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Expression and characterization of recombinant canine IL-13 receptor $\alpha 2$ protein and its biological activity in vitro

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Abstract

Our previous study showed that recombinant canine IL-13 (rcaIL-13) stimulated production of allergen-specific IgE in vitro by peripheral blood mononuclear cells (PBMC) from flea allergen-sensitized dogs. This has also been demonstrated using human IL-13 (huIL-13) and PBMC isolated from human allergy patients. The stimulatory activity of rcaIL-13 was specifically inhibited by a fusion protein of the extracellular domain of canine IL-13R $\alpha 2$ and the Fc fragment of canine IgG heavy chain (rcaIL-13R $\alpha 2$ -Fc). In this communication, we report the construction and expression of a non-fused recombinant extracellular domain of canine IL-13R $\alpha 2$ (rcaIL-13R $\alpha 2$) in an *E. coli* expression system. The *E. coli* expressed rcaIL-13R $\alpha 2$ was isolated in inclusion bodies, then solubilized in buffer containing denaturants and reducing agents. After refolding and purification, the biological activity of rcaIL-13R $\alpha 2$ was found in the monomer fraction resulting from gel filtration and ion exchange chromatographies. Biological activity of purified rcaIL-13R $\alpha 2$ was demonstrated by the specific inhibition of rcaIL-13 activity in a TF-1 cell proliferation assay. Additionally, rcaIL-13R $\alpha 2$ was found to be active in neutralizing rcaIL-13 induced upregulation of IgE mRNA levels in PBMCs of 'high IgE' dogs, which have been bred to exhibit a predisposition for high IgE production and are used as a model for allergic asthma. The data confirm our previous report that the regulatory effects of IL-13 on IgE production in canine PBMCs are similar to those reported in humans. Thus, allergic dogs, such as the 'high IgE' producing dogs, may be excellent models for research on IgE-mediated diseases in humans.

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Keywords: IL-13; IL-13 receptor; IgE; Canine cytokine

1. Introduction

Interleukin 13 (IL-13) is a cytokine produced by T cells and other cell types such as activated mast cells and basophils (Chomarat and Banchereau, 1998; Brinkmann and Kinzel, 1997; Burd et al., 1995). The biological functions of IL-13 include the stimulation of IgE production by human B cells (Corry, 1999) and the induction of eotaxin secretion

from the airway epithelial cells of mice (Li et al., 1999). Studies on human allergic patients indicate that the secretion of IL-13 by peripheral blood T cells after allergen stimulation was longer and at higher level in comparison to transient expression of IL-4 by the same cells (de Waal Malefyt et al., 1995; Minty et al., 1997). In mice, Wills-Karp et al. (1998) showed that IL-13, which induced many of the features of asthma in a mouse model, could be inhibited by the fusion protein of murine interleukin 13 receptor $\alpha 2$ chain and human IgG Fc-fragment (rmuIL-13R $\alpha 2$ -Fc).

Interleukin-13 receptor $\alpha 2$ chain (IL-13R $\alpha 2$) is a molecule that binds IL-13 specifically with high affinity (Caput et al., 1996; Donaldson et al., 1998). Over-expression of IL-13R $\alpha 2$ in some tumor cells has been reported (Caput et al., 1996; Debinski et al., 1995; Maini et al., 1997; Debinski et al., 1999). Transcripts for IL-13R $\alpha 2$ have been detected in many tissues of humans, mice and dogs (Donaldson et al., 1998; Tang, 2001). It is interesting to note that a soluble form of native IL-13R $\alpha 2$, which retained the high binding affinity to IL-13, was isolated from murine serum and

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urine (Zhang et al., 1997). Although the biological functions of IL-13R α 2 are not well defined, it has been proposed that the molecule might be involved in the regulation of IL-13 activity in vivo (Zhang et al., 1997; Chomarat and Banchereau, 1998). In order to better understand the interactions of IL-13R α 2 and IL-13, rmuIL-13R α 2-Fc was constructed and expressed in mammalian cells (Donaldson et al., 1998). After purification, rmuIL-13R α 2-Fc was used as a potent antagonist of mouse IL-13 in vitro and in vivo (Donaldson et al., 1998; Wills-Karp et al., 1998; Grünig et al., 1998).

We have recently cloned the cDNA encoding canine IL-13R α 2 (Tang, 2001) and constructed a recombinant fusion protein of the extracellular domain of canine IL-13R α 2 and Fc fragment of canine IgG (rcaIL-13R α 2-Fc) (Tang et al., 2001). Recombinant caIL-13R α 2-Fc was expressed in *E. coli*, refolded and purified, and shown to retain IL-13 binding specificity. Additionally, rcaIL-13R α 2-Fc inhibited the ability of canine IL-13 to stimulate IgE production by canine peripheral blood mononuclear cells (PBMCs) (Tang et al., 2001). The aim of the study reported here was to further characterize the biochemical and biological properties of canine IL-13R α 2. The cDNA encoding the extracellular domain of canine IL-13R α 2 was subcloned into p λ cro plasmid vector and expressed in *E. coli*. The biological activity of the recombinant protein was assessed in two in vitro assays.

2. Materials and methods

2.1. TF-1 cells culture and canine PBMC

TF-1 cells (R&D Systems, Minneapolis, MN) were maintained in RPMI-1640 culture media (Sigma Chemical Corporation, St. Louis, MO) as previously reported (Tang et al., 2001).

Peripheral blood mononuclear cells from four dogs were used in this study. Two were normal laboratory beagles (dog #4990 and #7689) and two were high IgE producing beagles (dog #1626B and #1626C) from Lovelace Respiratory Research Institute, Albuquerque, NM (Redman et al., 2001). The PBMCs were purified from whole blood by centrifugation through Ficoll (Density = 1.077, Amersham Pharmacia, Piscataway, NJ) at room temperature. The isolated PBMCs were washed in PBS pH 7.5 and cultured in RPMI media containing 5% normal dog serum (Gemini, Calabasas, CA), 200 mM L-glutamine, 50 μ g/ml gentamicin, 1 $\times$ non-essential amino acids, 1 $\times$ amino acids, 1 $\times$ sodium pyruvate and 5.5×10^{-5} M β -mercaptoethanol.

