Doctoral Thesis

The interplay of IgE and its high affinity receptor FceRI

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The Interplay of IgE and its High Affinity Receptor FcεRI

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

presented by
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2005
# Table of Contents

Summary .................................................................................................................. 3

Zusammenfassung .................................................................................................... 5

Abbreviations ........................................................................................................... 7

1 Introduction ......................................................................................................... 11

1.1 Immunoglobulin E (IgE) ................................................................................. 11

1.2 The high affinity IgE receptor, FcεRI .......................................................... 19

1.3 Allergy .............................................................................................................. 24

1.4 Central Question ............................................................................................ 30

2 Results Part I ...................................................................................................... 31

Induction of IgE and allergic-type responses in fur mite-infested mice ............ 31

2.1 Abstract .......................................................................................................... 31

2.2 Introduction .................................................................................................... 32

2.3 Materials and Methods .................................................................................. 34

2.4 Results ............................................................................................................ 36

2.5 Discussion ...................................................................................................... 46

3 Results Part II .................................................................................................... 51

Generation and characterization of hmFcεRIαβ Tg mice .................................. 51

3.1 Abstract .......................................................................................................... 51

3.2 Introduction .................................................................................................... 52

3.3 Materials and Methods .................................................................................. 54

3.4 Results ............................................................................................................ 60

3.5 Discussion ...................................................................................................... 76

4 Results Part III .................................................................................................. 79

Generation of a muFcεRIα-huIgG1Fc fusion protein ........................................ 79

4.1 Abstract .......................................................................................................... 79

4.2 Introduction .................................................................................................... 80

4.3 Materials and Methods .................................................................................. 82

4.4 Results ............................................................................................................ 86
4.5 Discussion ................................................................................................................. 97

5 Discussion .................................................................................................................... 101

5.1 The functions of IgE ............................................................................................... 101
5.2 The epidemic of allergy .......................................................................................... 102
5.3 Mechanisms of IgE-mediated immune responses ....................................................... 103
5.4 Remaining questions to be answered ....................................................................... 103
5.5 Questions addressed in this thesis .......................................................................... 106
5.6 Concluding remarks ............................................................................................... 108

6 References .................................................................................................................. 109

Acknowledgements ........................................................................................................ 127

Curriculum Vitae ............................................................................................................ 129
Allergic diseases, such as eczema, rhinitis, asthma, and food allergies, have greatly increased in the past two decades, mainly in developed countries. Immunoglobulin E (IgE) is known to play a crucial role in the development of allergies, however, the physiological function of IgE has most likely evolved to provide host defense mechanisms against parasite infections. IgE-mediated immune responses involve similar mechanisms during both allergic reactions and parasite clearance. Exposure to antigens leads to crosslinking of antigen-specific IgE molecules bound to high affinity IgE receptors, FcεRI, expressed predominantly on mast cells and basophils. Activation of FcεRI mediates the release of allergic mediators, which are responsible for symptoms of allergy. Understanding the mechanisms regulating IgE-dependent responses is therefore essential to provide effective therapy against allergic disease and to alleviate damage caused by parasite infections.

The goal of the present thesis was to investigate the initial requirements for IgE production and to elucidate the role of FcεRI expression on different cell subsets. Animal models that closely resemble the pathology of human allergic conditions and allow measurement of physiologic parameters are required to efficiently address these questions. We therefore established and characterized a novel allergy model based on infestation of mice with fur mites. The advantage of this model lies in the usage of natural murine ectoparasites allowing physiological exposure to antigens, which strongly resembles the human atopic sensitization. Fur mites were found to induce high levels of serum IgE in a T cell- and IL-4-dependent manner. Moreover, our results strongly suggest that mite antigen entry occurs via the skin of infested mice leading to T cell activation and B cell isotype switch to IgE in skin-draining lymph nodes. Furthermore, mite-induced mast cell degranulation in the skin was detected implying that some of the IgE produced in response to mite infestation was antigen-specific.

In the second part of this thesis we investigated the functions of FcεRI. An intriguing difference in the structure and cellular distribution of the human and murine high affinity IgE receptor was previously found. While murine FcεRI has an obligatory tetrameric structure and is only expressed on mast cells and basophils, the human receptor can be additionally expressed as a trimer found on eosinophils, dendritic cells, Langerhans’ cells, monocytes, macrophages, and platelets. To investigate the role of FcεRI expression on
these effector and antigen presenting cells, we generated a murine transgenic system (hmFceRIαβ Tg mice) that mirrors the human distribution pattern of FceRI. Although mRNA expression of the high affinity IgE receptor was successfully demonstrated in the targeted cells of the hmFceRIαβ Tg mice, FceRI protein expression could not be found. Further investigation of the model is therefore required to determine the reason for the absence of detectable FceRI protein expression and to evaluate how it may be overcome.

Lastly, to provide an additional tool for the investigation of FceRI we made an attempt to generate a specific antibody against the α chain of the murine receptor. Such an antibody is crucial for specific and efficient analysis of the expression of the murine FceRI, which was important for the study of FceRI transgenic mice. To achieve the production of an anti-muFceRIα antibody, a fusion protein composed of the extracellular domain of the murine FceRIα and the human IgG1 Fc portion was generated and used to immunize FceRIα-deficient mice. However, as the first series of immunizations did not result in the synthesis of anti-muFceRIα antibodies and because a commercial anti-mouse FceRIα antibody became available during the completion of this project, the investigation was interrupted. Nevertheless, the successfully generated and purified muFceRIα-huIgG1Fc fusion protein is available in large amounts and can be used in future if required.

In conclusion, the regulation of IgE production and the effector functions induced by IgE interactions with FceRI expressed on different cell types are still not fully understood. Analysis of novel animal models, such as those presented in this thesis, may provide answers to some of the still open questions and eventually lead to successful therapy of allergy and parasite-induced disease.
ZUSAMMENFASSUNG


Das Ziel der vorliegenden Dissertation war es, die Voraussetzungen für die IgE Produktion zu untersuchen, und die Rolle von FcεRI Expression auf diversen Zelltypen aufzuklären. Tiermodelle, welche die Pathologie von menschlichen allergischen Erkrankungen nachahmen und die es ermöglichen, physiologische Parameter zu messen, sind erforderlich um diese Fragen effizient anzugehen. Wir haben ein neues Allergiemodel etabliert und charakterisiert, das auf dem Befall von Mäusen durch Fellmilben basiert. Der Vorteil dieses Models liegt darin, dass natürliche Mausektoparasiten den Kontakt mit Antigenen in physiologischer Art und Weise ermöglichen, was wiederum der atopischen Sensibilisierung bei Menschen ähnlich ist. Wir haben gezeigt, dass Milbenantigene durch die Haut von befallenen Mäusen aufgenommen und über die Lymphgefäße in die dränierenden Lymphknoten transportiert werden. Dort führt das Antigen zur Aktivierung von T Zellen, die zur Produktion von IL-4 stimuliert werden. Diese Th2 Antwort führt zum IgE-Klassenwechsel in B Zellen und schliesslich zu stark erhöhten IgE Serumwerten. Zudem haben wir milbenabhängige Mastzelldegranulation in der Haut beobachtet, was darauf hindeutet, dass ein Teil an IgE, das als Antwort auf den Milbenbefall produziert wurde, Antigen-spezifisch sein muss.
Im zweiten Teil dieser Arbeit haben wir die Funktionen des FcεRI Rezeptors untersucht. Ein überraschender Unterschied in der Struktur und in der zellulären Verteilung zwischen dem IgE Rezeptor von Mensch und Maus wurde bereits vor einigen Jahren erkannt. Während FcεRI in der Maus eine ausschließlich tetramerische Struktur aufweist und nur auf Mastzellen und Basophilen exprimiert wird, kann FcεRI im Menschen eine trimerische Form annehmen, die auch auf eosinophilen Granulozyten, dendritischen Zellen, Langerhans-Zellen, Monozyten, Makrophagen und Thrombozyten exprimiert werden kann.

Um die Funktion von FcεRI Expression auf diesen Zellen zu untersuchen, haben wir ein transgenes System (hmFcεRIαβ Tg Maus) hergestellt, das die menschliche FcεRI Expression widerspiegelt. Obwohl die mRNA Expression des IgE Rezeptors in den Zellen der hmFcεRIαβ transgenen Mäuse erfolgreich gezeigt wurde, konnten wir keine FcεRI Protein Expression nachweisen. Das Modell erfordert deshalb weiterer Analyse, um den Grund für die fehlende FcεRI Protein Expression zu identifizieren, und zu beurteilen wie diese zu überwinden sei.

Darüber hinaus haben wir versucht, einen spezifischen Antikörper gegen die α Kette des FcεRI der Maus herzustellen. Ein solcher Antikörper ist erforderlich für eine spezifische und effiziente Untersuchung der FcεRI Expression, was für die Studie der FcεRI transgenen Mäuse ausschlaggebend war. Um die Produktion von anti-muFceR1α Antikörpern zu erzielen, haben wir ein Fusionsprotein hergestellt, das aus der extrazellulären Domäne der α Kette von FcεRI und dem Fc-Teil des menschlichen IgG1 Antikörpers bestand. Dieses Fusionsprotein wurde anschliessend zur Immunisierung von FcεRIα−/− Mäusen verwendet. Da aber die ersten Immunisierungs Serien nicht zur Synthese des erwünschten Antikörpers geführt hatten, und weil ein kommerzieller anti-muFceR1α Antikörper während der Durchführung dieses Projektes erhältlich wurde, haben wir die Versuche eingestellt. Dennoch steht das erfolgreich generierte und isolierte Fusionsprotein für allfällige weitere Studien zur Verfügung.

Schlussfolgernd ist festzuhalten, dass die Regulation der IgE Produktion und die Folgen der Interaktionen zwischen IgE und FcεRI noch nicht gänzlich geklärt sind. Analyse neuer Tiermodelle, wie z.B. solcher die in dieser Dissertation präsentiert wurden, könnten Antworten auf einige der offenen Fragen liefern und schliesslich zur erfolgreichen Therapie gegen Allergie und parasitäre Infektionen führen.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AID</td>
<td>Activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow-derived mast cell</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSS</td>
<td>Balanced salt solution</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining region</td>
</tr>
<tr>
<td>Ce</td>
<td>IgE heavy chain constant region</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CSR</td>
<td>Class-switch recombination</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Eos</td>
<td>Eosinophil</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>Fe</td>
<td>Crystallizable fragment</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FcεRI</td>
<td>High affinity IgE receptor</td>
</tr>
<tr>
<td>FcεRIα</td>
<td>Alpha chain of the high affinity IgE receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FcεRIβ</td>
<td>Beta chain of the high affinity IgE receptor</td>
</tr>
<tr>
<td>FcεRIγ</td>
<td>Gamma chain of the high affinity IgE receptor</td>
</tr>
<tr>
<td>FR</td>
<td>Framework</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLT</td>
<td>Germline transcript</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>Hyg</td>
<td>Hygromycin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's modified Dulbecco's media</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Association constant</td>
</tr>
<tr>
<td>Kana</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kₐ₀f</td>
<td>Dissociation rate</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans’ cell</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-assisted cell sorting</td>
</tr>
<tr>
<td>MAUD</td>
<td>Mite-associated ulcerative dermatitis</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MΦ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>N. brasiliensis</td>
<td><em>Nippostrongylus brasiliensis</em></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononucleoytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-PCR</td>
</tr>
<tr>
<td>S. mansoni</td>
<td><em>Schistosoma mansoni</em></td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>s.d.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Spl</td>
<td>Spleen</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T. spiralis</td>
<td><em>Trichinella spiralis</em></td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
1 INTRODUCTION

1.1 Immunoglobulin E (IgE)

Physiological properties and structure of IgE

The first demonstration to show that a serum factor (originally named “reagin”) was responsible for the development of allergic reactions was performed in 1921 by Carl Prausnitz and Heinz Küstner (Prausnitz, 1921). They injected serum from a fish-allergic individual (Küstner himself) into the skin of a non-allergic person (Prausnitz) and observed a typical weal and erythema reaction at the site of serum transfer after local administration of fish extract. Only decades later, in 1967, was reagin recognized as a new immunoglobulin class and was designated IgE due to the observed erythema reaction (Ishizaka and Ishizaka, 1967). The physiological characteristics of IgE differ from the other four known immunoglobulins, IgM, IgG, IgD, and IgA. First, IgE is the least abundant antibody class in serum, with concentrations of about 100 ng/ml in healthy individuals, compared with 10 mg/ml for IgG (Sutton and Gould, 1993). However, in atopic or parasitized individuals IgE concentrations can increase by up to 1000-fold (Barbera et al., 1977; Durmaz et al., 1998). Second, with a serum half-life of only about 2 days, IgE has the shortest biological half-life of all the immunoglobulins. However, if IgE is cell surface-bound, this half-life can be extended to 1-2 weeks (Tada et al., 1975). Similar to other immunoglobulins, IgE consists of heavy (H) and light (L) chains with variable (V) and constant (C) regions but it differs in that it is composed of ε H-chains, which contain four C domains (Cε1-Cε4). Such distribution of C regions is also found in IgM while the H-chains of IgG, IgD, and IgA are all composed of only three C domains (Gould et al., 2003) (Figure 1). The additional IgE domain (Cε2) replaces the hinge region found in the other antibody classes and is a critical determinant of the physical properties of IgE (See paragraph “IgE-FceRI interactions”). Recently, the crystal structure of the IgE-Fc fragment has been solved providing new insight into the conformation of IgE and the binding properties of IgE to its high affinity receptor, FceRI (Wan et al., 2002).
Figure 1. Structures of immunoglobulins. Immunoglobulins are composed of two heavy (H) and two light (L) chains. There are five types (isotypes) of H chains (α, γ, δ, ε, and μ) and two types of L chains (κ and λ). The amino-terminal (NH₃⁺) regions of each chain contain variable (V) domains responsible for antigen binding, while the carboxy-terminal (COO⁻) regions contain constant (C) domains responsible for the biological activity of the antibodies. The α, γ, and δ H chains contain three C domains (CH₁-CH₃) and a proline-rich hinge region (A), while the ε and μ H chains, which lack the hinge region, contain four C domains (CH₁-CH₄) (B). Immunoglobulins can also be subdivided into antigen-binding (Fab) and “crystallizable” fragments (Fc) by enzymatic digestion above the disulfide bonds (S-S) that link the H chains.

Regulation of IgE

The very low levels of serum IgE of healthy individuals suggest that IgE synthesis may be tightly regulated. This is thought to be important to prevent the potentially lethal consequences of IgE-dependent immune responses. Thus, multiple factors are involved in the induction of IgE synthesis.

