding. The established IL-4R allotype-specific mAbs and the PCR-based restriction analysis represent new tools to analyze the correlation between IL-4R allotype expression and disease phenotypes in mice.

It appears very unlikely that the newly discovered IL-4R allele arose spontaneously during the establishment or continuous breeding of the BALB/c inbred strain. First, the occurrence of 18 mutations within only 80 yr since the establishment of the BALB/c strain in 1913 is far from the known mutation rate of eukaryotic genes in evolution which is ~1 in 10^5 generations. Second, the fact that the sIL-4R sequences of AKR/J and SJL/J mice are identical to the allelic BALB/c IL-4R variant argue against a recent emergence of the IL-4R alleles, since no connection between dealers or breeders who established these three strains were found (Mouse Genome Database [MGD], Mouse Genome Informatics, The Jackson Laboratory [Bar Harbor, ME]. World Wide Web URL: http://www.informatics.jax.org/). Since the sIL-4R cDNAs of C3H, CBA, DBA/2, and FVB/N are identical to the published sequence, we speculate that the two defined murine allelic IL-4R variants existed before the establishment of inbred strains of mice and that a selective pressure had stabilized these IL-4R variants. At least in the molecules participating at the formation of the high-affinity IL-4R complex, the allelic variation of the IL-4R appears to be unique, because no sequence variation was found for the common γ chain. Our very limited analysis of human IL-4R sequences showed only a minor allotypic variation leading to a conservative amino acid substitution (Ile to Val), which is most likely irrelevant for the functioning of the human IL-4R. Nevertheless, more intense analysis of human IL-4R cDNAs is necessary to clarify whether functionally important IL-4R allotypes exist. Finding evidence of IL-4R allotypes in the human population and correlation with a disease such as allergy would be of obvious interest.

Focusing on the extracellular IL-4R domain the Cys34 substitution for the basic amino acid Arg in the BALB/c IL-4R alters not only the charge of the protein but may also lead to the loss of a potential disulfide bond. However, we consider the latter possibility unlikely because (*a*) the Cys34 is not conserved between the rat, human, and mouse IL-4Rs, and (*b*) the currently available model of the human IL-4R (27) does not display a disulfide bond at this part of the molecule. Two other exchanges in the extracellular part of the IL-4R lead to the substitution of a polar Thr against the nonpolar amino acids Ile or Met. However, the most important finding of our study is the fact that Asn47 is *N*-glycosylated in the C57BL/6 IL-4R and that the loss of this glycosylation site found either naturally in the BALB/c IL-4R or experimentally introduced by a single point mutation (C57BL/6-T49I variant) is responsible for the nearly threefold-reduced IL-4–neutralizing capacity of these IL-4R variants. Thus, we speculate that the glycan at Asn47 of the C57BL/6 IL-4R is either stabilizing the tertiary structure of the ligand-binding domain or influencing its accessibility for IL-4. Analogous findings have been reported for human CD2 where the elimination of the *N*-linked glycan, situated outside of the CD2/CD58 interface, resulted in a loss of the CD58-binding activity of CD2 (28). In the case of the human IFN-γ receptor, inhibition of *N*-linked glycosylation blocked human IFN-γ–binding capacity of the cell surface receptor molecules (29). A stringent requirement for *N*-linked glycosylation has also been shown for the basic fibroblast growth factor receptor (30). Moreover, at least partial glycosylation of the receptors for the epidermal growth factor (31) or insulin (32) was necessary to acquire ligand-binding capacity. On the other hand, inhibition of *N*-linked glycosylation or deglycosylation of LH receptors (33) or β-adrenergic receptors (34) did not influence ligand-binding properties.

Since none of the IL-4R allotype-specific mAbs block binding of IL-4, the allotypic amino acids appear not to be situated directly in the IL-4 binding groove of the IL-4R. However, like IL-4, the neutralizing rat mAb M1 displayed a threefold reduced binding to the sIL-4Rs lacking the *N*-glycosylation at Asn47. This is in line with the assumption that this mAb binds directly to the ligand-binding domain of the IL-4R, which is influenced by carbohydrate side chains.