2.2. Construction and expression of rcaIL-13R α 2

The cDNA encoding the extracellular domain of caIL-13R α 2 (Tang, 2001) was subcloned into p λ cro plasmid vector (Tripp et al., 1996). The plasmid containing the correct caIL-13R α 2 cDNA insertion (pcaIL-13R α 2- λ cro) was confirmed by DNA sequencing and transfected into

E. coli, strain BL21-DE3 (Novagen Inc., Madison, WI). Expression of rcaIL-13R α 2 was constitutive in this host strain. *E. coli* containing pcaIL-13R α 2- λ cro were grown in LB media (1% tryptone, 0.5% yeast extract, 1% NaCl, Becton Dickinson, Sparks, MD) with 100 μ g/ml ampicillin at 30 °C to an optical density of 15–20 in a 4 l fermenter (New Brunswick, Edison, NJ). The cells were harvested by centrifugation at $6000 \times g$ for 20 min and resuspended (10 ml buffer/g wet weight cells) in 25 mM Tris, pH 7.5, 10 mM EDTA (cracking buffer).

2.3. Cell processing and solubilization of inclusion bodies containing rcaIL-13R α 2

The cells were broken at 23,000–27,600 psi in a microfluidizer (Microfluidics, Newton, MA) using 50 recirculating pulses. Inclusion bodies containing rcaIL-13R α 2 were recovered by centrifugation at $2000 \times g$ for 20 min. The pellet containing the inclusion bodies was resuspended in cracking buffer (10.0 ml/g cells) and centrifuged at $2000 \times g$ for 20 min. The inclusion bodies were washed a total of four times by resuspending the inclusion bodies in cracking buffer (10.0 ml/g cells), rocking at 4 °C for 30 min and centrifuging at $15,000 \times g$ for 20 min. The first three washes were performed in cracking buffer containing 0.5% deoxycholate and the fourth wash omitted the detergent. Washed inclusion bodies were solubilized in 25 mM Tris, pH 9.5, 8 M urea, 50 mM β -mercaptoethanol (solubilization buffer) at 4 °C for 30 min. Soluble rcaIL-13R α 2 was recovered in the supernatant after centrifugation at $15,000 \times g$ for 20 min.

2.4. Isolation and refolding of rcaIL-13R α 2

Soluble rcaIL-13R α 2 (25 g cells in 250 ml of solubilization buffer) was loaded onto a 20 ml DEAE Sepharose column (1.6 cm $\times$ 10 cm, Amersham Pharmacia, Piscataway, NJ), previously equilibrated with 25 mM Tris, pH 9.0, 8 M urea, 10 mM DTT. Bound protein was eluted at a flow rate of 5.0 ml/min with 25 mM Tris, pH 9.0, 8 M urea, 10 mM DTT, 1 M NaCl (DEAE elution buffer) using a linear gradient to 0.7 M NaCl over 15 column volumes (CV). The receptor recovered in the DEAE pool (80–100 mg protein at 0.8 mg/ml) was reduced by the addition of 6 mM DTT, 0.05% Tween 20 and incubated at 30 °C for 2 h. Refolding was initiated by dilution of the reaction mixture with 50 mM Tris, pH 10.0 to yield a refolding reaction mixture containing a final protein concentration of 80–100 μ g/ml, 0.6 M urea, 45 mM Tris, pH 10.0, 1.0 mM EDTA, and 2.5 mM oxidized glutathione (GSSG). Refolding was performed at 4 °C overnight with gentle stirring. The refolding mixture was dialyzed extensively against phosphate buffered saline (PBS), pH 7.2, at 4 °C and clarified by centrifugation at $10,000 \times g$ for 30 min. The purified, refolded rcaIL-13R α 2 was fractionated by 12% SDS-PAGE under reducing conditions, and then transferred onto a ProBlotTM membrane (Applied Biosystems, Foster City, CA) following manufacturer's protocol. The dominant

protein band on the membrane with predicted molecular mass for rcaIL-13R α 2 was removed for N-terminal amino acid sequencing.

2.5. Purification of caIL-13R α 2 monomers

Aggregates present in the refolded product were removed by loading the refolded product (in PBS, pH 7.2, buffer A) onto an anion exchange column (1.6 cm $\times$ 5 cm, Q-Sepharose Fast Flow, Amersham Pharmacia) previously equilibrated with buffer A. Bound refolded rcaIL-13R α 2 was eluted with buffer B (buffer A + 1.0 M NaCl) using a step gradient: 0–20% buffer B over 2 column volumes (CV), 20% buffer B for 5 CV, 20–60% buffer B over 2 CV, 60% buffer B for 5 CV, 60–100% buffer B over 2 CV. Refolded monomers eluted at 20% buffer B, while the misfolded aggregates eluted at 60% buffer B. Final protein concentration of the pooled rcaIL-13R α 2 monomers was measured by Bradford Assay (Biorad, Hercules, CA). Aliquots of the product were assayed for biological activity.

2.6. Biological activities of rcaIL-13R α 2

TF-1 cells (R&D Systems, Minneapolis, MN), a human erythroleukemia cell line, were maintained in RPMI-1640 culture media (Sigma Chemical Corporation, St. Louis, MO) supplemented with 2 mM L-glutamine (Life Technologies, Gaithersburg, MD), 5 μ g/ml gentamicin (Sigma Chemical Corporation), 10% fetal bovine serum (Summit, Fort Collins, CO) and 2 ng/ml recombinant human GM-CSF (rhuGM-CSF, R&D Systems, Minneapolis, MN). This is referred to as complete medium. Complete medium lacking rhuGM-CSF is referred to as assay medium. Cells were cultured in complete medium, in a humidified incubator, maintained at 37 °C with a mixture of 5% CO₂ and 95% ambient air.

The ability of rcaIL-13R α 2 to inhibit the biological activity of rcaIL-13 was determined by proliferation of TF-1 cells. The assay was controlled with wells containing only TF-1 cells and either rcaIL-13 in assay medium or assay medium alone. A constant amount of rcaIL-13 (125 ng/ml) was added to serial dilutions of the refolded forms of rcaIL-13R α 2 (0.1–18.5 μ g/ml either crude refold prior to anion exchange chromatography, or monomers and aggregates separated by anion exchange chromatography) in a sterile, 96-well flat bottom tissue culture plate and allowed to preincubate for 60 min at 37 °C. Cytokine and receptor were tested in a range of molar ratios from 1:48 (cytokine: receptor) to 1:0.38. TF-1 cells were extensively washed in assay medium to remove rhuGM-CSF, then added at 1×10^4 cells per well. Cells were incubated with rcaIL-13 or rcaIL-13/rcaIL-13R α 2 mixtures for 72 h, then pulsed with 1.0 μ Ci/well H³-thymidine (ICN Pharmaceuticals, Irvine, CA) and incubation continued for an additional 4 h. Contents of the wells were harvested onto glass fiber filters and counted in a Wallac Trilux 1450 scintillation counter (Wallac Inc., Gaithersburg, MD).