One of the first steps leading to IgE production is specific binding of antigen to membrane-bound B cell receptors, which subsequently ensures the specificity of the IgE produced. B cells have the capacity to internalize, process and present antigenic peptides on MHC class II molecules (Vercelli et al., 1989). Peptide-MHC class II complexes are recognized by specific T cell receptors (TCRs) on CD4⁺ T cells. CD4⁺ T cells, also known as T helper cells, are crucial for regulation of both cellular and humoral immune responses by secreting cytokines and providing signals required for antibody production. Two subtypes of T helper
cells (Th1 and Th2) have been described based on their functional properties and the profile of cytokines they produce (Abbas et al., 1996). Th1 cells represent a major source of interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and other cytokines important for cell-mediated immune responses. Th1 responses antagonize IgE effector functions, as IL-12 and IFN-γ suppress IgE synthesis through direct effects on B cells (Coffman and Carty, 1986). On the other hand, Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, which are required for antibody responses, including IgE production (Aebischer and Stadler, 1996). After antigen-specific interaction of TCRs with peptide-MHC class II complexes, Th2 cells provide B cells with signals crucial for IgE synthesis. Such signals involve cytokines secreted by Th2 cells as well as cell-to-cell interactions between Th2 cells and B cells (Figure 2). The most important cytokines for induction of IgE are IL-4 and IL-13 (Finkelman et al., 1988; Minty et al., 1993; Punnonen et al., 1993). Binding of IL-4 and IL-13 to their receptors expressed on the surface of B cells leads to activation of the transcription factor signal transducer and activator of transcription 6 (STAT6), which provides the first signal for isotype switching to IgE (de Vries et al., 1993). Ligation of CD40 (expressed on B cells) to its ligand CD40L (expressed on Th2 cells) activates the transcription factor nuclear factor-κB (NF-κB), which induces the second signal leading to IgE synthesis. NF-κB acts in synergy with IL-4-induced STAT6. On one hand, STAT6 together with NF-κB induce production of Ce germline transcripts (GLTs) by binding to the Ie promoter located upstream of the Ce genes (Iciek et al., 1997). Although Ce GLTs lack the VDJ region and are “sterile”, meaning that they are not translated into protein because of stop codons in all reading frames, Ce GLT expression is a necessary step for the subsequent class-switch recombination (CSR) to IgE (Geha et al., 2003). On the other hand, synergy between STAT6 and NF-κB results in expression of activation-induced cytidine deaminase (AID) (Chaudhuri et al., 2003). AID has been shown to play a crucial role in CSR (Muramatsu et al., 2000) by a yet unresolved mechanism involving cytidine deamination of either RNA (Muramatsu et al., 1999) or single-stranded DNA (Chaudhuri et al., 2003). Thus, signals through both IL-4 and CD40 are required to induce Ce GLT expression and to reach a threshold level of AID expression necessary for switching to IgE (Messner et al., 1997). However, it has been reported that under some circumstances IgE can be produced independently of T cells (Jabara et al., 1990), IL-4 (Grunewald et al.,
2001; Morawetz et al., 1996), or CD40 (Litinskiy et al., 2002) implying that regulation of IgE synthesis is a complex process involving additional molecules and signaling pathways.

**Figure 2. Major T cell – B cell interactions regulating induction of IgE.** Specific binding of antigen by B cells via surface IgM (a) leads to processing and presentation of antigens by MHC class II molecules and allows antigen-specific interactions with T cells via TCRs (b). Activated CD4+ Th2 cells express high levels of CD40L and produce the cytokines IL-4 and IL-13. Interaction of CD40L with CD40 expressed by B cells (c) activates NF-κB (d), while binding of IL-4 and IL-13 to their receptors (e) recruits STAT6 (f). Synergy between NF-κB and STAT6 leads to transcription of C\v{c} GLT and AID (g), both required for subsequent IgE synthesis (h).
Introduction

**Effector functions of IgE**

*Type I immediate hypersensitivity*

The immune system attempts to protect individuals from pathogens by inducing localized inflammation. However, an inflammatory response can also have deleterious effects. Inappropriate immune responses resulting in host damage can derive either from a reaction against self-antigens (autoimmunity) or from an overreaction against environmental antigens (hypersensitivity). Such an “overreaction” was first described by Paul Portier and Charles Richet in 1902 (Cohen and Zelaya-Quesada, 2002; Haas, 2001). These two French scientists discovered that a second injection of fluids isolated from jellyfish induced a violent reaction in dogs that showed no symptoms to an earlier injection. They called this phenomenon “anaphylaxis” to mean the opposite of prophylaxis. Anaphylactic reactions are currently referred to as immediate or type I hypersensitivity. Hypersensitivity reactions have been classified into four types by Gell and Coombs (Coombs RRA, 1963): type I, IgE-mediated hypersensitivity; type II, IgG-mediated cytotoxic hypersensitivity; type III, immune complex-mediated hypersensitivity; and type IV, cell-mediated hypersensitivity. Type I hypersensitivity is induced by antigens that elicit secretion of specific IgE. High affinity IgE receptors, FcεRI, highly expressed on mast cells and basophils, capture secreted IgE (Wedemeyer et al., 2000). Such sensitized cells can be activated within minutes following re-exposure to the same antigen (Figure 3). Signaling pathways induced by antigen-dependent crosslinking of IgE bound to FcεRI trigger degranulation of the cell and result in the release of preformed inflammatory mediators including histamine, neutral proteases, and proteoglycans (Boyce, 2004). Additionally, activated mast cells and basophils secrete newly synthesized lipid mediators such as prostaglandin D₂, leukotriene C₄, and platelet-activating factor as well as numerous proinflammatory cytokines, chemokines and growth factors, including TNF-α, IL-4, IL-13, and eotaxin (Prussin and Metcalfe, 2003).

Clinical manifestations related to mediators released during IgE-induced degranulation include enhanced local vascular permeability, increased cutaneous blood flow, erythema, and other effects such as itching (Kay, 2001). Pathology induced by type I hypersensitivity can range from moderate allergic reactions including hay fever and eczema, to life-threatening conditions, such as systemic anaphylaxis and asthma.
**Figure 3. Activation and degranulation of mast cells.** Soluble IgE binds to FceRI expressed on mast cells. Crosslinking of receptor-bound IgE by specific antigen triggers the release of preformed mediators (e.g. histamine) from the granules and induces synthesis of additional inflammatory factors (e.g. lipids and cytokines).

*Regulation of mast cell survival and FceRI expression*

Recent reports have suggested that binding of monomeric IgE to FceRI, i.e. in the absence of antigen, can have immuno-regulatory functions. It has been demonstrated that monomeric IgE can promote mouse mast cell proliferation induced by IL-3 or SCF *in vitro* (Asai et al., 2001; Kalesnikoff et al., 2001; Kitaura et al., 2003). The mechanism of monomeric IgE-mediated mast cell survival has been shown to involve anti-apoptotic effects, however, its biological relevance remains to be determined. In addition, it has been reported that IgE binding to FceRI in the absence of specific antigen induces upregulation of FceRI surface expression on mast cells and basophils (Lantz et al., 1997; Yamaguchi et al., 1997). The enhanced expression of FceRI by IgE has been shown to be achieved by receptor stabilization at the membrane as well as recruitment of a preformed pool of receptors in the presence of continual basal protein synthesis (Borkowski et al., 2001). IgE binding to FceRI might also make the receptor resistant to degradation via conformational changes induced in the α chain (Kubo et al., 2001). The biological relevance of FceRI upregulation induced by newly produced IgE may be an enhanced sensitivity to pathogens due to reactivity to an increased number of antigens. This also implies that prevention of
IgE-mediated FcεRI upregulation may be a useful target to diminish the impact of allergic disorders.

**Protection against parasites**

The first observation that parasites, particularly helminthes, induce strong primary and secondary IgE responses was described by Ogilvie in 1964 (Ogilvie, 1964). Numerous reports have since accumulated suggesting that the biological role of IgE-associated immune responses is to provide host defense against parasites (Hagan, 1993; Jarrett and Miller, 1982; Levy, 2004). However, different degrees of importance of IgE in parasite clearance have been shown and the requirement of IgE for protective host immunity to parasites has remained controversial. Investigation of *Schistosoma mansoni* (*S. mansoni*) infection in humans revealed host defense mechanisms involving IgE-dependent killing of larval schistosomes by platelets and macrophages (Capron et al., 1987). Moreover, resistance to reinfection with *S. mansoni* was associated with high anti-schistosomular IgE levels (Rihet et al., 1991). Convincing evidence that protection against reinfection is associated with high levels of parasite-specific IgE was also provided by studies of *Schistosoma haematobium* infection (Hagan et al., 1991). Additionally, a positive correlation of parasite-specific IgE levels and protection in rodents was found for *Trichinella spiralis* (*T. spiralis*) (Ahmad et al., 1991; Bell, 1998; Dessein et al., 1981). Interestingly, a powerful IgE response, with over 60% parasite-specific IgE, was found within the intestinal lumen of *T. spiralis* infected mice, while only 10% of serum IgE was found to be specific (Negrao-Correa, 2001; Negrao-Correa et al., 1996). A recent study of IgE-deficient mice infected with *T. spiralis* revealed that IgE was required for both the elimination of adult worms from the intestine and the killing of larvae present in the skeletal muscle through mechanisms involving mast cell homeostasis and secretion of mast cell protease-1 (Gurish et al., 2004).

However, other reports have speculated that IgE production in response to helminth infection is merely the consequence of a strong Th2 response induced by the parasites but plays no crucial role in parasite expulsion. In some cases this may indeed be true. For example, it has been shown that mice lacking functional IgE receptors expel *Trichuris muris* with normal kinetics (Betts and Else, 1999).
Intriguingly, the presence of disproportionately high levels of total (non-parasite specific) IgE has been observed in response to many parasitic helminthes (Dessaint et al., 1975; Turner et al., 1979). The generation of such polyvalent IgE has been explained in two ways (Pritchard, 1993). First, the presence of non-specific IgE might imply the existence of a protective strategy used by the parasite whereby competition with specific IgE is induced. Second, high amounts of unrelated IgE could possibly reduce the risk of anaphylaxis, thereby protecting the host from potentially lethal consequences of hyperreactivity to parasite antigens.

Taken together, IgE participation in the protective mechanisms involved in parasite infections may strongly depend on the parasite species. Complex life cycles involving different tissue localizations of helminthes, such as lung, stomach, or intestine, may induce various mechanisms leading to protective immunity of the host. For example, it has been proposed that mucosal penetration by the nematode may be required for induction of an IgE response that could participate locally in worm elimination. Thus, parasite infections result in multifactoral and redundant immune responses depending on the parasite species and infection site. While a Th2-associated immune response is known to be a common feature in immunity to most parasites, the exact effector elements, their regulation and modes of action that are important for protection against each individual parasite, remain to be determined.
1.2 The high affinity IgE receptor, FceRI

Structure and expression of FceRI

FceRI is a multimeric cell surface receptor that exists in two isoforms. It can be expressed either as a heterotetramer composed of one α, one β, and two γ chains (αβγ2), or as a heterotrimer lacking the β chain (αγ2) (Kinet, 1999) (Figure 4). The αβγ2 form of FceRI is abundantly expressed on IgE-associated effector cells, mast cells and basophils, reaching 3x10^5 molecules/cell (Liu et al., 2001). In humans, the trimeric form of FceRI has been demonstrated to be expressed on Langerhans’ cells (Bieber et al., 1992; Wang et al., 1992), monocytes (Maurer et al., 1994), eosinophils (Gounni et al., 1994), peripheral blood dendritic cells (Maurer et al., 1996), and platelets (Joseph et al., 1997). Interestingly however, mice lack expression of the αγ2 form of FceRI and therefore do not express the receptor on cells other than mast cells and basophils. The α chain of the receptor (FceRIα) is a type I integral membrane protein with two Ig-like domains (D1 and D2) in the extracellular (N-terminal) region, a single transmembrane domain, and a short cytoplasmic tail (Garman et al., 1998). FceRIα is heavily glycosylated due to seven N-linked glycosylation sites involved in interactions between the α chain and the folding machinery of the endoplasmic reticulum (Letourneur et al., 1995b). Crystal structure analysis of IgE-Fc bound to FceRIα revealed that the Cε3 domains of IgE-Fc bind two distinct sites located in the D2 domain of FceRIα (Garman et al., 2000). The β subunit (FceRIβ) has four transmembrane domains separating amino and carboxy terminal cytoplasmic tails containing an immunoreceptor tyrosine-based activation motif (ITAM) near the carboxy terminus (Kinet et al., 1988). Two major functions of FceRIβ are known. First, FceRIβ has been shown to amplify signals produced by the γ chain of the receptor, even though β does not appear to possess any signaling capacity through its own ITAM (Jouvin et al., 1994; Lin et al., 1996). Second, it has been demonstrated that FceRIβ facilitates surface expression of the receptor by promoting the processing and export of the α chain to the cell surface and by enhancing the stability of the receptor complexes (Donnadieu et al., 2000). The γ chains (FceRIγ) form a disulfide-linked homodimer with short extracellular portions and long cytoplasmic tails containing an ITAM on each subunit (Kuster et al., 1990). Several other receptors, including FcγRI, FcγRIIIA, FcαR, and the CD3 complex of the T
cell receptor (TCR), share the ubiquitously expressed γ subunit, which is crucial for induction of signaling cascades through phosphorylation of the ITAMs (Daeron, 1997).

**Figure 4. Structure and cellular distribution of the high affinity IgE receptor, FcεRI.** FcεRI is expressed either as a αβγ2-tetramer (A) or a αβγ2-trimer (B). The αβγ2 form of FcεRI is expressed on mast cells and basophils of both humans and mice, while the αγ2 structure is found only in humans on Langerhans' cells, monocytes, eosinophils, peripheral blood dendritic cells, and platelets. The extracellular region of the α chain contains two Ig-like domains (D1 and D2) responsible for binding of IgE. The β subunit has four transmembrane domains and an ITAM in the cytoplasmic tail. The γ chains form a disulfide-linked (S-S) homodimer with long cytoplasmic tails containing an ITAM on each subunit.

**IgE-FcεRI interactions**

The unique binding kinetic of IgE to FcεRI is the most characteristic feature of this high affinity receptor. FcεRI binds monomeric IgE with an association constant $K_a$ of $10^{10}$ M$^{-1}$ (Kulczycki and Metzger, 1974) which is considerably higher than binding of IgG to FcγRIII, the closest homologue of FcεRI, characterized by a $K_a$ of $10^5$ M$^{-1}$ (Maenaka et al., 2001). This exceptionally high affinity of IgE binding to FcεRI reflects a very slow dissociation rate, $k_{off} \sim 10^{-5}$ s$^{-1}$ for FcεRI, compared with $k_{off} \sim 1$ s$^{-1}$ for FcγRIII (Ishizaka et al., 1986). The most important implication for the biological function of IgE resulting from such a slow dissociation rate is the long half-life of receptor-bound IgE, reaching 1-2 weeks
(Tada et al., 1975). This results in persistent sensitization of cells expressing FcεRI, such as mast cells and basophils, and is responsible for their capacity to react very fast to antigen challenge, a feature characteristic of allergic responses. Recent investigation of crystal structures of IgE and its high affinity receptor provided explanations for their unusual binding kinetics (Wan et al., 2002). Binding of the Cε3 domain of IgE-Fc to the extracellular domain of FcεRIα results in a profound conformational change of the adjacent Cε2 domain. This conformational change is believed to greatly stabilize the IgE-FcεRI complex and therefore lead to the slow dissociation rate (Novak and Bieber, 2002).

**Biological functions of FcεRI**

The best-known role of FcεRI is the induction of mast cell and basophil degranulation when multivalent antigens crosslink the receptor by interacting with specific receptor-bound IgE. The resulting processes, involving rapid release of granule-stored mediators as well as synthesis and secretion of cytokines and chemokines, have been widely described and shown to initiate allergic disorders and mediate immune responses against parasites (Kawakami and Galli, 2002). The requirement for FcεRI in induction of IgE-dependent allergic reactions has been shown in mice that lack either the α or β chain gene of the receptor as these mice do not exhibit cutaneous or systemic anaphylaxis (Dombrowicz et al., 1993; Dombrowicz et al., 1998). In addition, FcεRI-deficient mice have an impaired immunity to *S. mansoni* (Jankovic et al., 1997). In the *S. mansoni* model IgE-FcεRI interactions are not directly involved in development of the Th2 response or resistance to primary infection, but have been proposed to play a role in down-regulation of host pathology by controlling hepatic fibrosis and egg granuloma formation. Moreover, investigation of FcεRI on human eosinophils has revealed that during *S. mansoni* infection FcεRI participates in eosinophil-mediated cytotoxicity against the parasite by promoting eosinophil degranulation (Gounni et al., 1994).