The reduced IL-4–neutralizing capacity of the BALB/c sIL-4R may be best explained by the increased dissociation rate as compared to the C57BL/6 sIL-4R, as previously shown for other cytokine receptors. For example, the decreased IL-2–neutralizing ability of the soluble IL-2R β chain alone when compared to β/γ chain heterodimers is due to a higher dissociation rate (35). Although surface plasmon resonance measurements revealed a slightly faster dissociation rate for the BALB/c IL-4R, these differences were much more pronounced when receptor complexes expressed on the cell membrane were analyzed. This may be due to the different experimental settings (immobilized receptor versus immobilized ligand) and/or the participation of the common γ chain on the cell membrane leading to slower kinetics and higher K_d values.

Thus far, we have not analyzed the functional consequences of the five allotypic amino acid substitutions found in the cytoplasmic region of the murine IL-4R. Since these include two nonconservative substitutions in a domain crit-

ically involved in IL-4R signaling (Gessner, A., and M. Röllinghof, unpublished data) the effect of the intracellular IL-4R differences is in the center of ongoing investigations.

In the 10 yr since the original description of Th1 and Th2, the study of the frequencies of these populations and their role in various infectious and autoimmune diseases has become a major focus. The clinical outcome of several diseases is critically dependent on the type of Th response dominating and thereby controlling the effector functions of other cell types such as macrophages and B cells. The development of Th2 is induced by IL-4 (for review see reference 36), which dominates over IL-12, the main inducer of Th1 development, when both cytokines are present during the early Th differentiation (37). For BALB/c mice, the existing data suggest a defect in $CD4^+$ subset priming such that IL-4 is oversensitizing or overabundant. Several experimental findings support this hypothesis. First, during the early phase of *L. major* infection, there is a higher expression of IL-4 in BALB/c mice compared to other inbred strains, which is not downregulated during the subsequent weeks (38, 39). Second, priming of ovalbumin-specific TCR-transgenic $CD4^+$ T cells in vitro in the absence of exogenously added cytokines caused a much more Th2-like phenotype in cells on the BALB/c background than with precursors from the B10.D2 background (40). Third, using such TCR transgenic T cells, only 200 U/ml of IL-4 could drive full Th2 differentiation in vitro when cells were on the BALB/c background (41) in contrast to TCR transgenic T cells from B10.A mice, which required fivefold more IL-4 for Th2 differentiation (42).

The inherited differences in the capability to generate either a Th1 or Th2 response in vivo appear to be multigenically controlled (43, 44). In the case of EAE, the clinical severity correlates with the expression of Th1 cytokines (45) and the induction of Th2 results in clinical protection (11). Microsatellite exclusion mapping of backcrossed mice revealed that the disease susceptibility for EAE was most strongly linked to mouse chromosome 7 ($P < 0.001$; reference 44). Since the murine IL-4R gene is located on chromosome 7, it will be interesting to analyze the contribution of the IL-4R allotypes defined in our study to the Th1/Th2 development and disease susceptibility in this model of autoimmunity. In lupus-like renal disease of New Zealand White mice, another model of autoimmunity, one of the most strongly associated loci was also found to be located on mouse chromosome 7 ($P < 0.008$) within an interval containing the IL-4R gene (46).

For several reasons we speculate that the allotypic differences of the murine IL-4R may influence the function of IL-4 in vivo in several ways partially causing the IL-4 hyperresponsiveness of the BALB/c mouse. First, the reduced neutralizing activity of the newly identified IL-4R allotype could be of importance in situations with a molar excess of the sIL-4R to the ligand (less efficient IL-4 antagonist). Second, during the early phase of a specific T cell response, when the concentrations of IL-4 exceed those of the sIL-4R, the sIL-4R may act as a transport molecule for IL-4 (47). The higher dissociation rate of the BALB/c sIL-4R allotype could result in a more rapid release of IL-4 in microenvironments with IL-4–responsive cells, prolonging the half-life of IL-4 (transporter function). Third, the enhanced dissociation rate of IL-4 from the membrane-bound BALB/c IL-4R as detected in this study could result in more receptor signals per IL-4 molecule, since the shorter contact time of IL-4 might enable the ligand to contact more receptor molecules in a given period of time. In sum, the resulting inborn differences of IL-4 responsiveness might contribute to the observed differential tendencies of inbred mice to generate either a Th1- or a Th2-dominated immune response.

We thank Drs. Christian Bogdan and Klaus Schröppel for discussions and critical reading of the manuscript and Dr. Jürgen Bernhagen for performing the protein quantification by the amino acid derivatization method.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 263/A6).

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Received for publication 28 May 1997 and in revised form 11 August 1997.

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