Data were collected in triplicate and averaged for each assay condition tested. The percent neutralization was calculated from the following formula: 100% – (competed average cpm/uncompeted average cpm $\times$ 100).

The specificity of rcaIL-13R α 2 in the assay was measured by substituting either recombinant canine IL-4 (rcaIL-4) (Heska Corporation, Ft. Collins, CO) or rhuGM-CSF (R&D Systems) for IL-13 in the same TF-1 cell culture. The concentrations of rcaIL-4 and rhuGM-CSF used in the study were 25 and 0.25 ng/ml, respectively.

2.7. Effects of rcaIL-13 and rcaIL-13R α 2 on total IgE mRNA levels produced by PBMC in culture

Canine PBMCs (2×10^6 /1.5 ml/well) isolated as described above were cultured in (a) RPMI medium only, (b) RPMI medium containing rcaIL-13 (50 ng/ml), (c) RPMI medium containing rcaIL-13 and monomeric rcaIL-13R α 2 (450 ng/ml) that had been incubated at 4 °C overnight or (d) RPMI medium containing rcaIL-13 and monomeric rcaIL-13R α 2 pre-mixed at room temperature immediately before addition to the cell cultures. Cells were incubated for 1 week under the conditions described. PBMCs from each culture condition were harvested and total RNA purified using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol.

2.8. Detection of total IgE mRNA in canine PBMC samples by RT-PCR

The first strand cDNAs of canine PBMC samples were synthesized using T-primed First-strand Kit (Ready To GoTM, Pharmacia Biotech Inc., USA). Canine hypoxanthine phosphoribosyl transferase (cHPRT) transcripts were used as the house keeping gene control in the RT-PCR. The canine HPRT primers cHPRT-3F (5'-TCAAGGGAG-GCTATAAATTC-3') and HPRT-9R (5'-GAACTCTCATC-TTAGGCT-3') were used in the PCR as previously reported (Tang, 2001). Specific canine IgE primers, cIgE226F (5'-TGTGGATCCAACCTTCATTCCGCCTACCGTG-3') and cIgE380R (5'-CCAGAATTCACAGGTCCACTACCAGGCA-GGT-3'), were designed based on the reported canine IgE cDNA sequence (GenBank Accession L36872). Amplification of cDNA samples was performed as follows: 95 °C for 8 min; then 38 cycles consisting of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min; followed by a single final extension at 72 °C for 5 min. The PCR products were analyzed by using 1% agarose gel electrophoresis.

3. Results

3.1. Expression and identification of rcaIL-13R α 2

DNA sequencing confirmed that the fragment encoding the caIL-13R α 2 subcloned into p λ cro matched the predicted

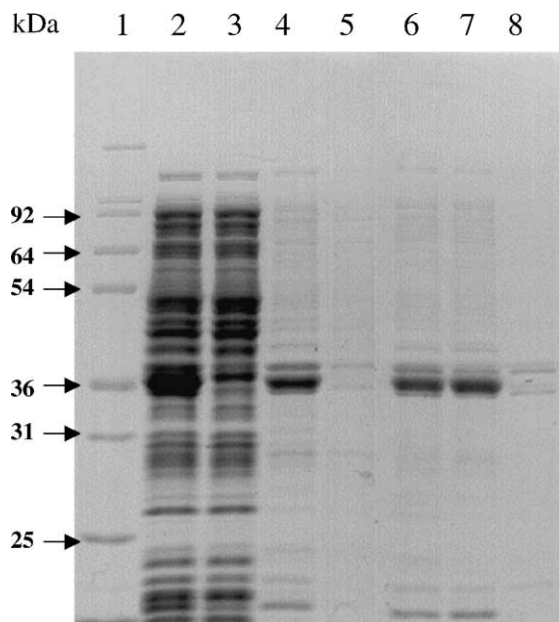


Fig. 1. Expression and subcellular localization of rcaIL-13R α 2. SDS-PAGE analysis of *E. coli* extract and fractions expressing rcaIL-13R α 2. Lanes: (1) molecular weight markers, (2) *E. coli* extract, (3) soluble *E. coli* fraction post lysis, (4) inclusion bodies, (5) soluble fraction post detergent wash, (6) inclusion bodies post detergent wash, (7) urea solubilized inclusion bodies, and (8) inclusion body fraction post urea solubilization.

sequence (data not shown). SDS-PAGE analysis of *E. coli* cells constitutively expressing rcaIL-13R α 2 showed the presence of a prominent band at 37 kDa corresponding to rcaIL-13R α 2 (Fig. 1, lane 2). Processing of the recombinant product showed that it was localized in insoluble inclusion bodies and could be solubilized in 8 M urea, 50 mM Tris, pH 9.5, 50 mM β -ME (Fig. 1, lanes 4 and 7). Solubilized rcaIL13R α 2 bound the DEAE anion exchange column, and SDS-PAGE analysis of the fractions showed that the product eluted at 0.2–0.25 M NaCl as a single 37 kDa band on SDS-PAGE under reducing conditions (data not shown). Amino acid sequencing of the purified 37 kDa protein indicated that the molecule had the correct amino acid sequence at the N-terminus as predicted for canine IL-13R α 2 (data not shown).