Demonstration of FcεRI expression on human antigen presenting cells (APCs) has inferred a novel function of FcεRI in IgE-mediated antigen presentation pathways. Important differences between FcεRI present on the classical IgE-associated effector cells (mast cells and basophils) and professional APCs have to be pointed out. APCs express the trimeric form of the receptor (αγ2), which shows substantially lower expression density and mediates weaker signals (Novak et al., 2003a). This suggests a distinct function for the high
affinity IgE receptor on APCs. The importance of FcεRI in IgE-dependent antigen presentation to T cells has been demonstrated on monocytes and circulating dendritic cells (Maurer et al., 1995; Maurer et al., 1998). Studies of FcεRI-positive monocytes revealed that high affinity IgE receptor-mediated endocytosis is more efficient than pinocytotic allergen uptake. Thus, allergen presentation to T cells is highly enhanced when allergen-IgE complexes are targeted to FcεRI-bearing APCs compared to T cell activation in absence of IgE. This mechanism may therefore critically lower the atopic individual’s threshold to mount allergen-specific T cell responses. Interestingly, APCs of patients with atopic diseases have been shown to express increased levels of FcεRI correlating with high serum IgE concentrations (Kraft et al., 1998). Thus, FcεRI-mediated antigen presentation has been proposed to mediate delayed-type hypersensitivity reactions (in contrast to immediate-type hypersensitivity mediated by mast cells and basophils) involved in diseases, such as atopic dermatitis (AD) and atopic eczema/dermatitis syndrome (AEDS), in which aeroallergens penetrating the skin barrier can be directly taken up by FcεRI-bearing Langerhans’ cells (LCs) leading to T cell activation and inflammation (Figure 5). Moreover, a surprising functional difference in LCs from normal individuals and individuals with AD has been observed: calcium mobilization upon crosslinking of FcεRI can only be detected in LCs freshly isolated from patients with AD, but not in those from skin of healthy individuals, despite the presence of significant amounts of the receptor (Jürgens et al., 1995). The differential regulation of FcεRI expressed on LCs from atopic patients has been reported to involve upregulated expression and activity of protein tyrosine kinases (PTK) leading to activation of phospholipase C-γ (PLCγ) induced after IgE and antigen ligation to FcεRI (Kraft et al., 2002b). Furthermore, signals through FcεRI in LCs of atopic patients have been shown to induce expression of NF-κB resulting in activation of pro-inflammatory cytokines, such as TNF-α and monocyte chemoattractant protein-1 (MCP-1) (Kraft et al., 2002a). These findings support the view that FcεRI-expressing APCs may contribute to the inflammatory reactions found in atopic disease.

Taken together, recent investigations into the role of FcεRI on APCs have shed new light on mechanisms controlling allergic and inflammatory hypersensitivity reactions. Additional studies need to be undertaken to elucidate the detailed function of the high affinity IgE
receptor expressed on different cell types in order to determine whether intervention with FceRI-mediated signaling cascades might be used for therapeutic strategies.

Figure 5. Contribution of FceRI⁺ LCs in development of AD. Allergen taken up through the skin (a) binds to specific IgE present in high amounts in atopic patients. FceRI expressed on LCs facilitates the uptake of allergens via IgE bound to the receptor leading to a more efficient presentation of antigens and activation of LCs (b). In the regional lymph node LCs present the antigen to naïve T cells resulting in activation of the T cells (c). Effector T cells migrate to the skin where they trigger allergic inflammatory reactions, e.g. mediated by type-2 cytokines, which may lead to dermatitis (d).
1.3 Allergy

Definition of allergy

The term “allergy” was introduced by von Priquet in 1906 and it originally referred to all forms of altered reactivity to antigenic stimulation, which may result in a protective response (immunity) or an adverse clinical reaction (hypersensitivity) (Priquet, 1963). However, the current usage of the term allergy describes potentially harmful immune responses to environmental antigens, known as allergens. The pathology of allergic diseases reflects activation of allergen-specific T cells, especially Th2 cells, and generation of allergen-specific antibodies, mostly IgE. The term “atopy” (from Greek atopos, meaning out of place) is used to describe IgE-mediated diseases. Patients with atopy produce IgE antibodies against common environmental allergens and suffer from atopic diseases, such as rhinitis, asthma, and eczema. Importantly, some allergic diseases, such as contact dermatitis and hypersensitivity pneumonitis, develop through IgE-independent mechanisms and can be therefore considered as nonatopic allergic conditions (Kay, 2001). Allergic disease can usually be divided into three types of responses induced by allergen challenge: (i) acute allergic responses, which can be elicited within minutes after exposure to allergen reflecting the actions of mediators released from mast cells; (ii) late-phase reactions, which develop several hours after allergen challenge and result in the recruitment of circulating leukocytes; and (iii) chronic allergic inflammation, which occurs in response to repeated allergen challenge and is characterized by long-lasting changes in the tissue and enhanced propensity for Th2 cell-driven immune responses.

Allergens

An important question in the field of allergic disorders was asked by Aas in 1978: “What makes an allergen an allergen?” (Aas, 1978). Although the first investigation of allergens took place as early as the 1870s when Charles Blackley identified grass pollens as the cause of his own disease, it is still not clear whether allergens are a specific subset of antigens or whether any antigen can potentially become an allergen (Mohapatra and Lockey, 2001). Molecular cloning and characterization of a large number of allergens has allowed the identification of certain general characteristics of allergens. Allergens typically are proteins, of which many show enzymatic activity (Cromwell, 1997). This led to the hypothesis that Th2-type responses to enzymes evolved because of selection pressure that favored the
development of immune responses to parasites that often require enzymes for successful invasion of host tissues (Stewart et al., 1993). Other allergens are glycoproteins and it has been proposed that oligosaccharide side chains may favor the binding to lectin receptors on APCs and promote allergen uptake (Cromwell, 1997). The clinical classification of allergens is based on their origin or patterns of distribution within the environment. Thus, aeroallergens, further subdivided into indoor and outdoor aeroallergens, constitute airborne particles, such as pollen grains, dust mite feces, and animal dander. Food allergens refer to allergens ingested with food and the most prominent derive from peanuts, fish, cow’s milk, and cereal grains. Insect venoms represent an important group of allergens, because of their high risk of causing anaphylaxis (Valentine, 1992). Although about 70 major allergens, meaning allergens that induce immediate skin test responses in >90% of allergic individuals, have been identified, it has not yet been revealed why these substances induce Th2 cell-driven, IgE-associated responses. Thus, given such a wide range of allergens, further research is required to understand the complexity of allergic disorders.

Factors involved in the development of allergy

Atopic diseases such as asthma, hives, eczema, atopic dermatitis, allergic rhinitis, and food allergy have increased remarkably over the last two decades, and are now a major source of disability worldwide, affecting mainly industrialized countries (1998; Holgate, 1999; Sunyer et al., 1999). Due to the large number of people affected and the high annual costs of treating allergic disorders, a large body of literature has accumulated discussing factors that promote development of allergy.

Genetics

Investigation of asthma and eczema within families has revealed that allergy has a genetic basis. Twin studies have shown that the heritability of asthma may be as high as 75% (Duffy et al., 1990) and the presence of eczema-specific genes has been suggested from the observation that parental eczema confers a higher risk of eczema in offspring than parental asthma or rhinitis (Dold et al., 1992). Techniques used to identify genes that are relevant to allergy and asthma include the candidate-gene approach, which depends on the identification of polymorphisms in a known gene, and positional cloning, which links the inheritance of a specific chromosomal region with the inheritance of a disease (Cookson, 1999). Studies using the candidate-gene method have revealed an association between an
allele of the human leukocyte antigen (HLA)-DR locus and reactivity to the ragweed allergen Ra 5 (Marsh et al., 1982), and the linkage of atopy to a polymorphism of FcεRIβ (Hill and Cookson, 1996) and to the IL-4 family of cytokine genes on chromosome 5 (Marsh et al., 1994). Positional cloning has shown that chromosomes 2q, 5q, 6q, 12q, and 13q contain loci linked both to asthma and atopy (Cookson, 1999). However, the clinical relevance of these findings remains to be determined.

_Feto-maternal events_

It is widely recognized that most atopic disorders originate in childhood and therefore research on the early development of the immune system is important to understand the chronology of events leading to allergic reactions. Experimental data has suggested that infants can develop an immune response to common environmental antigens whilst in utero (Warner et al., 2000). Specific allergen-induced responses have been shown to occur as early as 22 weeks of pregnancy and maternal exposure to birch pollen from this point onwards results in increased infant allergen-specific responses at birth, suggesting that antigen passage can occur across the placenta (Jones et al., 1996). Furthermore, peripheral blood mononucleocytes (PBMCs) from babies with a family history of allergy have been demonstrated to lack production of the Th1 cytokine IFN-γ (Holt et al., 1992; Tang et al., 1994). Interestingly, several reports have proposed that during pregnancy the immune response at the materno-fetal level is biased towards a Th2 response (Prescott et al., 1998). This implies that fetal IFN-γ production may be required to counterbalance the Th2 environment of the placenta in order to prevent an allergic Th2 phenotype in infants. However, if the mother is atopic and exposed to allergens that trigger her own allergy during pregnancy, Th2-type cytokines could potentially cross the placenta and hamper the fetal IFN-γ response. Early exposure of the infant to the same allergens may additionally influence the immune response, resulting in atopic disease, as shown by the observation that the level of house dust mite exposure during the first year of life correlates with the subsequent risk of childhood asthma (Sporik et al., 1990).

_Lifestyle_

Results of international epidemiological studies have provided ample evidence that the increase in the prevalence of allergic disease correlates with western lifestyle. Substantial regional differences in importance of atopy have been shown over the past 20 years (1998)
including studies of West and East Germany (von Mutius et al., 1998) as well as West and East Europe (Bjorksten et al., 1998). Furthermore, rural lifestyle has been shown to have protective effects on the development of allergies (Braun-Fahrlander et al., 1999). Large families or animal pets have also been listed amongst the factors protecting against atopy leading to the hypothesis that an excessively clean environment may result in inappropriate immune responses (Rook and Stanford, 1998). It has been proposed that microbial stimulation in the gastrointestinal tract could possibly explain the association of atopic disease with clean western lifestyle as microbial antigens stimulate Th1 cell activation. An example of a link between bacteria colonizing the gastrointestinal tract and atopic sensitizations has been described in a study of Swedish and Estonian children (Bjorksten et al., 1999; Sepp et al., 1997). While lactobacilli and eubacteria were predominant in non-atopic individuals, coliforms and Staphylococcus aureus dominated in allergic children. The findings that atopic sensitization might be influenced by the bacterial species colonizing the gut may explain why living in a rural community, which increases the likelihood of exposure to bacteria found in barns, might protect from allergic disease.

The hygiene hypothesis versus the counter-regulatory model

The argument that bacterial and viral infections during early life direct the maturing immune system toward Th1 responses, which counterbalance allergy-associated responses of Th2 cells, has led to the so-called hygiene hypothesis, first postulated in 1989 by David Strachan (Strachan, 1989). The hygiene hypothesis argues that the increased prevalence of allergic disease results from a reduced exposure to microbes that are more common on farms and in less developed countries. This view has been supported by findings suggesting that childhood infections show a negative association with atopy. In particular, exposure to food and orofecal pathogens, such as hepatitis A, Toxoplasma gondii, and Helibacter pylori, has been shown to reduce the risk of allergic disease by >60% (Matricardi et al., 2000). However, studies reporting that the prevalence of Th1-dependent autoimmune diseases is also increasing and that Th2-associated parasite infections do not increase the risk of allergy, contradict the hygiene hypothesis and imply that the Th1/Th2 balance model should be re-evaluated (Allen and Maizels, 1997; Gor et al., 2003). Thus, recent reviews have favored a novel, so called “counter-regulatory” model, which offers a unifying explanation for the increasing incidence of both allergy and autoimmunity in the absence of persistent immune challenge characteristic for a westernized lifestyle (Wills-Karp et al.,
Anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β, have been suggested to be the key players in the counter-regulatory model. As most pathogenic infections have been shown to upregulate IL-10 production, consequently suppressing allergic and autoimmune disease, decreased infectious pressure found in developed countries might result in decreased levels of anti-inflammatory cytokines required for prevention of deleterious immune responses (Moore et al., 2001; van den Biggelaar et al., 2000). This is supported by a previous finding of decreased IL-10 production in asthmatic patients (Borish et al., 1996).

In summary, the interrelation between pathogenic infections and development of atopic disorders seems to involve complex regulatory mechanisms that remain to be experimentally proven. Factors required for counter-regulation, such as cell types and anatomical locations involved, should be addressed in animal models to increase our understanding of the mechanisms regulating the development of allergic disease.

**Animal models used in the study of allergy**

Various animal models, including mice (Levine and Vaz, 1970), rats (Byars and Ferraresi, 1976), guinea pigs (Tanaka et al., 1988), monkeys (Turner et al., 1996), and dogs (Ermel et al., 1997), have been established in an attempt to provide insights into the immunology and pathophysiology of human atopic disorders. The murine system has been widely used in immunologic research because of the availability of a multitude of genetically engineered mice as well as detailed knowledge of the murine genome and immune system. Additionally, various techniques and reagents have been developed to assess immunological responses in mice. Models to investigate allergic-type diseases require close resemblance to the pathology of the disease in humans and should allow measurement of physiologic parameters. Numerous sensitization protocols with food- and aero-allergens have been used to induce an atopic phenotype in mice (Lloyd et al., 2001). Generation of recombinant allergens has been shown to be advantageous to obtain reliable and reproducible experimental data (Herz et al., 2004). Sensitization to ovalbumin (OVA) and challenge with aerosolized OVA represents the most widely used protocol used in mice to study human bronchial asthma (Neuhaus-Steinmetz et al., 2000). Nevertheless, methods based on immunization with high amounts of protein antigen do not reflect the natural pathway of atopic sensitization leading to allergic reactions in humans. Thus, natural
murine infections inducing a Th2-type immune response including high levels of IgE, such as helminth infections, have also been used to investigate mechanisms involved in development of allergy (Finkelman et al., 1997). Although helminth infections have provided important insight into the regulation of Th2 responses, they are often cleared by the mouse and therefore do not induce a persistent IgE response that would mimic chronic allergic diseases in humans. In consequence, novel improved murine models that would resemble more closely the features of human allergy are required to allow investigation of all aspects of the disease.
1.4 Central Question

The main objective of this thesis was to investigate mechanisms involved in the regulation of IgE production and to analyze the role of IgE interactions with its high affinity receptor, FceRI. To reach this aim, a new model of a murine infection resulting in allergy-like symptoms was established and characterized (Results Part I). In particular, natural murine ectoparasites, the fur mites *Myocoptes musculinus* and *Myobia musculi*, were used and their value as a model for atopic disorders evaluated. Furthermore, a transgenic mouse model mimicking the expression pattern of the human FceRI was generated in order to provide a tool to study the role of FceRI expression on the same cell types as found in humans (Results Part II). Lastly, an attempt to generate a monoclonal antibody against the α chain of the murine FceRI was made to allow specific analysis of the receptor expression (Results Part III).
2 RESULTS PART I

Induction of IgE and allergic-type responses in fur mite-infested mice

2.1 Abstract

High serum IgE levels are characteristic of allergic diseases and immune responses to most parasites. A murine allergy model based on infestation with the fur mites *Myocoptes musculinus* and *Myobia musculi* was investigated. Analysis of mite infestation in various knockout mice revealed that IgE production in response to these ectoparasites was dependent on T cells, IL-4, and CD40L interaction. Secretion of IL-4 by CD4+ T cells obtained from peripheral lymph nodes draining mite-infested skin sites was increased with progressing mite infestation and correlated with the serum IgE induction. A time course analysis of the mRNA expression of ε germline, switched IgE, and AID transcripts suggested that switching to IgE in response to fur mites occurred initially in skin-draining lymph nodes. In addition, mite infestation induced mast cell degranulation in the skin as well as mast cell infiltrations into skin-draining lymph nodes. Analysis of the immune response generated in mite-infested mice should provide information useful for better understanding of mechanisms involved in IgE induction and regulation. Due to the physiological way of allergen-exposure resembling the atopic sensitization in humans, mite infestation might offer a highly valuable model for the investigation of allergic disorders.
2.2 Introduction

IgE is the main antibody isotype involved in allergic disease and immune responses against parasites. The direct effects of IgE during an immediate-type hypersensitivity reaction are well characterized (Corry and Kheradmand, 1999; Kay, 2001; Kinet, 1999). However, many of the specific factors involved in the initiation and induction of IgE are yet to be determined. Many components influencing development of atopy have been proposed, such as lifestyle, diet, and genetic background (Cookson, 1999). Nevertheless, it is still unclear why some individuals develop allergy while others remain allergy-free. Investigation of factors regulating the induction of IgE is therefore of crucial importance to elucidate the etiology of allergic diseases.

Nematode infection models have been widely used to study IgE-related immune responses (Finkelman et al., 1997; Shea-Donohue and Urban, 2004). However, in contrast to allergic diseases, which are chronic, frequently used parasites, such as Trichinella spiralis and Nippostrongylus brasiliensis, induce Th2-type responses leading to protection. Other frequently employed methods to study allergic disorders have been based on immunization with high amounts of purified protein antigens in adjuvants, which induce some of the symptoms of allergy or asthma (Herz et al., 2004). While this can provide information on the effects of IgE after induction, it is important to investigate additional in vivo models, which could yield physiologically relevant information on the factors regulating the initiation of allergic responses.

Murine fur mite infestation was previously suggested to closely mimic the development of allergic type responses (Jungmann et al., 1996a; Laltoo et al., 1979; Weisbroth et al., 1976), however, little research has been done on the immune response to ascariasis. Most common mouse fur mite species are Myocoptes musculinus and Myobia musculi (Gambles, 1952). These mites are natural parasites of mice and live their entire life cycle on the same host (Friedman and Weisbroth, 1977). Interestingly, despite the fact that these mites are classified as ectoparasites and do not penetrate the deeper layers of the skin, infested mice develop greatly increased total serum IgE levels (Jungmann et al., 1996b; Laltoo et al., 1979). Following prolonged periods of infestation, mice develop skin lesions known as mite-associated ulcerative dermatitis (MAUD) of which severity is influenced by the
genetic background of the infested mice (Dawson et al., 1986; Friedman and Weisbroth, 1975).

In the present study we characterized the initial requirements of IgE induction in mite-infested mice. We show that fur mite infestation represents a good animal model for investigation of allergic-type immune responses involving activation of Th2 cells and secretion of IL-4. T cell activation and B cell isotype switching to IgE appear to occur within skin-draining LN. Furthermore, mast cell degranulation was greatly increased in the skin and increased mast cell infiltration in skin-draining LN was observed. These data suggest that mite antigens enter through the skin of infested animals leading to induction of a Th2-type response and B cell switch to IgE in skin-draining LN. Murine mite infestation is therefore a promising physiological animal model in which to study induction of allergic responses.
2.3 Materials and Methods

Mice and mite infestation

C57BL/6, CD40L<sup>−/−</sup> (Renshaw et al., 1994), IL4<sup>−/−</sup> (Kopf et al., 1993), IL5<sup>−/−</sup> (Kopf et al., 1996), and TCRβδ<sup>−/−</sup> (Mombaerts et al., 1993) mice were bred and maintained at the Institute of Laboratory Animal Science of the University of Zurich. BALB/c mice were purchased from Harlan (Netherlands).

For mite infestation female mice at 6-8 weeks of age were put into a cage with a previously infested mouse carrying a mixed population of *Myocoptes musculinus* and *Myobia musculi*. Hereafter infestation occurred by direct contact. Transgenic mice were housed in the same cage with respective control mice in order to avoid cage-cage variation.

Serum IgE and IgG<sub>1</sub> measurement

Blood was collected weekly and total serum IgE and IgG<sub>1</sub> concentrations were determined by enzyme-linked immunosorbent assays (ELISA). ELISA plates were coated with 5 µg/ml rat anti-mouse IgE (clone 6HD5) or with 1 µg/ml goat anti-mouse IgG<sub>1</sub> (Southern Biotechnologies) in 0.1 M NaHCO<sub>3</sub> (pH 9.6) and blocked with 5% (w/v) bovine serum albumin (BSA). Serially diluted serum or standards, IgE (clone TIB-141) and IgG<sub>1</sub> (Zymed), were added and incubated for 2 hours. Bound IgE was detected with biotinylated anti-mouse IgE (clone RIE-4) at 2.5 µg/ml followed by horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) at 1 µg/ml. Bound IgG<sub>1</sub> was detected with biotinylated anti-mouse IgG (Sigma). ABTS peroxidase solution (<sup>2</sup>,<sup>2</sup>′-azino-bis-[3-ethylbenzthiazidine] 6-sulfonic acid) 0.1 mg/ml, 0.05% H<sub>2</sub>O<sub>2</sub>, and 0.1 M NaH<sub>2</sub>P<sub>4</sub> pH 4.0) was used for the peroxidase-catalyzed color reaction. Color reaction was read at 405 nm in a Bio-Rad microplate reader and standard curves and antibody concentrations analyzed with Microplate Manger III software (Bio-Rad).

Flow cytometry

To obtain single cell suspensions, lymph nodes and spleens were smashed though nylon or metal grids whereas lungs were digested for 1 hour at 37°C in DNase I (50 µg/ml, Fluka Chemie) and Collagenase (500 µg/ml, Invitrogen). Cells were restimulated for 6 hours with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of Brefeldin A (5 µg/ml).
Collected cells were surface-stained with anti-CD4-FITC (5 μg/ml, BD Biosciences), fixed with 2% paraformaldehyde and permeabilized in 0.1% saponin. Intracellular staining was performed with anti-IL-4-APC (2 μg/ml, BD Biosciences), anti-IL-5-PE (2 μg/ml, BD Biosciences), or anti-IFN-γ-APC (1 μg/ml, BD Biosciences).

**Histochemistry**

Freshly removed organs were immersed in Carnoy’s fixative (60% Ethanol, 20% Chloroform, 20% Acetic acid). Sections of 5 μm thickness were cut in paraffin, and stained with toluidine blue using standard procedures.

**RT-PCR and sequencing**

B cells were purified to > 95% from spleen and lymph nodes by magnetic-assisted cell sorting (MACS) (Miltenyi Biotec) using anti-B220 microbeads. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. First-strand cDNA synthesis of 1 μg total RNA was performed with random hexamers and SuperScript II reverse transcriptase (Invitrogen Life Technologies). 1 μl of the resulting cDNA was amplified by PCR. Primer sequences and PCR conditions were as follows: mature IgE switched transcripts (962 bp): 5’-CCTGCCCTCGGTTCGTTCTGA-3’ and 5’-CTAGGCAGACTGAAGATGAAG-3’ (94°5’; [94°1’; 58°1’; 72°1’]x35; 72°10’; 10°), ε germline transcripts (300 bp): 5’-GCAGAAGATGGCTTCGATTAAGAAGACGT-3’ and 5’-TCGGCTGAGGATGAGGATGTCGTACGT-3’ (94°5’; [94°1’; 60°1’; 72°1’]x35; 72°10’; 10°), μ germline transcripts (325 bp): 5’-CTCTGGCCCTGTTATTGTTCG-3’ and 5’-ATGGTGCTGGGAGGAAGTC-3’ (94°5’; [94°1’; 60°1’; 72°1’]x35; 72°10’; 10°), AID (300 bp): 5’-GGCTGAGGTTAGGGTTCCATTCAG-3’ and 5’-GAGGGAGTCAAGAAAGTCACTCAG-3’ (94°5’; [94°1’; 60°1’; 72°1’]x35; 72°10’; 10°).

For sequence analysis cDNA was synthesized from B cells purified from spleens of day 80 mite-infested mice. V(D)J_{H}-Cs transcripts were amplified by PCR using a degenerative primer mix (Krebber et al., 1997) specific for the V_{H} region and a reverse primer that anneals within exon 1 of the Cs gene (IgEc, 5’-TCTGAATACCAGGTCACAGTC-3’). PCR products were purified using the agarose gel extraction kit (Qiagen) and subcloned directly into the pGEM-T vector (Promega) for automated sequencing.
2.4 Results

Mite infestation induces IgE

To investigate the antibody response induced by ascariasis, total serum IgE levels were analyzed in C57BL/6 and BALB/c mice infested with two common species of mouse fur mites, *Myobia musculi* and *Myocoptes musculinus*. Blood was collected at weekly intervals following infestation to determine the kinetics of IgE induction. Upregulation of total serum IgE was first observed 15 days after mite infestation (Figure 6A) followed by a gradual increase for about ten weeks. Around 90 days post infestation IgE levels reached a 1000-fold increase from about 3 ng/ml to 2000 ng/ml in C57BL/6 mice and 60 ng/ml to 10,000 ng/ml in BALB/c mice. These high serum IgE levels then remained stable up to two years after first contact with the ectoparasite (data not shown). In addition, total serum IgG1 levels were also determined (Figure 6B). The kinetics for IgG1 induction showed a similar course as observed for IgE, however the increase (10-fold in C57BL/6 and 20-fold in BALB/c) was less prominent. Thus, fur mite infestation parallels an allergic situation where high serum IgE levels are chronically induced in response to the constant presence of naturally occurring allergens.

![Figure 6](image_url)

**Figure 6. Mite infestation induces IgE and IgG1.** C57BL/6 mice (closed symbols) or BALB/c mice (open symbols) were infested with fur mites at day 0. Blood was collected at weekly intervals and total serum IgE (A) and IgG1 (B) was determined by ELISA. The data points represent the mean ± standard deviation (s.d.) of 11 C57BL/6 and 8 BALB/c mice. Results are representative of five separate experiments. The dotted line in A indicates the lower limit sensitivity (0.7 ng/ml) of the IgE ELISA.
Induction of IgE in response to mite infestation requires T cells, CD40L, and IL-4

To investigate which factors are required for the immune response induced against mites, several different knockout mice were infested. At various time points thereafter, blood was collected and total serum IgE and IgG1 concentrations were analyzed. A requirement for T cells for mite-induced IgE was confirmed by investigating TCRβΔΔ mice (Mombaerts et al., 1993), as IgE levels remained below detection limit throughout the time of mite infestation (Figure 7, upper panel). T cells induce isotype switch to IgE by providing cytokines and through direct cognate interaction with B cells. To investigate if both forms of T cell help were required, mice deficient in CD40L, IL-4 or IL-5 were infested with mites and serum IgE levels monitored. As expected, no increase in serum IgE levels was observed in either IL-4 (Kopf et al., 1993) or CD40L (Renshaw et al., 1994) deficient mice, however, the absence of IL-5 (Kopf et al., 1996) did not alter IgE production in response to mites (Figure 7, upper panel). IgG1 measurements led to comparable observations (Figure 7, lower panel).

These data suggest that mite infestation induces B cells to switch to IgE or IgG1 under the influence of signals through CD40 and the Th2-type cytokine IL-4. Thus, the immune response to fur mites requires T cells and IL-4, as observed for IgE induction during allergic reactions. Therefore, mite infestation may serve as a suitable model for investigation of allergic responses causing high serum IgE levels.
Figure 7. Absence of IgE or IgG1 induction in response to mite infestation in mice deficient in T cells, CD40L or IL-4. Knock-out (open symbols) and WT control mice (closed symbols) were infested with mites by direct contact and housed together in the same cage. Total serum IgE (upper panel) and IgG1 (lower panel) of TCRβδ<sup>-/-</sup>, CD40L<sup>-/-</sup>, IL-4<sup>-/-</sup>, and IL-5<sup>-/-</sup> mice were determined by ELISA at different time points after infestation with mites. The data points represent the mean ± s.d. of 4-5 mice per group. Results are representative of two separate experiments. The dotted line in the upper panel indicates the lower limit sensitivity (0.7 ng/ml) of the IgE ELISA.
Mite infestation induces IL-4 secretion in skin-draining LN

To further characterize the immune response mounted against fur mites, we investigated the site of T cell activation, which could provide insight into the site of antigen entry. For this purpose, we determined the cytokine profile of T cells in spleen, lung, and LN draining the skin (brachial and cervical LN), the gut (mesenteric LN), and the lung (mediastinal LN). Cells were isolated at several time points after mite infestation and re-stimulated in vitro with PMA and Ionomycin. Percentages of IL-4-, IL-5-, and IFNγ-producing cells were determined by intracellular staining. Representative dot plots of IL-4+ CD4+ T cells from three different time points post mite infestation are shown in Figure 8A. IL-4 secretion in response to mite infestation was observed in CD4+ T cells from skin-draining LN, mediastinal LN, and lung, but not spleen or mesenteric LN (Figure 8B). Importantly, while significant increase in IL-4 production in mediastinal LN and lung occurred only late after mite infestation (>150 days), the increase in IL-4 levels in skin-draining LN was observed already 30 days post infestation, corresponding to the time of IgE upregulation. These data suggest that the IL-4 responsible for B cell switch to IgE in mite-infested mice was produced in skin-draining LN. In contrast, CD4+ T cell secretion of IL-5 was slightly increased above background levels only at very late time points (>150 days, data not shown). This correlates well with the very low levels of peripheral blood eosinophilia observed in infested mice. In our animal facility the percentage of eosinophils in peripheral blood of naïve C57BL/6 mice averages 0.6% (±0.1% SEM, n=38) and was increased to 2.5% (±0.2% SEM, n=38) on day 60 post infestation. After this time eosinophil levels remained stable or even declined. T cell secretion of IFNγ remained stable at background levels except late time points after mite infestation (data not shown).

Taken together, these results show that T cell activation in response to contact with fur mites was induced in skin-draining LN, suggesting that mite antigens enter initially via the skin and not through other possible routes, such as the lung or the gut. Moreover, skin-draining LN were increased in size following mite infestation while the size of other LN remained stable (data not shown). This provides additional evidence that after uptake of mite antigen T cells are activated in skin-draining LN.
Figure 8. **CD4⁺ T cells in skin-draining LN produce IL-4 in response to mite infestation.** A) Representative dot plots of brachial and cervical LN isolated on day 0, 60, and 320 post mite infestation after PMA/lonomycin restimulation. The percentage of IL-4⁺ CD4⁺ cells is indicated. B) Cells isolated from LN, spleen, and lung on indicated time points after mite infestation and stimulated with PMA/lonomycin (light bars) or with medium alone as controls (dark bars). Gates were set on live CD4⁺ cells and IL-4 expression was analyzed. Results represent the mean ± s.d. of 5 mice per group. Results are representative of two separate experiments.