3.2. Refolding and purification of rcaIL-13R α 2

Recombinant canine IL-13R α 2 was refolded after partial purification as described in Section 2. Analysis of refolded rcaIL-13R α 2 by SDS-PAGE under non-reducing conditions showed the presence of high molecular weight bands (~70 kDa) suggesting that disulfide linked aggregates were present in the refolded mixture (data not shown). Gel filtration chromatography of the refolded product (1 $\times$ 30 HR Superdex S-200) showed that high molecular weight aggregates eluted at 13.0 ml (Fig. 2B, lane 2) while monomers eluted at 15.0 ml (Fig. 2B, lane 3) with the predicted molecular masses of 70 and 37 kDa, respectively. Q-Sepharose

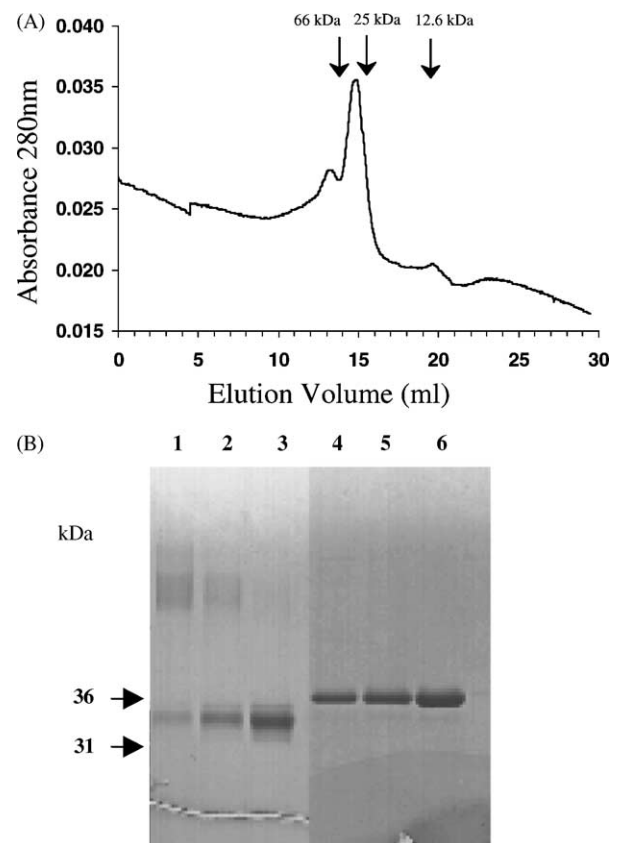


Fig. 2. Gel filtration chromatography of refolded rcaIL-13R α 2. (A) Refolded rcaIL-13R α 2 (200 mg in 500 ml) was loaded onto a Superdex S-200 column previously equilibrated with PBS, pH 7.2 as described in the text. Elution volume of bovine serum albumin (66 kDa), chymotrypsinogen (25 kDa) and cytochrome C (12.6 kDa) indicated by arrows. (B) SDS-PAGE analysis (14% Tris-glycine) of the S-200 fractions of refolded rcaIL-13R α 2. Lanes 1–3 are fractions at 13–15 ml elution volume under non-reducing conditions; lanes 4–6 are fractions at 13–15 ml elution volume under reducing conditions.

chromatography of refolded rcaIL-13R α 2 separated differently refolded forms of rcaIL-13R α 2 (Fig. 3). Two major peaks were eluted at 0.2 M NaCl (Peak A) and 0.6 M NaCl (Peak B) (Fig. 3A). SDS-PAGE analysis under reducing and non-reducing conditions showed that rcaIL-13R α 2 contained in Peak A migrated as a single band (Fig. 3A, Peak A; Fig. 3B, lanes 2 and 4). In comparison, the rcaIL-13R α 2 contained in Peak B migrated as a single 37 kDa band under reducing conditions on SDS-PAGE (Fig. 3A, Peak B; Fig. 3B, lane 3). However, under non-reducing conditions, the bulk of this material migrated as diffuse, high molecular weight aggregates (Fig. 3B, lane 5). The apparent molecular mass of protein in Peak A, under reducing conditions on SDS-PAGE, was approximately 37 kDa. However, under non-reducing conditions its apparent molecular weight was 33 kDa (Fig. 3B, lanes 2 and 4). These data strongly suggest that the refolded monomers present in Peak A contain intact intra-molecular disulfide bonds and are correctly refolded. Both Peak A and Peak B pools were assayed for bioactivity.

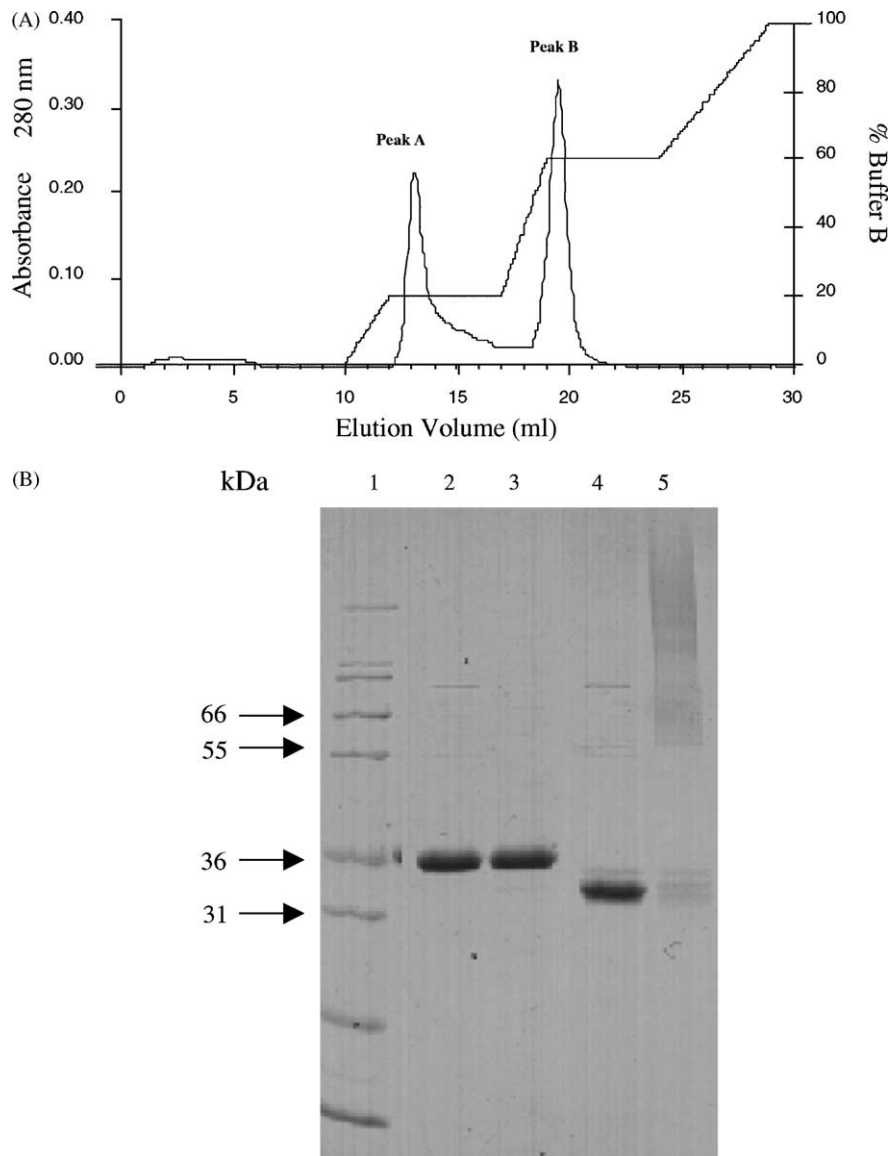


Fig. 3. Q-Sepharose chromatography of refolded rcaIL-13-R α 2. Refolded rcaIL-13-R α 2 was loaded onto an anion exchange column (1.6 cm $\times$ 5 cm, Q-Sepharose fast flow, Amersham Pharmacia, Piscataway, NJ) previously equilibrated with PBS, pH 7.2. (A) Bound protein was eluted as described in the text. (B) SDS-PAGE analysis of Peak A and B. Lane 1, Molecular weight marker; lane 2, Peak A under reducing conditions; lane 4, Peak A under non-reducing conditions; lane 3, Peak B under reducing conditions; lane 5, Peak B under non-reducing conditions.

3.3. Inhibition of IL-13 activity on TF-1 cells

Both rcaIL-13 and rhuIL-13 have been shown previously to stimulate TF-1 cell proliferation in culture (Yang et al., 2000; Tang et al., 2001). Pre-incubation of refolded and purified rcaIL-13R α 2 with rcaIL-13 inhibited rcaIL-13 induced stimulation of TF-1 cell proliferation in a dose dependent manner (Fig. 4). The refolded monomers of rcaIL-13R α 2 (Peak A) isolated by Q-Sepharose chromatography inhibited rcaIL-13 induced proliferation to a greater extent than either the crude refolded or the aggregated form of rcaIL-13R α 2 (Peak B) (Fig. 4). The concentration of the refolded form required to inhibit TF-1 cell proliferation by 50% (ND 50) were 10 μ g/ml, 0.3 μ g/ml, 0.15 μ g/ml for

the Peak B, crude refold, and Peak A, respectively (Fig. 4). The concentration in which rcaIL-13R α 2 inhibited more than 90% of rcaIL-13 dependent TF-1 cell proliferation was 0.6 μ g/ml which represented a molar ratio of rcaIL-13 to rcaIL-13R α 2 around 1:1.5. Purified rcaIL-13R α 2 did not inhibit rcaIL-4 and rhuGM-CSF in the stimulation of TF-1 cell proliferation (Fig. 4).

3.4. Inhibition of total IgE mRNA levels in PBMC stimulated by rcaIL-13

To test whether rcaIL-13R α 2 inhibited rcaIL-13 stimulated IgE mRNA synthesis from canine PBMCs, equivalent amounts of the first strand cDNAs from different dog PBMC

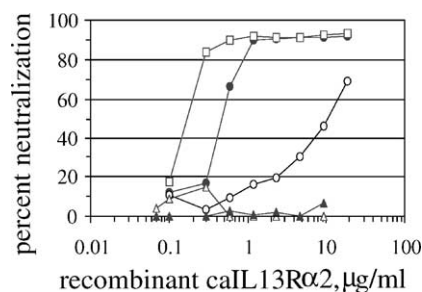


Fig. 4. Inhibition of rcaIL-13 induced proliferation of TF-1 cells by rcaIL-13R α 2. Varying concentrations of the Peak A refolded form of rcaIL-13R α 2 (0.1–18.5 μ g/ml) were pre-incubated with 125 ng/ml rcaIL-13, 25 ng/ml rcaIL-4, or 0.25 ng/ml rhuGM-CSF. TF-1 cells were incubated with the cytokine/rcaIL-13R α 2 mixtures for 72 h, then pulsed with H³-thymidine as described in the text. The percent neutralization of the IL-13 induced proliferation of TF-1 cells was calculated as described in the text. (●) Crude refolded rcaIL-13R α 2 with rcaIL-13; (□) purified rcaIL-13R α 2 (Peak A) with rcaIL-13; (○) purified rcaIL-13R α 2 (Peak B) with rcaIL-13; (Δ) rcaIL-13R α 2 (Peak A) with rcaIL-4 and (▲) rcaIL-13R α 2 (Peak A) with rhuGM-CSF.

samples were used in RT-PCR analysis, based upon the band intensity of control cHPRT DNA (Fig. 5). The RT-PCR results showed that total canine IgE mRNA levels were increased after stimulation of canine PBMCs with rcaIL-13 in

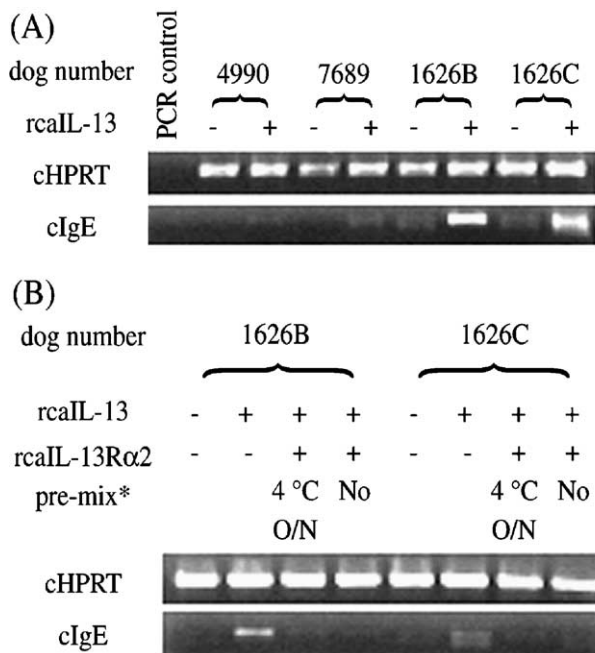


Fig. 5. Effects of rcaIL-13 and rcaIL-13R α 2 on the transcription of IgE mRNA by PBMC from allergic dogs. First strand cDNAs were prepared from total RNAs of canine PBMCs cultured in either culture medium alone, medium containing rcaIL-13 or medium containing rcaIL-13 and rcaIL-13R α 2. The levels of canine IgE mRNA in PBMCs were measured by RT-PCR using the first strand cDNA as the templates and canine IgE specific primers in the reactions as described in Section 2. Canine recombinant IL-13 stimulated the production of IgE mRNA in PBMCs (A). The activity of rcaIL-13 on the stimulation of IgE mRNA production by PBMCs was inhibited by rcaIL-13R α 2 with or without overnight pre-incubation of rcaIL-13R α 2 and rcaIL-13 at 4 °C (B).

culture (Fig. 5A). It is interesting to note that the level of total IgE mRNA was greatly increased in PBMC samples from 'high IgE' dogs (Fig. 5A, dog #1626B and #1626C). Addition of rcaIL-13R α 2 to rcaIL-13 with or without overnight preincubation at 4 °C abolished the ability of rcaIL-13 to up-regulate canine IgE mRNA levels in cultured canine PBMCs (Fig. 5B).