**Switching to IgE is induced in skin-draining lymph nodes**

In order to determine at which sites B cell switching to IgE was occurring, B cells were purified from the spleen and LN draining the skin, lung, and gut of naïve and mite-infested mice. RT-PCR was then performed to detect ε germline transcripts (GLT), μGLT, mature switched IgE, and activation-induced cytidine deaminase (AID). The presence of εGLT provides evidence for the initial steps leading to IgE class-switch recombination (CSR). The “sterile” εGLT, which lacks the VDJ region, is an essential prerequisite for CSR. AID
has been shown to play a crucial role in CSR (Muramatsu et al., 2000) and its expression indicates initiation of CSR. Mature IgE transcripts, resulting from DNA excision and ligation of the VDJ sequences to the constant ε locus, indicate the presence of IgE switched B cells. μGLT expression was also analyzed and used as an internal control as μGLT result from heavy chain VDJ rearrangement during B cell development and characterize functional B cells. Our results show an upregulation of εGLT and mature IgE transcripts induced in B cells isolated from skin-draining LN, while in B cells from spleen and mesenteric LN the expression of all transcripts remained comparable over the time of infestation (Figure 9). AID expression was also upregulated in skin-draining LN but to a lesser extent. Importantly, the levels of μGLT remained constant, indicating that B cell isolation and cDNA synthesis were comparable in all samples (Figure 9).

Thus, these data suggest that initial B cell switching to IgE in response to mite infestation occurs in skin-draining LN, corresponding to the site of initial IL-4 production.

![Figure 9](image.png)

**Figure 9.** Messenger RNA expression of εGLT and mature switched IgE transcripts is upregulated in skin-draining LN upon mite infestation. B cells were isolated from spleen (Spl), brachial and cervical LN (sLN), and mesenteric LN (mLN) of naïve or fur mite-infested C57BL/6 mice at 15, 23, and 35 days post infestation. Positive controls (+) derive from RNA isolated from B cells stimulated in vitro with IL-4 and anti-CD40 for 40 hours. Negative controls (-) are H2O. Results are representative of two independent experiments.
IgE induced by mite infestation is polyclonal

Parasite infections are known to induce an increase in total IgE although the levels of parasite-specific IgE are usually low (Hagan, 1993; Pritchard, 1993; Turner et al., 1979). This implies that parasites may induce a polyclonal non-specific response. In order to investigate the diversity of the IgE repertoire induced by mite infestation, we determined the usage of the V, D, and J segments of IgE heavy chains present in B cells of mite-infested mice. For this purpose, B cells were isolated from spleens of infested mice and V(D)J Cε transcripts were amplified by RT-PCR and sequenced. Sequences containing an open reading frame and the correct Cε sequence were then aligned to the closest germ-line sequence using the DNAplot database (www.dnaplot.de) to determine the Vh, Dh, and Jh gene usage and the number of mutations within the framework (FR) and the complementarity-determining regions (CDR). Analysis of twelve sequences obtained from three C57BL/6 mice is shown in Table 1 and 2. The majority (11/12) of analyzed clones were found to align to different germline sequences suggesting that the antibody response to fur mites is not directed against a single antigen. Furthermore, the sequences of Vε transcripts of mite-infested mice were not highly mutated suggesting that they may not have undergone somatic hypermutation. This reveals the possibility that the IgE produced in response to mite infestation might not result from a germinal center reaction. However, many more sequences need to be analyzed to allow for representative conclusions.

Table 1. V(D)J Cε rearrangements in mite-infested C57BL/6 mice.

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*Closest germline Vh families of clones isolated from three mite-infested C57BL/6 mice (B6#1-3) were assigned using the DNAplot database (www.dnaplot.de). Numbers of mutations in the framework (FR) and the complementarity-determining region (CDR) were determined for each sequence. Ratio of replacement to silent mutations (R/S) was calculated unless not applicable (NA). Sequence was isolated in two clones after PCR reaction.
Results Part I

Table 2. V(D)J Cε sequences in mite-infested C57BL/6 mice.

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Nucleotides inserted between the VH, DH, and JH sequences. Differences to the closest germline sequence are underlined.

Mite infestation induces mast cell degranulation in skin and mast cell recruitment to skin-draining LN

To investigate the effects of the high IgE concentrations induced upon mite infestation, we performed histochemistry analysis of mast cells by staining sections of several organs with toluidine blue. Total mast cell numbers as well as numbers of degranulated mast cells were determined. While total mast cell numbers in skin sections did not change throughout the time of mite infestation (Figure 10A), the numbers of degranulated mast cells increased greatly upon exposure to mites (Figure 10B). The dramatic increase in degranulation is clearly visible in figures 10C-F, while no such increase was observed in mice deficient in T cells (Figure 10G) or CD40L (Figure 10H) even late after mite infestation. Induction of mast cell degranulation suggests the presence of mite-specific IgE, which would be crosslinked by mite antigens. It also demonstrates that scratching alone resulting from mite presence on the fur cannot be responsible for mast cell degranulation as scratching was also observed in TCRβε−/− and CD40L−/− mice. Thus, our results strongly suggest that the immune response resulting from mite infestation is directed against specific antigens. Moreover, we observed an accumulation of mast cells in skin-draining LN (Figure 10I-L) but not in gut-draining LN (Figure 10M-N). No mast cell infiltration was found in any other organs such as spleen, lung, jejunum, ileum or colon (data not shown). Mast cells infiltrating skin-draining LN upon mite infestation may interact with lymphocytes and influence mite-induced immune responses. However, the relevance of such interactions remains to be determined.
Figure 10. Mast cell degranulation occurs in skin of mite-infested mice. Total numbers ± s.d. of mast cells per 10 high power fields (HPF) of a 20x magnification (A) and the percentage ± s.d. of degranulated mast cells (B) were determined in skin sections of WT C57BL/6 mice at different time points after mite infestation. Tissue sections stained with Toluidine blue are depicted as follows: skin of naïve (C) or mite-infested WT C57BL/6 mice on day 45 (D), 165 (E), and 320 (F) post mite infestation; skin of TCRß-deficient (G) and CD40L-deficient (H) mice on day 200 post mite infestation; cervical LN of naïve (I) or mite-infested WT C57BL/6 mice on day 45 (J), 165 (K), and 320 (L) post mite infestation; mesenteric LN of naïve (M) or mite-infested WT C57BL/6 mice on day 320 (N) post mite infestation. The arrows indicate examples of degranulated mast cells. Results are representative of three mice per group and two independent experiments.
Additionally, we investigated whether mucus production was induced in the lung of mite-infested mice by staining lung sections with periodic acid Schiff (PAS). Accumulation of mucus was never observed in the lungs of mice at various time points post mite infestation (data not shown), indicating absence of lung inflammation in this model.

Induction of mast cell degranulation in the skin as well as infiltration of mast cells in skin-draining LN, in agreement with the observations of IL-4 production and B cell switch to IgE, provide strong evidence that after mite infestation antigen uptake takes place in the skin and an immune response is induced in skin-draining LN.
2.5 Discussion

In the present study we showed that murine ectoparasite infestation can serve as a valuable model to investigate allergic disorders. We characterized the immune response against *Myocoptes musculinus* and *Myobia musculi*, two fur mite species naturally occurring in mice. Our results demonstrate that mite infestation induces a strong Th2-type response resulting in persistent high serum IgE levels. The fact that mites cause a chronic IgE-associated response is of particular interest because of its resemblance with chronically induced atopic reactions in humans that cannot be easily reproduced by common allergy models based on peptide immunizations.

In this report we provide evidence that induction of the immune response against fur mites, involving IL-4 secretion by CD4+ T cell and B cell switching to IgE, takes place in LN draining mite-infested skin. Our results thus suggest that mite antigen is taken up via the skin. Although other antigen entry sites, such as ingestion through the gut or inhalation through the lung, may also be possible, the absence of marked signs of inflammation in these organs indicates that priming of the allergic responses occurs in other tissues. Upregulation of the expression of eGLT and switched IgE transcripts during the course of mite infestation was explicitly shown in B cells isolated from skin-draining LN. Some increase of eGLT and switched IgE transcription was also observed in the mesenteric LN, however, this increase was only detected on day 15 after mite infestation, while in skin-draining LN the upregulation of eGLT and IgE expression was maintained until at least day 35. The induction of high serum IgE levels observed between 15 and 60 days post mite infestation correlates therefore with the class switch recombination to IgE in skin-draining LN. This provides additional evidence for antigen entry to occur via the skin.

As no mite antigen has been identified thus far, we could not directly prove that any of the IgE produced in response to mite infestation was antigen-specific. However, highly increased mast cell degranulation in skin of infested mice strongly suggests that at least part of the total IgE induced was mite antigen specific. This view is supported by a previous report in which passive and active cutaneous anaphylaxis as well as mast cell degranulation in the skin have been demonstrated in response to mite extract (Laltoo et al., 1979). Nevertheless, a large component of the mite-mediated IgE might be antigen non-specific as sequence analysis of the mature IgE transcripts suggested a polyclonal response to fur mite
infestation. Interestingly, the analyzed IgE was not highly mutated from germline sequences, suggesting that the mite-induced IgE (whether specific or non-specific) had not undergone somatic hypermutation. Analysis of additional sequences should provide detailed insight into the nature of the IgE repertoire induced by mites.

In recent years, fur mites have been investigated in the context of atopic dermatitis in Nishiki Cinnamon (NC) mice. The Japanese NC mice were initially believed to spontaneously develop dermatitis-like syndromes accompanied by high serum IgE levels and were therefore suggested as a good model to study human atopic dermatitis (AD) (Matsuda et al., 1997). Later however, the fur mites *Myocoptes musculinus* and *Myobia musculi* were reported to be the cause of skin lesions and increased IgE production in NC mice (Iijima et al., 2000; Morita et al., 1999). Mite antigen-specific IgE was demonstrated in the sera of mite-infested NC mice by histamine release from bone marrow-derived mast cells stimulated with mite extract (Morita et al., 1999). Additionally, histochemical examination of mite-induced skin lesions has revealed significantly increased numbers of mast cells, eosinophils, and T cells as well as the presence of IL-4 (Matsuda et al., 1997). This suggests that IL-4 released from skin CD4+ T cells and mast cells may be involved in the pathogenesis of the AD-like lesions occurring in mite-infested NC mice. However, the mechanism regulating the development of mite-dependent dermatitis has not yet been clarified. Genetic background has been previously accounted for different susceptibilities to mite-associated ulcerative dermatitis (MAUD) (Dawson et al., 1986; Friedman and Weisbroth, 1975). The NC strain appears to be particularly susceptible showing rapid development of severe skin lesions (at ~12 weeks of age) (Matsuda et al., 1997). In the present study, we investigated mite infestation mainly on C57BL/6 mice. MAUD symptoms were observed in about 5% of these mice when mite infestation was maintained for at least one year, which is comparable to previously published data (Dawson et al., 1986). The choice of an adequate mouse strain seems therefore to be of major importance depending on the questions investigated with the mite infestation model. NC mice, prone to readily develop skin lesions, may be most appropriate to study atopic dermatitis. In the present report however, C57BL/6 mice were chosen as a suitable strain to investigate the mechanisms required for the induction and regulation of mite-dependent IgE.

The physiological environment of fur mite infestation makes this model highly suitable for the investigation of initial events inducing IgE production and IgE-mediated
pathophysiology leading to allergic disease. The way of antigen exposure resulting from mite infestation resembles natural allergen sensitization responsible for atopy in humans. Noteworthy, the importance of house dust mites in the development of human atopic dermatitis has been suggested by several studies (Cameron, 1997; Tan et al., 1996). House dust mites are known to trigger airway responses which led to the hypothesis that the respiratory route may be relevant for the induction and exacerbation of dermatitis after house dust mite inhalation in patients with high IgE levels and a history of asthma. In the fur mite model antigens appear to enter the host through the skin and may directly trigger dermatitis-like responses.

Histochemical analysis of organ sections isolated from mite-infested mice revealed both mite-dependent mast cell degranulation in the skin and accumulation of large numbers of mast cells in skin-draining LN. Mast cell degranulation in the skin is likely due to mite antigen-induced crosslinking of IgE bound to the high affinity IgE receptor (FceRI). The effects of inflammatory mediators released by mast cell degranulation are known to induce symptoms of allergy and may eventually contribute to the dermatitis-like lesions occurring in mite-infested mice. However, the role of mast cell accumulation in skin-draining LN has remained controversial. Mite-induced mast cell infiltrations have been previously reported by Jungmann et al. who speculated that mast cells might recirculate into the lymphatics and contribute to antigen transport and presentation in draining LN (Jungmann et al., 1996b). Several studies have shown that mast cells can enhance the development of T cell-associated responses. For example, mast cells have been shown to be able to present antigen in vitro through MHC class I- and class II-dependent pathways (Fox et al., 1994; Frandji et al., 1993; Malaviya et al., 1996). Moreover, mast cells have been demonstrated to express a number of molecules that may allow them to interact with lymphocytes. These molecules include the costimulatory molecules CD40L (Gauchat et al., 1993) and CD80/CD86 (Frandji et al., 1996) and the accessory molecules intercellular adhesion molecule (ICAM)-1 (Valent et al., 1991), ICAM-3 (Babina et al., 1999), and lymphocyte function-associated antigen (LFA)-1 (Toru et al., 1997). Thus, mast cells may contribute to B cell switching to IgE by inducing IL-4- and CD40L-mediated pathways. Interestingly, mast cell migration to LN has also been demonstrated during dinitrofluorobenzene (DNFB)-induced contact hypersensitivity in mice (Wang et al., 1998). Wang et al. have suggested that mast cells may contribute to the induction of primary immune responses by
migration from the site of antigen encounter to draining lymph nodes, where they may promote T cell recruitment by secretion of the chemokine macrophage inflammatory protein (MIP)-1β. In fur mite-infested mice mast cell infiltration was observed exclusively in skin-draining LN. The absence of mast cell accumulation in any other organs suggests the involvement of mite antigens taken up through the skin leading to activation of immune responses in the skin-draining LN. Further investigation is required to evaluate the significance of mast cell accumulation in the skin-draining LN and its possible involvement in mite-induced responses.