4. Discussion

Molecules encoding IL-13R α 2 have been identified and cloned from humans (huIL-13R α 2), mice (muIL-13R α 2) and dogs (caIL-13R α 2) (Caput et al., 1996; Donaldson et al., 1998; Tang, 2001). The overall amino acid identities among IL-13R α 2s were calculated as 59% for huIL-13R α 2 versus muIL-13R α 2, 62.6% for huIL-13R α 2 versus caIL-13R α 2 and 47.5% for caIL-13R α 2 versus muIL-13R α 2. The full-length protein contains an extracellular domain, a trans-membrane domain and a short cytoplasmic tail, although a soluble form of IL-13R α 2 that has high binding affinity to murine IL-13 has been reported in the serum and urine of mice (Zhang et al., 1997). Previous studies into the interaction between IL-13 and its binding proteins from humans and mice showed that IL-13 had much higher binding affinity to IL-13R α 2 than to IL-13R α 1 (Caput et al., 1996; Donaldson et al., 1998). Although the real functions of IL-13R α 2 in vivo are not well elucidated, it is generally believed that the molecule might function as a decoy for IL-13 (Zhang et al., 1997; Donaldson et al., 1998; Chomarat and Banchereau, 1998).

Investigations on the interactions between rmuIL-13 and its respective receptor indicated that the binding affinity of IL-13 to IL-13R α 2, produced in mammalian expression systems, was in the range of 0.25–1.2 nM (Caput et al., 1996; Donaldson et al., 1998). The studies also showed that the binding of rmuIL-13R α 2 was rmuIL-13 specific and not inhibited by IL-4 (Caput et al., 1996; Donaldson et al., 1998). Due to its high affinity for IL-13, the fusion protein of mouse IL-13R α 2 and Fc-fragment of human IgG1 (rmuIL-13R α 2-Fc) was constructed, expressed in mammalian cells and tested for its binding in vitro (Donaldson et al., 1998), and for its biological activity in vivo (Wills-Karp et al., 1998; Grünig et al., 1998; Chiaramonte et al., 1999). The in vitro study showed that rmuIL-13R α 2-Fc is 100-fold more effective than rmuIL-13R α 1-Fc at neutralizing IL-13 (Donaldson et al., 1998). Administration of the rmuIL-13R α 2-Fc fusion protein to mice inhibited biological activity of IL-13 in vivo (Wills-Karp et al., 1998; Grünig et al., 1998; Chiaramonte et al., 1999). We have recently expressed canine IL-13R α 2 in the form of a fusion protein with the Fc-fragment of canine IgG (rcaIL-13R α 2-Fc) in an *E. coli* expression system (Tang et al., 2001). The purified and refolded rcaIL-13R α 2-Fc was able to neutralize over 90% of rcaIL-13 induced TF-1 cell proliferation at a molar ratio of approximately 1:3, rcaIL-13

to rcaIL-13R α 2-Fc. The fusion protein also inhibited the stimulatory effect of rcaIL-13 on the allergen-specific IgE production by PBMCs from allergic dogs (Tang et al., 2001).

It is well documented that IgE is an important mediator involved in allergic disease, such as allergic asthma, allergic rhinitis and atopic dermatitis. Blocking the binding of IgE to its high affinity receptor (Fc ϵ RI) with the humanized monoclonal antibody (rhuMab-E25) was found to be effective at improving symptoms and reducing medication in human patients with allergic disease (Shields et al., 1995; Casale et al., 1997; Milgrom et al., 1999; Fick, 1999). Interestingly, studies on the effects of rhuMab-E25 in allergic patients indicated that the antibody not only reduced serum IgE levels, but also inhibited expression of Fc ϵ RI on the surface of basophils and the late phase reaction in allergic patients after allergen challenge (MacGlashan et al., 1997; Fahy et al., 1997; Jardieu and Fick, 1999; Fahy, 2000). These studies indicated that IgE and IgE-mediated immune responses play an important role in the progression and maintenance of allergic disease. Research into the regulatory mechanisms of IgE production by B cells showed that IL-4 and IL-13 are the key elements that control immunoglobulin class switching and enhance IgE production in humans (Corry, 1999). The soluble form of IL-4R α has been used as a therapeutic reagent in clinical trials for the treatment of moderate persistent asthma (Borish et al., 1999, 2001). The important role of IL-13 in allergic disease has been supported by studies showing prolonged expression of IL-13 at high levels, but not IL-4, at allergic skin lesions of allergic patients (de Waal Malefyt et al., 1995; Minty et al., 1997; Huang et al., 1995). Additionally, investigations have shown that IL-13 was the key mediator for the exaggeration of asthmatic symptoms in the mice of a lung asthma model (Wills-Karp et al., 1998; Grünig et al., 1998). Based on the current understanding of the regulatory mechanisms of IL-4 and IL-13, it seems that IL-4 is important to the initiation of Th2 type immune responses, and IL-13 may be more important in the progression and maintenance of allergic disease. Targeting IL-13 in vivo with a soluble form of high affinity IL-13 binding protein, such as IL-13R α 2-Fc, represents an alternative approach for the control of IgE-mediated allergic disease.

The expression, purification and characterization of active rcaIL-13R α 2 from an *E. coli* expression system is important, because it provides a necessary reagent in the research of allergic disease. Recently, we have expressed rcaIL-13R α 2-Fc in *E. coli* and have shown that the fusion protein was biologically active in the neutralization of canine IL-13 activity (Tang et al., 2001). However, further investigation on the ability of rcaIL-13R α 2-Fc to bind protein A indicated that the Fc portion of the fusion protein was either not properly refolded, or other components of the Fc-fragment, such as carbohydrates, might be needed for a functional Fc region in the fusion protein. In order to investigate the biological properties of rcaIL-13R α 2, without interference of a potentially misfolded Fc-region,

we expressed the extracellular domain of caIL-13R α 2 in *E. coli*. Similar to the expression of rcaIL-13R α 2-Fc (Tang et al., 2001), rcaIL-13R α 2 was expressed as an insoluble form in the inclusion bodies of transfected *E. coli*. Using the refolding protocol as described in Section 2, rcaIL-13R α 2 was found to be soluble in PBS after refolding. However, based on the SDS-PAGE of refolded rcaIL-13R α 2 under non-reducing conditions, not all soluble rcaIL-13R α 2 was folded into the same conformation. Utilizing gel filtration chromatography, the refolded rcaIL-13R α 2 was fractionated into two protein peaks with molecular masses of approximately 70 and 37 kDa, respectively (Fig. 2A). Fraction number 15 contained a single protein band under reducing and non-reducing SDS-PAGE conditions, indicating that the rcaIL-13R α 2 was a monomer (Fig. 2B). Following further purification of rcaIL-13R α 2 using ion exchange chromatography, the properly folded rcaIL-13R α 2 (Peak A) was separated from its aggregated form (Peak B) (Fig. 3).

The abilities of different rcaIL-13R α 2 preparations to inhibit rcaIL-13 in stimulation of TF-1 cell proliferation was tested (Fig. 4). It was interesting to note that, in order of decreasing potency, refolded rcaIL-13R α 2 inhibited rcaIL-13 activity in the following manner: rcaIL-13R α 2 (Peak A) > rcaIL-13R α 2 (Crude Refold) > rcaIL-13R α 2 (Peak B) (Fig. 4). In comparison to rcaIL-13R α 2-Fc, purified rcaIL-13R α 2 (Peak A) was approximately five times more effective at neutralizing rcaIL-13 activity. The minimum molar ratio of rcaIL-13R α 2 to rcaIL-13 which demonstrated at least 90% neutralization was 1.2:1 for rcaIL-13R α 2 and 6:1 for rcaIL-13R α 2-Fc (Tang et al., 2001).

Purified rcaIL-13R α 2 (Peak A) had no effect on the activities of rcaIL-4 and rhuGM-CSF in stimulation of TF-1 cell proliferation (Fig. 4). This was also demonstrated by comparing the average cpm values for each cytokine alone versus cytokine plus rcaIL-13R α 2 at a given molar ratio (rcaIL-13R α 2:cytokine). For rcaIL-13 these values were 4,424 cpm versus 398 cpm (24:1); for rcaIL-4 50,239 versus 61,815 (128:1); and for rhuGM-CSF 293,969 cpm versus 273,375 cpm (13,398:1). The concentration of each cytokine used in this study was chosen from the linear region of the individual dose response curve. These results indicated that rcaIL-13R α 2 has high binding specificity to rcaIL-13, which was in agreement with the reported observations for human and mouse IL-13R α 2s (Caput et al., 1996; Donaldson et al., 1998). The high and specific inhibitory effect of rcaIL-13R α 2 on rcaIL-13 activity and the small rcaIL-13R α 2 to rcaIL-13 molar ratio (1.2:1) imply that the purified rcaIL-13R α 2 (Peak A) was correctly folded.

Biological activity of rcaIL-13R α 2 was further evaluated by assaying its ability to regulate IgE mRNA levels in PBMCs from dogs. Increased IgE mRNA levels were found in PBMCs from both normal laboratory beagles and high IgE producing beagles, after exposure to rcaIL-13 (Fig. 5A). It is interesting to note that the upregulation of IgE transcripts by rcaIL-13 was much greater in the high IgE dogs (dog numbers: 1626B and 1626C)

than in the normal laboratory beagles (dog numbers: 4990 and 7689), even though the 'high IgE' dogs had a higher starting baseline for IgE transcripts (Fig. 5A). The ability of PBMCs from the 'high IgE' dogs to produce high levels of IgE mRNA in response to the stimulation of rcaIL-13 is consistent with the characteristic dog breeds which produce high levels of IgE and eosinophilia after sensitization by allergens (Redman et al., 2001). The stimulatory effect of rcaIL-13 on the upregulation of IgE mRNA in PBMCs from 'high IgE' dogs was neutralized by addition of rcaIL-13R α 2, with or without preincubation of rcaIL-13 with rcaIL13-Ra2 (Fig. 5B). This result is consistent with our previous finding that rcaIL-13R α 2-Fc could inhibit the rcaIL-13 stimulated allergen-specific IgE production by PBMCs of allergic dogs in culture (Tang et al., 2001).