Great increase in the prevalence in allergic diseases has been observed over the past two decades and has been recognized to positively correlate with westernized lifestyle (1998). Ample epidemiologic data has suggested that allergy may to some extent result from diminished infectious burden in childhood (Cookson, 1999). The mechanism behind this hypothesis has recently been proposed to involve anti-inflammatory cytokines, such as IL-10 or TGF-β, induced by exposure to pathogens and responsible for suppression of allergic and autoimmune diseases (Wills-Karp et al., 2001). The fur mite infestation model presented in this report could serve to evaluate the regulatory theory of protection against atopic reactions. Mite infestation could be investigated following exposure to viral or bacterial pathogens to study their effect in naturally occurring IgE-mediated immune responses. Thus, the mite allergy model could provide insights into factors regulating the development of atopic disease.
3 RESULTS PART II

Generation and characterization of hmFcεRIαβ Tg mice

3.1 Abstract

Major differences in the expression pattern of the high affinity IgE receptor, FcεRI, exist between humans and rodents. While the tetrameric form of the receptor (αβγ2) is expressed on mast cells and basophils of both mice and humans, the trimeric complex (αγ2) has only been found to be expressed in humans and is present on eosinophils, dendritic cells, Langerhans’ cells, monocytes, macrophages, and platelets. To elucidate the role of FcεRI expression on these multiple potential effector and antigen presenting cells, we generated two transgenic (Tg) mouse models that mimic the human situation. The hmFcεRIα Tg mouse expresses the murine α chain of the receptor under control of the human FcεRIα promoter, while the hmFcεRIβ Tg mouse expresses the murine β chain under control of the human FcεRIα promoter. hmFcεRIαβ double Tg mice were generated by crossing the two Tg lines together. mRNA expression of the transgenes was successfully demonstrated by RT-PCR and Northern blot, which revealed the intended distribution of all three chains of the high affinity IgE receptor on same cells as found in humans. Nevertheless, FcεRI protein expression, investigated by flow cytometry, Western blot, and immunohistochemistry, could not be found in the targeted cells of the hmFcεRIαβ Tg mice. Thus, the generated mouse model requires further investigation to determine the reason of the absence of FcεRI protein expression and to evaluate whether this drawback can be corrected.
3.2 Introduction

The high affinity receptor for IgE, FcεRI, is an essential component of IgE-mediated diseases, such as allergy and asthma. After specific receptor crosslinking via IgE/antigen interactions, FcεRI signal transduction leads to cell degranulation and release of pro-inflammatory mediators. In both humans and mice FcεRI is expressed on mast cells and basophils as a tetramer composed of one α, one β, and two γ chains (αβγ2) (Perez-Montfort et al., 1983). The extracellular domain of the α chain, containing two Ig-like domains, is responsible for binding to IgE (Blank et al., 1991). Although both the β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAMs), β is believed to act merely as an amplifier of signals generated by γ (Jouvin et al., 1994; Lin et al., 1996). Additionally, the β chain has been demonstrated to have a second amplifier function by stabilizing the receptor complex on the cell surface and facilitating maturation of the α chain (Donnadieu et al., 2000). In rodents, the β chain has been reported to be indispensable for efficient surface expression of the receptor (Blank et al., 1989; Ra et al., 1989). In contrast, the human FcεRI can also be found in a trimeric structure, composed only of one α and two γ chains (αγ2), expressed on Langerhans’ cells (Bieber et al., 1992; Wang et al., 1992), monocytes (Maurer et al., 1994), eosinophils (Gounni et al., 1994), peripheral blood dendritic cells (Maurer et al., 1996), and platelets (Joseph et al., 1997). The discovery of FcεRI expression on cells other than mast cells and basophils raised many questions regarding the role of the high affinity IgE receptor, in particular concerning its function in antigen presentation (Novak et al., 2003a; von Bubnoff et al., 2001). However, the lack of FcεRI expression on antigen presenting cells in mice makes it difficult to use murine models for investigation of the biological relevance of FcεRI-mediated antigen presentation. Thus, a “FcεRI-humanized” mouse model was generated with the aim of assessing the functions of FcεRI on antigen presenting cells in a situation more resembling humans (Dombrowicz et al., 1996; Dombrowicz et al., 1998). In these human FcεRIα transgenic mice (hFcεRIα Tg) the endogenous gene encoding the α chain of the FcεRI was replaced with its human homologue. The human FcεRIα promoter used in hFcεRIα Tg mice controlled expression of the transgene such that it retained the cell specificity and structure as found in humans. However, the presence of the human FcεRIα protein in these
mice complicates assessment of the physiological role of the trimeric FcεRI because binding of mouse IgE to the human α chain is characterized by lower affinity than that of human IgE (Pruzansky and Patterson, 1986). Thus, our aim was to generate a different FcεRI Tg mouse model, where the murine FcεRIα was expressed under control of the human FcεRIα promoter, such that in the resulting hmFcεRIα Tg mice the murine high affinity IgE receptor should be expressed with the same cell distribution as observed for the human FcεRI. Such a transgenic animal model should allow investigation of the role of FcεRI expression in host defense or development of allergic diseases using in vivo murine assays.
3.3 Materials and Methods

Mice

C57BL/6 mice were bred and maintained at the Institute of Laboratory Animal Science of the University of Zurich. FcεRIα−/− mice (Dombrowicz et al., 1993) were kindly provided by Jean-Pierre Kinet (Boston, USA). BALB/c mice were purchased from Harlan (Netherlands).

Generation of the transgenic mice

hmFcεRIα Tg mice:

The pGL2 expression vector containing the 2.9 kb sequence of the human FcεRIα promoter, subcloned into Sma I and Xho I restriction digestion sites, was kindly provided by Jean-Pierre Kinet (Boston, USA). The pGEM-7 expression vector containing the pα1H6.8 sequence coding for all 5 exons of the murine FcεRIα genomic DNA, subcloned into Hind III restriction digestion sites, was kindly provided by William E. Paul (Bethesda, USA). A Xho I restriction site was inserted immediately upstream of the ATG codon of the pα1H6.8 sequence to allow cloning of the murine FcεRIα genomic DNA into the pGL2 vector downstream of the 2.9 kb human FcεRIα promoter. The correct cloning of the resulting pGL2 vector (14.8 kb) containing the 2.9 kb human FcεRIα promoter followed by the 6.3 kb murine FcεRIα sequence was verified by sequencing. Thereafter, the vector was digested with Sma I and Nar I resulting in excision of a 9.2 kb sequence coding for the human FcεRIα promoter and the murine FcεRIα. This sequence was microinjected into male pronuclei of fertilized C57BL/6 oocytes at the Institute of Laboratory Animal Science of the University of Zurich.

hmFcεRIβ Tg mice:

The complete cDNA sequence of the murine FcεRIβ sequence was kindly provided by Chisei Ra (Tokyo, Japan) and was subcloned into the pGL2-phFcεRIα-mFcεRIα vector replacing the mFcεRIα sequence. Microinjection was performed as described above.
Bone marrow-derived mast cell (BMMC) culture

Femur and tibia bones were collected from C57BL/6 mice and bone marrow cells were flushed out with BSS. Cells were washed and resuspended at an initial concentration of 10^6 cells/ml in IMDM containing 10% FCS, 50 μM 2-Mercaptoethanol, 20 ng/ml recombinant mouse stem cell factor (SCF, R&D Systems), and 5% X63-IL-3 cell-conditioned medium corresponding to about 5 ng/ml IL-3. Cells were maintained in tissue culture petri dishes at 20 ml/dish in a humidified incubator at 37°C with 5% CO₂. Non-adherent cells were initially transferred to new dishes every other day, later once a week and subsequently expanded as necessary. Fresh SCF and IL-3 were provided weekly. 6 week cultures contained >80% mast cells as confirmed by FACS analysis of CD117 expression.

Cell isolation

Macrophages

Peritoneal macrophages were induced by injection of 1 ml of 5% thioglycollate i.p. The peritoneal cavity was washed with 10 ml ice-cold BSS 72 hours post thioglycollate injection. Macrophages were purified by MACS (Miltenyi Biotec) using anti-CD11b microbeads.

Dendritic cells

To obtain single cell suspensions, spleens or lymph nodes were smashed on metal or nylon grids, respectively. Dendritic cells were then purified by MACS (Miltenyi Biotec) using anti-CD11c microbeads.

Langerhans’ cells

Epidermal cells were isolated as previously described (Schuler and Steinman, 1985). Briefly, ears were collected, split and digested with trypsin at 37°C (dorsal halves in 0.6% trypsin for 20 min and ventral halves in 1% trypsin for 45 min). Epidermal sheets were peeled from the underlying dermis and epidermal cells were collected into HBSS containing 10% FCS. Langerhans’ cells were purified from the epidermal cell suspension by MACS (Miltenyi Biotec) using anti-MHC II microbeads.
**Eosinophils**

Bronchoalveolar lavage (BAL) was performed on *Nippostrongylus brasiliensis*-infected mice on day 14 post infection by inserting a blunted 18G plastic needle into the trachea and flushing the lung three times with 1 ml ice-cold PBS. The recovered cells were analyzed directly without any further purifying step because of low cell yields.

**RT-PCR**

Total RNA was isolated from about 5×10^6 cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed with random hexamers and SuperScript II reverse transcriptase (Invitrogen Life Technologies). Transcripts encoding mFceRI were amplified using the following primers and conditions: α chain (467 bp band) 5’-CGATGGTCGTAAGGTCTGCTGC-3’ and 5’-ATAACTGTAGTTGAAAGCATGG-3’ (94°5’; [94°1’; 54°30”; 72°1’]x35; 72°10’; 10°), β chain (306 bp band) 5’-AACATTGCTAGTAGCATTGATGC-3’ and 5’-CCCTTTGCTCCATCCACTAC-3’ (94°5’; [94°1’; 58°30”; 72°1’]x35; 72°10’; 10°), and γ chain (402 bp band) 5’-CAGCCGTGATCTTTCTGTTTCTG-3’ and 5’-TTAACGGAGATGGGGACCTTC-3’ (94°5’; [94°1’; 58°30”; 72°1’]x35; 72°10’; 10°).

**Northern blot**

Total RNA was isolated from 10^7 cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Equal amounts of RNA (5 μg/lane) were separated on a 1.5% agarose-formaldehyde gel. The RNA was transferred onto a Hybond-N+ nucleic acid transfer membrane (Amersham Biosciences) using the Turbo Blotter system (Schleicher&Schuell). The specific mRNA was detected by hybridization of the membrane with a 32P-labeled RNA probe specific for the murine FceRIα gene. The DNA template for the riboprobe was generated by cloning a 630 bp FceRIα RT-PCR product (primers: FceRIαNhe 5’-CTAGCTAGCATGGTCAGTGGAAGGTCTG-3’ and FceRIαXba 5’-GCTCTAGAAGTTGAGCCAATAATAACTGC-3’, conditions: 94°5’; [94°1’; 54°30”; 72°1’]x35; 72°10’; 10°) into the pGEM-T vector (Promega) and subsequently linearizing the vector by Nde I restriction digestion. The probe was synthesized by incubating 1 μg of the DNA template for 3 h at 37°C in presence of 50 μCi [α-32P]UTP (800 Ci/mmol, Amersham Bioscience), 1 U T7 polymerase, and 10 μM
Ribonuclease Triphosphate set (rATP, rCTP, and rGTP) in a total volume of 25 µl of T7 buffer (Roche). The RNA-containing membrane was prehybridized for 1 h at 68°C in presence of the ULTRAhyb hybridization buffer (Ambion). 5-15x10^6 cpm of the probe was then added and hybridized overnight at 68°C. The membrane was washed twice in 2xSSC and twice in 0.2xSSC, both containing 0.2% SDS, for 15 min at 60°C and exposed overnight to a BIOMAX MR film (Kodak).

**Western blot**

Cells were lysed at 5x10^7 cells/ml in lysis buffer containing 1% digitonin, 150 mM NaCl, 50 mM Hepes, and complete protease inhibitor cocktail (one tablet per 50 ml lysis buffer, Roche). 500 µl lysate was immunoprecipitated with biotinylated anti-FcεRIα antibody (10 µg/ml, eBioscience) for 2 h on a helicopter rotor at 4°C. Streptavidin-conjugated sepharose (Amersham Biosciences) was added and incubated for an additional 2 h on a helicopter rotor at 4°C. The sepharose beads were then washed 3x with PBS and samples denaturated by incubating for 5 min at 95°C in SDS reducing loading buffer. After centrifugation (3 min, 13000 RPM) proteins were separated by running on a 12.5% SDS-polyacrylamide Tris gel (Bio-Rad Laboratories) and then electrotransferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a semi-dry transfer unit (SemiPhor Hoefer). Membranes were blocked in 4% milk/PBS overnight at 4°C. Detection of FcεRIβ was performed with mouse anti-mouse FcεRIβ antibody (Rivera et al., 1988) (1 µg/ml, clone JRK, kindly provided by Juan Rivera, Bethesda, USA) followed by goat anti-mouse IgG antibody (1 µg/ml, EY Laboratories). Detection of FcεRIγ was performed with rabbit anti-mouse FcεRIγ antibody (1 µg/ml, Upstate Biotech) followed by goat anti-rabbit IgG (1 µg/ml, Sigma) antibody. All antibodies were diluted in 1% milk/PBS and incubated with the membranes for 2 h. For both β and γ chains HRPO-conjugated donkey anti-goat IgG (1 µg/ml, Abcam) was used as tertiary antibody and incubated with the membranes for 1 h. Revelation was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions.

**Flow cytometry**

Purified cells were stained using standard procedures with the following antibodies: anti-mouse CD117-APC (0.7 µg/ml, BD Pharmingen), anti-mouse CD11b-PE (0.4 µg/ml, BD
Pharmingen), anti-mouse CD11c-PE (0.7 μg/ml, BD Pharmingen), anti-mouse I-A^b-PE (0.7 μg/ml, BD Pharmingen), anti-mouse GR-1-PE (2 μg/ml, BD Pharmingen), anti-mouse FceRIα-FITC (15 μg/ml, eBiosciences), and FITC-labeled Golden Syrian Hamster isotype control (15 μg/ml, eBiosciences). For intracellular staining cells were fixed with 2% paraformaldehyde and permeabilized in 0.1% saponin. Cells were acquired in a FACS Calibur (BD) and analyzed using FlowJo software.

**Immunofluorescence**

Cytospins of 10^5 cells were performed in a Shandon Southern centrifuge. Cells were fixed in acetone, air-dried, and rehydrated in PBS immediately prior to staining. Anti-mouse CD117-APC (2 μg/ml, BD Pharmingen) or anti-mouse FceRIα-biotin (10 μg/ml, eBiosciences) antibodies were applied onto the fixed cells and incubated for 45 min at room temperature (RT). When staining for FceRIα, streptavidin-Cy3 (1.3 μg/ml, Jackson Immunoresearch) was applied for 30 min at RT. Nuclei were stained with DAPI (1 μg/ml, Sigma) for 5 min at RT. After each staining step slides were washed 3x in PBS. Slide covers were mounted using DakoCytomation fluorescent mounting medium. Fluorescence was monitored on a Zeiss Axiophot microscope with a JVC KYF70 camera, using the Analysis software (Soft Imaging System, Münster, Germany).

**Nippostrongylus brasiliensis infection**

**Parasite maintenance and injection**

*N. brasiliensis* was maintained by serial passage through 6 week-old Lewis rats. To establish rat infections, 5000 infective L3 stage larvae were resuspended in 0.5 ml H2O and injected s.c. Rat fecal pellets were collected daily between day 7 and 11 post infection and cultured with granular charcoal at 27°C for 14-28 days. Exsheathed L3 stage larvae were harvested and resuspended in H2O. Mice were injected s.c. with 550 L3 larvae in 200 μl H2O.

**Determination of parasite egg numbers**

Infected mice were transferred to gridded cages lined with moist paper and fecal pellets were collected daily between day 5 and 11 post *N. brasiliensis* infection. Pellets were resuspended in equal volumes of H2O and saturated NaCl solution, vortexed and a sample
quickly removed and transferred to a McMaster Worm Egg Counting Chamber (Weber Scientific International Ltd.) for quantification. The number eggs per gram feces was calculated knowing that the counting chamber holds 150 μl volume and that 25 mouse fecal pellets correspond to 1 gram.