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References

- Borish, L.C., Nelson, H.S., Lanz, M.J., Claussen, L., Whitmore, J.B., Agosti, J.M., Garrison, L., 1999. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am. J. Respir. Crit. Care Med.* 160, 1816–1823.
- Borish, L.C., Nelson, H.S., Corren, J., Bensch, G., Busse, W.W., Whitmore, J.B., Agosti, J.M., 2001. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J. Allergy Clin. Immunol.* 107, 963–970.
- Brinkmann, V., Kinzel, B., 1997. IL-4 and IL-13 in atopy: regulation of production and effects. In: Vercelli, D. (Ed.), *IgE Regulation: Molecular Mechanisms*. Wiley, New York.
- Burd, P.R., Thompson, W.C., Max, E.E., Mills, F.C., 1995. Activated mast cells produce interleukin 13. *J. Exp. Med.* 181, 1373–1380.
- Caput, D., Laurent, P., Kaghad, M., Lelias, J.M., Lefort, S., Vita, N., Ferrara, P., 1996. Cloning and characterization of a specific interleukin (IL)-13 binding protein structurally related to the IL-5 receptor alpha chain. *J. Biol. Chem.* 271, 16921–16926.
- Casale, T.B., Bernstein, I.L., Busse, W.W., LaForce, C.F., Tinkelman, D.G., Stoltz, R.R., Dockhorn, R.J., Reimann, J., Su, J.Q., Fick, R.B., Adelman, D.C., 1997. Use of an anti-IgE humanized monoclonal antibody in ragweed-induced allergic rhinitis. *J. Allergy Clin. Immunol.* 100, 110–121.
- Chiaromonte, M.G., Schopf, L.R., Neben, T.Y., Cheever, A.W., Donaldson, D.D., Wynn, T.A., 1999. IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. *J. Immunol.* 162, 920–930.
- Chomarat, P., Banchereau, J., 1998. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int. Rev. Immunol.* 17, 1–52.
- Corry, D.B., 1999. IL-13 in allergy: home at last. *Curr. Opin. Immunol.* 11, 610–614.
- Debinski, W., Obiri, N.I., Powers, S.K., Pastan, I., Puri, R.K., 1995. Human glioma cells overexpress receptors for interleukin 13 and are extremely sensitive to a novel chimeric protein composed of interleukin 13 and pseudomonas exotoxin. *Clin. Cancer Res.* 1, 1253–1258.
- Debinski, W., Gibo, D.M., Hulet, S.W., Connor, J.R., Gillespie, G.Y., 1999. Receptor for interleukin 13 is a marker and therapeutic target for human high-grade gliomas. *Clin. Cancer Res.* 5, 985–990.
- de Waal Malefyt, R., Abrams, J.S., Zurawski, S.M., Lecron, J.C., Mohan-Peterson, S., Sanjanwala, B., Bennett, B., Silver, J., de Vries, J.E., Yssel, H., 1995. Differential regulation of IL-13 and IL-4 production by human CD8+ and CD4+ Th0, Th1 and Th2 T cell clones and EBV-transformed B cells. *Int. Immunol.* 7, 1405–1416.
- Donaldson, D.D., Whitters, M.J., Fitz, L.J., Neben, T.Y., Finnerty, H., Henderson, S.L., O'Hara Jr., R.M., Beier, D.R., Turner, K.J., Wood, C.R., Collins, M., 1998. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. *J. Immunol.* 161, 2317–2324.
- Fahy, J.V., 2000. Reducing IgE levels as a strategy for the treatment of asthma. *Clin. Exp. Allergy (Suppl. 1)*, 16–21.
- Fahy, J.V., Fleming, H.E., Wong, H.H., Liu, J.T., Su, J.Q., Reimann, J., Fick, R.B., Boushey, H.A., 1997. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am. J. Respir. Crit. Care Med.* 155, 1828–1834.
- Fick, R.B., 1999. Anti-IgE as novel therapy for the treatment of asthma. *Curr. Opin. Pulm. Med.* 5, 76–80.
- Grünig, G., Warnock, M., Wakil, A.E., Venkayya, R., Brombacher, F., Rennick, D.M., Sheppard, D., Mohrs, M., Donaldson, D.D., Locksley, R.M., Corry, D.B., 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282, 2261–2263.
- Huang, S.K., Xiao, H.Q., Kleine-Tebbe, J., Paciotti, G., Marsh, D.G., Lichtenstein, L.M., Liu, M.C., 1995. IL-13 expression at the sites of allergen challenge in patients with asthma. *J. Immunol.* 155, 2688–2694.
- Jardieu, P.M., Fick, R.B., 1999. IgE inhibition as a therapy for allergic disease. *Int. Arch. Allergy Immunol.* 118, 2–4.
- Li, L., Xia, Y., Nguyen, A., Lai, Y.H., Feng, L., Mosmann, T.R., Lo, D., 1999. Effects of Th2 in the lung: IL-13 potentially induces eotaxin expression by airway epithelial cells. *J. Immunol.* 162, 2477–2487.
- MacGlashan, D.W., Bochner, B.S., Adelman, D.C., Jardieu, P.M., Togias, A., McKenzie-White, J., Sterbinsky, S.A., Hamilton, R.G., Lichtenstein, L.M., 1997. Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J. Immunol.* 158, 1438–1445.
- Maini, A., Hillman, G., Haas, G.P., Wang, C.Y., Montecillo, E., Hamzavi, F., Pontes, J.E., Leland, P., Pastan, I., Debinski, W., Puri, R.K., 1997. Interleukin-13 receptors on human prostate carcinoma cell lines represent a novel target for a chimeric protein composed of IL-13 and a mutated form of Pseudomonas exotoxin. *J. Urol.* 158, 948–953.
- Milgrom, H., Fick, R.B., Su, J.Q., Reimann, J.D., Bush, R.K., Watrous, M.L., Metzger, W.J., 1999. Treatment of allergic asthma with monoclonal anti-IgE antibody. *rhumAb-E25 Study Group. N. Engl. J. Med.* 341, 1966–1973.
- Minty, A., Asselin, S., Bensussan, A., Shire, D., Vita, N., Vyakarnam, A., Wijdenes, J., Ferrara, P., Caput, D., 1997. The related cytokines interleukin-13 and interleukin-4 are distinguished by differential production and differential effects on T lymphocytes. *Eur. Cytokine Netw.* 8, 203–213.
- Redman, T.K., Rudolph, K., Barr, E.B., Bowen, L.E., Muggenburg, B.A., Bice, D.E., 2001. Pulmonary immunity to ragweed in a beagle dog model of allergic asthma. *Exp. Lung Res.* 27, 433–451.
- Shields, R.L., Whether, W.R., Zioncheck, K., O'Connell, L., Fendly, B., Presta, L.G., Thomas, D., Saban, R., Jardieu, P., 1995. Inhibition of allergic reactions with antibodies to IgE. *Int. Arch. Allergy Immunol.* 107, 308–312.
- Tang, L., 2001. Molecular cloning of canine IL-13 receptor α chain (α 1 and α 2) cDNAs and detection of corresponding mRNAs in canine tissues. *Vet. Immunol. Immunopath.* 79, 181–195.
- Tang, L., Borroughs, K.L., Morales, T., Stedman, K., Sellins, K., Clarke, K., McDermott, M., Yang, S., McCall, C., 2001. Recombinant canine IL-13 receptor (2-Fc fusion protein inhibits canine allergen-specific-IgE production in vitro by peripheral blood mononuclear cells from allergic dogs. *Vet. Immunol. Immunopathol.* 83, 115–122.
- Tripp, C.A., Selkirk, M.E., Grieve, R.B., 1996. *Dirofilaria immitis* Gp29 proteins, nucleic acid molecules and uses thereof. US Patent No. 5,569,603, issued 29 October 1996.

- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258–2261.
- Yang, S., Boroughs, K., McDermott, M.J., 2000. Canine Interleukin-13: molecular cloning of full-length cDNA and expression of biologically active recombinant protein. *J. Interf. Cytokine Res.* 20, 779–785.
- Zhang, J.G., Hilton, D.J., Willson, T.A., McFarlane, C., Roberts, B.A., Moritz, R.L., Simpson, R.J., Alexander, W.S., Metcalf, D., Nicola, N.A., 1997. Identification, purification, and characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned IL-13 receptor and IL-4 receptor alpha-chains. *J. Biol. Chem.* 272, 9474–9480.