**Determination of peripheral blood eosinophilia**

Blood smears were prepared from a drop of freshly collected blood, air-dried, and stained with Diff-Quik (Dade Behring) for differential cell counts of lymphocytes, macrophages, neutrophils, and eosinophils. A total of 200 cells were counted and the percentage of eosinophils determined.

**Determination of total serum IgE levels**

Total serum IgE concentrations were determined by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner Microlon) were coated with 5 μg/ml rat anti-mouse IgE (clone 6HD5) in 0.1 M NaHCO3 (pH 9.6) and blocked with 5% (w/v) bovine serum albumin (BSA). Serially diluted serum or standards (IgE clone TIB-141) were added and incubated for 2 hours. Bound IgE was detected with biotinylated anti-mouse IgE (clone RIE-4) at 2.5 μg/ml followed by horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) at 1 μg/ml. ABTS peroxidase solution (2,2’-azino-bis-[3-ethylbenzthiazidine-6-sulfonic acid] 0.1 mg/ml, 0.05% H2O2, and 0.1 M NaH2PO4 pH 4.0) was used for the peroxidase-catalyzed color reaction. Color reaction was read at 405 nm in a Bio-Rad microplate reader and standard curves and IgE concentrations analyzed with Microplate Manger III software (Bio-Rad).
3.4 Results

**Generation of hmFceRIαβ Tg mice**

The strategy used to generate mice with FceRI expression patterns comparable to those found in humans was based on the results obtained by Dombrowicz et al. (Dombrowicz et al., 1996). Thus, to obtain the hmFceRIα Tg mouse, the 2.9 kb promoter region of the human FceRIα encoding the information necessary for cell-specific expression was subcloned into the pGL2 expression vector. The murine FceRIα genomic DNA fragment of 6.3 kb was then subcloned downstream of the promoter into the Xho I and Hind III restriction digestion sites (Figure 11A). For microinjection into C57BL/6 oocytes the promoter and gene were excised from the vector by Sma I and Nar I restriction digestion. Integration and germline transmission of the transgene were monitored by PCR and Southern blot analysis (data not shown).

Expression of the human FceRIα chain in the previously described hFceRIα Tg mice (Dombrowicz et al., 1996; Dombrowicz et al., 1998) was reported to be independent of the presence of the β chain and trimeric αγ2 complexes were identified on peritoneal macrophages isolated from hFceRIα Tg mice. However, in the present approach we used the murine FceRIα chain, which has been demonstrated to require the presence of the β chain for correct surface expression of FceRI (Blank et al., 1989; Ra et al., 1989). Thus, in order to ensure proper expression, in addition to the hmFceRIα Tg mouse we generated an hmFceRIβ Tg mouse, which should express the β chain of the murine FceRI with the same cellular distribution characteristics as found in humans. The transgene construct for the hmFceRIβ Tg mouse was generated by replacing the mFceRIα gene in the pGL2-phFceRIα-mFceRIα with the mFceRIβ sequence (Figure 11B). Lastly, the two generated Tg strains, hmFceRIα and hmFceRIβ, were crossed to obtain double transgenic hmFceRIαβ mice. The phenotype of the double transgenic mice was analyzed by RT-PCR, Northern blot, Western blot, FACS analysis, and immunohistofluorescence as described below. Additionally, the immune response of hmFceRIαβ mice to *Nippostrongylus brasiliensis* infection was assessed.
Figure 11. Transgene constructs for hmFceRIα and hmFceRIβ Tg mice. The 2.9 kb human FceRIα promoter was subcloned into the Sma I and Xho I restriction sites of the pGL2 expression vector. For the hmFceRIα mouse, the 6.3 kb murine FceRIα genomic DNA was subcloned downstream of the promoter into the Xho I and Hind III restriction sites (A). For the hmFceRIβ mouse, the 0.7 kb murine FceRIβ cDNA was subcloned downstream of the promoter into the Xho I and Nar I restriction sites (B). Both vectors were digested with Sma I and Nar I to excise the transgene, which was then used for microinjections into male pronuclei of fertilized C57BL/6 oocytes to generate hmFceRIα and hmFceRIβ Tg mice.
RT-PCR analysis of FcεRI expression in hmFcεRIαβ Tg mice

In order to investigate mRNA expression of FcεRI in different cell types of hmFcεRIα and hmFcεRIαβ Tg mice, RT-PCR primers were generated for each of the chains of the receptor. The primers were tested on the MC/9 mast cell line as well as on bone marrow-derived mast cells (BMMC) of WT C57BL/6, hmFcεRIα Tg, and FcεRIα-deficient mice (Figure 12A). All mast cell samples expressed all three chains of FcεRI (α, β, and γ) except BMMC from FcεRIα−/− mice (Dombrowicz et al., 1993) in which FcεRIα was not detected. As expected, peritoneal macrophages of C57BL/6 mice, used as negative control, expressed only the γ chain of the FcεRI, which is known to be ubiquitously expressed.

Analysis of hmFcεRIα Tg mice revealed mRNA expression of the α chain of the high affinity IgE receptor in macrophages, dendritic cells, eosinophils, and Langerhans’ cells, while no such expression could be detected in WT C57BL/6 mice (Figure 12B). The quality of the cDNA was controlled by the presence of γ chain transcripts in all samples. mRNA expression of both FcεRIα and FcεRIβ was confirmed in macrophages and dendritic cells of the double transgenic hmFcεRIαβ mice (Figure 12C). We have not yet confirmed expression of FcεRIα or FcεRIβ in Langerhans’ cells and eosinophils due to the large numbers of cells required for RNA isolation coupled with limited availability of the double transgenic mice. Surprisingly, we also detected FcεRIα mRNA expression in splenic B cells isolated from hmFcεRIα Tg mice after infection with N. brasiliensis and in splenic B cells of naïve double transgenic mice but at very low levels (data not shown). Further investigation is required to confirm expression of the transgenic high affinity IgE receptor in B cells. As expected, investigation of T cells isolated from both hmFcεRIα and hmFcεRIαβ Tg mice did not reveal any FcεRIα mRNA expression (data not shown).

Taken together, these data indicate that mRNA expression of the high affinity receptor was successfully achieved on the targeted cells of the hmFcεRIαβ Tg mice.
Figure 12. mRNA expression of the individual chains of FcεRI in hmFcεRIα and hmFcεRIαβ Tg mice. RT-PCR for detection of the α, β, and γ chains of FcεRI was performed on mast cells (MC/9 line and BMMC), thioglycollate-induced peritoneal macrophages (MΦ), naïve splenic dendritic cells (spl DC), DC from lymph nodes (LN DC), eosinophils (eos) from the BAL of N. brasiliensis-infected mice, and naïve Langerhans' cells (LC). Cells were isolated from WT C57BL/6 (B6), FcεRIα−/− (α−), hmFcεRIα Tg (αTg), or hmFcεRIαβ Tg (αβ) mice. Controls (A) as well as analysis of hmFcεRIα (B) and hmFcεRIαβ (C) Tg mice are depicted. H2O was used as negative control. The size of each PCR product is indicated. The 100 bp Ladder (L) was used for the size marker and the 500 bp band is indicated. Results are representative of two to four separate experiments.
Northern blot analysis of FcεRI expression in hmFcεRIαβ Tg mice

To provide a quantitative method to investigate mRNA expression of the high affinity IgE receptor, Northern blot analysis for FcεRIα expression was developed. Initially, Northern blot analysis was used to quantify FcεRIα expression levels in different hmFcεRIα Tg lines derived from founders 2, 3, and 14 (Figure 13A). The Tg line with the highest FcεRIα expression was identified (founder 3) and this line was then crossed with hmFcεRIβ Tg mice to generate double transgenic mice.

Northern blot analysis was also performed in order to confirm the RT-PCR results showing that the murine α chain gene was expressed in hmFcεRIα and hmFcεRIαβ Tg mice in the same cells as found in humans. mRNA of the α chain of the receptor was detected in splenic dendritic cells from both hmFcεRIα and hmFcεRIαβ Tg mice, as indicated by the presence of a 1 kb band corresponding to the 1005 bp of the murine FcεRIα mRNA (Ra et al., 1989) (Figure 13B). 18 S rRNA bands were also observed due to unspecific binding of the probe and could be eliminated by more stringent washing (data not shown). However, as they served as a convenient internal control for the amount of loaded RNA they were not routinely washed away. Contrary to what was observed by RT-PCR no expression of FcεRIα was observed in macrophages of either single or double Tg mice. The lack of detection by Northern blot analysis is likely to be due to sensitivity of this assay. It is possible that the levels of FcεRIα expression in macrophages may be lower than in dendritic cells and therefore detectable only by RT-PCR and not by Northern blot. As Northern blot analysis requires large cell numbers to obtain sufficient RNA, investigation of other cell types, such as Langerhans’ cells and eosinophils, could not be performed due to limited availability of the transgenic mice.
Figure 13. Northern blot analysis of FcεRlα expression. FcεRlα expression levels in splenic dendritic cells (spl DC) purified from hmFcεRlα Tg lines derived from founders 2, 3, and 14 were investigated using a FcεRlα-specific radioactive probe (A). Thioglycollate-induced peritoneal macrophages (MΦ) and naïve spl DC purified from WT C57BL/6 (B6), hmFcεRlα Tg (αTg), or hmFcεRlαβ Tg (αβTg) mice were equally analyzed (B). BMMC from WT C57BL/6 mice were used as positive control. The 18 S rRNA bands reflect the amount of loaded RNA. The 0.16-1.77 Kb RNA Ladder was used as marker and the positions of the 1.77 Kb and 1.28 Kb bands are indicated. Results are representative of two (A) or four (B) separate experiments.
Western blot analysis of FcεRI expression in hmFcεRIαβ Tg mice

The results presented thus far confirmed mRNA expression of FcεRI in hmFcεRIαβ Tg mice. We next investigated whether the mRNA was also transcribed into protein in the different cell types of the transgenic mice. For this purpose a method for Western blot analysis of the murine high affinity IgE receptor was developed and tested on mast cells. To assess cytosolic proteins, cells were lysed and lysates were immunoprecipitated with a monoclonal anti-mouse FcεRIα antibody to subsequently identify proteins that were bound to and therefore co-immunoprecipitated with FcεRIα. Precipitated proteins were then analyzed for the presence of FcεRIβ and FcεRIγ by probing with specific antibodies against the β and γ chains of FcεRI. This method allowed demonstration of the presence of the β and γ chains and their association with the α chain. As expected, both the β (Figure 14A) and γ (Figure 14B) chains were found to be associated with the α chain in the mast cell line MC/9 as well as in BMMC of WT mice. Importantly, anti-α chain antibody-mediated immunoprecipitation of BMMC lysates derived from FcεRIα-deficient mice did not reveal the presence of neither β nor γ chains, indicating the specificity of the co-precipitation of the β and γ chains. Thus far we did not succeed in detecting the presence of the α chain in cell lysates by probing membranes containing fractionated proteins with a specific anti-FcεRIα antibody. This may be due to conformational changes induced in FcεRIα while running the samples on a reducing SDS-gel or transferring them to nitrocellulose membranes. Such conformational changes may mask or destroy binding sites of the anti-FcεRIα antibody and therefore inhibit its binding to the α chain.

Analysis of peritoneal macrophages and splenic dendritic cells derived from either hmFcεRIα or hmFcεRIαβ Tg mice did not reveal co-precipitation of the β nor the γ chain with the α chain of FcεRI (Figure 14C). Weak detection of a protein corresponding to the size of the γ chain in the macrophage samples was present, as shown by the weak bands in the lower panel of Figure 14C. However, this protein was also detected in macrophages isolated from FcεRIα-deficient mice, suggesting that the detected band did not result from co-immunoprecipitation with the α chain. It is possible that this band reflects non-specific binding of the anti-γ antibody to a protein present in macrophages. Alternatively, this band may indicate the ability of the γ chain from macrophages to non-specifically “stick” to the sepharose beads used in the immunoprecipitation. Thus, although we could detect mRNA
expression of the subunits composing the high affinity IgE receptor in macrophages and
dendritic cells of the generated transgenic mice, we were not able to show their association
at the protein level. Importantly, this method was based on immunoprecipitation with
FceRIα leaving it open whether the absence of FceRI protein detection in the Tg mice was
due to lack of α chain protein expression in the peritoneal macrophages and dendritic cells
of the Tg mice or to the lack of receptor complex formation in these cells. Alternative
approaches may be envisaged to answer this question and possibly demonstrate the
presence of FceRI complexes in the cells of hmFceRIα and hmFceRIαβ Tg mice.
Therefore, we are currently developing methods for Western blot analysis where cell
lysates are immunoprecipitated with either the β or the γ chain of FceRI and fractionated
proteins are subsequently probed with specific antibodies against the α, β, and γ chains of
the receptor.

Taken together, further investigation is required to determine whether translation of the
FceRI chains from mRNA into protein or their association into receptor complexes was
inhibited in the targeted cells of hmFceRIα and hmFceRIαβ Tg mice. Furthermore, it is
possible that the expression level of FceRI in the transgenic mice is below the detection
limit of the Western blot, implying that additional assays are required to confirm the data.
Figure 14. Immunoprecipitation and Western blot of FcεRI. Digitonin-solubilized protein extracts were prepared from the mast cell line MC/9, BMMC, thioglycollate-induced peritoneal macrophages (Mφ) or naïve splenic dendritic cells (spl DC) isolated from WT C57BL/6 (B6), hmFcεRIα Tg (α), hmFcεRIαβ Tg (αβ) or FcεRIα-deficient (α<sup>−/−</sup>) mice. Immunoprecipitation was carried out with anti-mFcεRIα Ab and co-precipitation of β and γ chain proteins was analyzed by Western blot using antibodies specific for the mFcεRIβ and mFcεRIγ. The molecular mass is indicated in Kilodaltons. Results are representative of four (mast cells) or one (Mφ and DC) separate experiments.
FACS analysis of FcεRI expression in hmFcεRIαβ Tg mice

To further investigate whether hmFcεRIαβ Tg mice express FcεRI, flow cytometry analysis was performed to detect both intra- and extracellular protein expression of FcεRIα. In contrast to the Western blot analysis, which detects the association of the receptor subunits, this approach provides direct information on the presence of translated FcεRIα. Several different cell types were isolated from WT C57BL/6, hmFcεRIα Tg, and hmFcεRIαβ Tg mice and FcεRIα expression was assessed on each cell population. In contrast to BMMC, which were found to express high amounts of the receptor (Figure 15, top panel), FcεRIα protein expression was not detected in macrophages, Langerhans’ cells or dendritic cells isolated from naive hmFcεRIα or hmFcεRIαβ Tg mice (Figure 15, middle panel).

A positive correlation between FcεRI cell surface expression and serum IgE concentrations has been shown previously (Yamaguchi et al., 1997). The mechanisms controlling upregulation of FcεRI by IgE involve receptor stabilization at the membrane and continued basal synthesis of receptors (Borkowski et al., 2001). It has also been reported that in humans FcεRI expression on APCs is greatly upregulated in atopic individuals, which are characterized by high levels of IgE (Maurer et al., 1994; Novak et al., 2003b). Therefore, it was possible that the inability to detect expression of FcεRI in APCs isolated from hmFcεRIα and hmFcεRIαβ Tg mice may reflect the lack of significant levels of serum IgE. Thus, in order to achieve maximal expression of FcεRI we attempted to induce high levels of serum IgE in the transgenic mice prior to cell isolation. Mice were therefore infected with the nematode *Nipponstrongylus brasiliensis*, which is known to induce up to 1000-fold increase in total serum IgE (Finkelman et al., 1988). Cells were then isolated 15 days following *N. brasiliensis* infection when IgE levels had reached a peak of 1769 ng/ml (±166.7 SEM, n=50). Besides macrophages, dendritic cells, and Langerhans’ cells, FcεRIα expression was additionally investigated in eosinophils isolated from BAL of *N. brasiliensis*-infected mice. However, despite the presence of high levels of serum IgE, FcεRIα expression could not be detected in any of the analyzed cells (Figure 15, lower panel).
Figure 15. Intracellular FACS analysis of FcεRIα expression. BMMC, thioglycollate-induced peritoneal macrophages (Mφ), splenic dendritic cells (spl DC), DC from lymph nodes (LN DC), Langerhans’ cells (LC), and eosinophils (eos) from the BAL isolated from either naïve or *N. brasiliensis*-infected WT C57BL/6 (black), hmFcεRIα Tg (αTg, green), or hmFcεRIαβ Tg (αβTg, red) mice were analyzed for FcεRIα expression by intracellular flow cytometry. Cells were gated for the specific cell type markers of each cell population as indicated in brackets. Control stains were performed using BMMC from FcεRIα−/− (blue) and with an isotype control antibody (grey). Results are representative of three (naïve) or two (*N. brasiliensis*-infected) separate experiments.

These data therefore suggest that although there is expression of the α chain of FcεRI at the mRNA level, there does not appear to be expression of FcεRIα protein in the analyzed cells from either hmFcεRIα or hmFcεRIαβ Tg mice. Intracellular FACS analysis should allow detection of both intracellular FcεRIα protein expression and FcεRIα expressed on the cell surface as part of the receptor complex. Thus, these results could be explained by a potential inhibition of the translation of FcεRIα in macrophages, dendritic cells, Langerhans’ cells, and eosinophils of the generated transgenic mice. The reason for such inhibition remains to be determined. Alternatively, it is possible that the α chain is translated into protein but then retained in the endoplasmic reticulum (ER) and rapidly
Results Part II

degraded, which would hinder its detection by FACS. The retention in the ER could be due to incomplete interactions of the β with the α chain, which in mice is required to release FcεRIα from the ER (Ra et al., 1989). Possible mutations in the transgene could lead to structural changes of the receptor chains inhibiting their interactions. Thus, sequence analysis of the transgenic FcεRI may provide further insight into the factors responsible for the absence of FcεRI protein expression in hmFcεRIαβ Tg mice.

**Immunofluorescence analysis of FcεRI expression in hmFcεRIαβ Tg mice**

Although the data from flowcytometry suggested that FcεRIα protein was not expressed in the targeted cells of hmFcεRIα and hmFcεRIαβ Tg mice, we decided to confirm these results by an additional method. For this purpose cytospins of several cell types isolated from WT C57BL/6, hmFcεRIα or hmFcεRIαβ Tg mice were investigated for FcεRIα protein expression by immunofluorescence. To begin with the method was tested on BMMC from WT C57BL/6 and FcεRIα-/- mice, which were used as positive and negative controls, respectively. The results showed clear expression of the mast cell marker CD117 on both cell types, while FcεRIα expression was detected only on WT cells (Figure 16A). However, no FcεRIα expression could be detected in macrophages, dendritic cells, or Langerhans’ cells isolated from either WT or transgenic mice as shown by fluorescence comparable to background levels (Figure 16B). As not all cell surface maker antibodies were available for immunofluorescence analysis, the purity and viability of each cell sample was confirmed by FACS prior to preparation of the cytospins. Analysis of FcεRIα expression in cells isolated from *N. brasiliensis*-infected mice equally revealed only background levels of fluorescence (data not shown), implying that even in presence of high serum IgE levels no detectable FcεRIα expression was induced.

Taken together, these results are consistent with the FACS data leading to the conclusion that despite of significant FcεRIα mRNA expression in the targeted cells of hmFcεRIα and hmFcεRIαβ Tg mice no α chain protein could be detected.
Figure 16. Immunofluorescence of cytopsins. Cytospins of BMMC isolated from WT C57BL/6 (B6) and FcεRIα/− (α−) mice were analyzed for CD117 and FcεRIα expression using specific fluorescent antibodies; nuclei were stained with DAPI (A). FcεRIα expression was investigated in thioglycollate-induced peritoneal macrophages (Mφ), splenic dendritic cells (spl DC), dendritic cells from lymph lodes (LN DC), and Langerhans' cells (LC) isolated from WT C57BL/6 (B6), hmFcεRIα Tg (αTg) or hmFcεRIαβ Tg (αβTg) (B). Results are representative of two separate experiments.
Immune response of hmFcεRIαβ Tg mice to *Nippostrongylus brasiliensis* infection

hmFcεRIα and hmFcεRIαβ Tg mice were infected with the nematode parasite *N. brasiliensis* to induce high serum levels of IgE and to drive maximal FcεRI expression. Additionally, the immune response of the transgenic mice to *N. brasiliensis* was assessed. This approach was based on the hypothesis that the presence of FcεRI on the surface of APCs or eosinophils could provide mechanisms leading to a more efficient immune response against the parasite. For example, FcεRI-mediated antigen uptake by APCs could lead to enhanced antigen presentation and therefore increased T cell activation. Furthermore, activation of eosinophils via FcεRI could improve killing of the parasitic larvae during their passage through the lung. Thus, the level of protection against *N. brasiliensis* was assessed in hmFcεRIα and hmFcεRIαβ Tg mice and compared to that in WT C57BL/6 mice.

Within 10 days post *N. brasiliensis* infection total serum IgE levels increased greatly and remained high in both WT and transgenic mice (Figure 17A). There was no significant difference in the kinetics nor magnitude of IgE induction between WT and transgenic mice. Peripheral blood eosinophil levels were also investigated as *N. brasiliensis* infection is known to induce blood and tissue eosinophilia, which has been suggested to play a role in host protection and expulsion of the parasite (Shin et al., 1997). The percentage of eosinophils in the blood started to increase at day 7 post infection and reached a peak of about 10% around day 15 (Figure 17B). Comparable results were observed for WT as well as hmFcεRIα and hmFcεRIαβ Tg mice. In WT mice the immune response induced by *N. brasiliensis* leads to expulsion of the worms within two weeks after infection. During that period infecting larvae develop into the adult stage within the gut lumen and produce eggs that are expelled via the fecal pellets. The numbers of eggs in the feces are inversely proportional to the strength of the protective immune response. Fecal worm egg counts were therefore monitored in *N. brasiliensis*-infected WT as well as hmFcεRIα and hmFcεRIαβ Tg mice. Comparable worm egg numbers and comparable kinetics of worm expulsion were found between WT and both Tg mouse strains (Figure 17C). The eggs found in hmFcεRIαβ Tg mice had a slightly delayed onset and were cleared one day earlier, however, data of several experiments showed no significant difference between the analyzed mouse strains indicating the they had a comparable degree of protection to *N.
*Nippostrongylus brasiliensis*. Although our results were variable, especially the range of eosinophilia and egg counts was very wide, repeated *N. brasiliensis* injections demonstrated that the overall response did not differ between WT, hmFceRIα, and hmFceRIαβ Tg mice.

**Figure 17. Nippostrongylus brasiliensis (Nb) infection of hmFceRIαβ Tg mice.** C57BL/6 (closed circles), hmFceRIα Tg (open triangles), and hmFceRIαβ Tg (open squares) mice were infected with 550 Nb L$_3$ larvae on day 0. Blood was collected on several time points after infection (as indicated) and total serum IgE levels (A) and blood eosinophilia (B) were determined. The data points represent the mean ± s.d. of 3-5 mice per group. The dotted line in A indicates the lower limit sensitivity (0.7 ng/ml) of the IgE ELISA. Numbers of Nb eggs per gram feces were monitored for each mouse strain (C). Results are representative of five separate experiments.
It is possible that FcεRI expressed on APCs may only play a role in secondary parasite infections, where IgE produced during the primary response has already bound to the receptor. To investigate this hypothesis we performed a booster infection of *N. brasiliensis* ten weeks after the primary infection. Analysis of IgE serum levels during the secondary immune response did however not reveal any significant differences between WT, hmFcεRIα, and hmFcεRIαβ Tg mice (data not shown).
3.5 Discussion

FcεRI transgenic mice were generated to provide a novel tool for the investigation of various aspects of the biology of the high affinity IgE receptor. Using the human FcεRIα promoter we generated transgenic mice with the aim of expressing the murine FcεRI on the same cell types as demonstrated for the human FcεRI. We first generated hmFcεRIα Tg mice that express the murine α chain of the receptor under control of the human FcεRIα promoter. Although previously reported transgenic mice generated with the same technique but using the human α chain of the receptor have been shown to express the αγ2-trimeric receptor complex (Dombrowicz et al., 1998), in the present approach important species differences between the murine and human FcεRIα chain had to be considered, such as the different requirements for receptor surface expression. Transfection studies had previously demonstrated that the murine α chain contains endoplasmic reticulum (ER) retention signals located in the cytoplasmic tail (Letourneur et al., 1995a). The other chains of the FcεRI complex appear to prevent retention and degradation of the α chain in the ER by masking the ER retention signals and allowing export of the receptor to the Golgi apparatus and subsequently to the plasma membrane. While the γ chain has been shown to be sufficient to mask the ER retention signals of the human α chain, the murine α chain requires both the β and γ chains to allow receptor release from the ER (Ra et al., 1989). Therefore, in order to achieve surface expression of FcεRI in hmFcεRIα Tg mice, we were obliged to induce expression of the β chain in the cell types that express the transgenic α chain. For this purpose, hmFcεRIβ Tg mice were generated. Nevertheless, even after crossing the hmFcεRIα and hmFcεRIβ strains together to obtain double transgenic mice, detectable levels of receptor expression could not be found in antigen presenting cells. This result was unexpected as FcεRI mRNA expression was observed in these cells. Several reasons for the lack of detectable protein expression of the high affinity IgE receptor in the generated transgenic mice can be considered. First, a considerable difference in the expression density of FcεRI between the classical IgE effector cells (mast cells and basophils) and other FcεRI-expressing cells has been reported (Donnadieu et al., 2000). It was therefore possible that very low expression levels of the receptor in the targeted cells of our transgenic models could have prevented us from detecting the receptor by commonly
used methods. However, this expression density difference has been accredited to the lack of the β chain on cells other than mast cells and basophils, which was overcome in our approach by additional generation of hmFcεRIβ Tg mice. Second, as Western blot analysis did not show any association of the receptor chains, it can be speculated that regardless of the addition of the β chain to the system, the receptor complex might still be retained and degraded in the ER. Additional analysis, such as confocal microscopy, would be required to determine the exact location of the FcεRI chains in the cytosol. Moreover, to generate the hmFcεRIβ Tg strain rather than using the genomic sequence of FcεRIβ for the transgene construct, β chain cDNA was employed. Thus, it cannot be excluded that the absence of normally occurring mRNA maturation events, such as splicing, could modify the cell processes and prevent the transgene from being translated. Lack of the β chain would consequently lead to degradation of the α chain in the ER. Lastly, it has to be envisaged that mRNA of both the α and β FcεRI chains detected by RT-PCR or Northern blot may have not been translated into protein. This would explain the complete lack of FcεRIα detection by both surface and intracellular FACS analysis. At the moment we do not have an explanation for the absence of FcεRI translation. Many factors are involved in the machinery of gene expression and translation and can account for absence of an expected phenotype in transgenic mouse models. Mutations of the sequence could inhibit transgene expression. Therefore, we are currently analyzing the mRNA sequences of the α and β chains in macrophages of hmFcεRIαβ Tg mice.

*Ne brasiliensis* infection did not reveal any differences in protection against the parasite between WT and hmFcεRIαβ Tg mice. This may obviously be due to the lack of FcεRI protein expression in the Tg mice. However, it is also important to evaluate whether *N. brasiliensis* infection was the appropriate model to investigate hmFcεRIαβ Tg mice. Clearance of *N. brasiliensis* has been suggested to be IgE-independent (Watanabe et al., 1988) and may therefore not involve FcεRI-mediated mechanisms. This would easily explain the absence of differences in protection against *N. brasiliensis* between WT and hmFcεRIαβ Tg mice. Thus, additional models should be investigated to analyze the immune response in hmFcεRIαβ Tg mice, such as parasite infections where IgE is required for protection (e.g. *T. spiralis* (Gurish et al., 2004)).
Taken together, we generated two mouse models, the hmFceRIα and the hmFceRIβ Tg mice, which were crossed together to obtain double transgenic hmFceRIαβ mice. Although presence of the transgenes was detected in the genomic DNA of all these models and their mRNA expression was shown in the targeted cell types, no detectable protein expression was observed. Additional investigation is required to determine the exact reason for lack of protein expression in order to modify the transgenic mice and to enable them to be suitable models for investigation of the role of FceRI expression on APCs and eosinophils.
4 RESULTS PART III

Generation of a muFcεRIα-huIgG₁Fc fusion protein

4.1 Abstract

Studies investigating the interaction of IgE and its high affinity receptor, FcεRI, have revealed novel functions and expression patterns of FcεRI. Mouse models have been widely used to study the downstream effects of IgE binding to FcεRI and their impact on immune responses. However, the lack of a specific anti-mouse FcεRIα antibody hampers investigations of the role of FcεRI in mice. Here we describe the successful generation of a fusion protein composed of the extracellular domain of murine FcεRIα and the human IgG₁ Fc domain. This fusion protein was used for immunization of FcεRIα⁻/⁻ mice in an attempt to induce production of anti-mouse FcεRIα antibodies.
4.2 Introduction

Cell surface receptors for immunoglobulin (Ig) Fc regions, Fc receptors (FcRs), are involved in a broad range of tasks within the immune system and are important regulators of immune responses by inducing either activation or inhibition of cellular effector functions. FcRs are widely expressed on various hematopoietic cells allowing interactions of the Fc domain of antibodies with many specialized cells leading to a diverse range of immune responses (Daeron, 1997). Thus, FcRs are able to provide a link between humoral and cell-mediated immunity. The functions of FcRs can be divided into three groups (Takai, 2002). First, the most important function of FcRs is the regulation of cellular immune responses. Triggering of FcRs leads to biological events such as degranulation of mast cells (Metzger et al., 1986), phagocytosis by macrophages (Anderson et al., 1990), proliferation of B lymphocytes (Phillips and Parker, 1983), or induction of cytokine and chemokine expression (Turner and Kinet, 1999). Although most FcRs induce activating signals through immunoreceptor tyrosine-based activation motifs (ITAMs), there is one inhibitory FcR, FcγRIIB, which contains in its cytoplasmic domain an inhibitory motif (ITIM) allowing down-regulation of many of the above cited effector responses (Ravetch and Bolland, 2001). Second, FcRs play an important role in internalization of immune complexes leading to enhanced antigen presentation (Regnault et al., 1999). The third major function of FcRs involves transcellular transfer of Igs, such as transplacental transfer of maternal IgG (Simister and Mostov, 1989) or transfer of IgA to mucosal surfaces (Phalipon and Corthesy, 2003).

Two Fc receptors for IgE have been identified, the high affinity IgE receptor, FcεRI, and the low affinity IgE receptor, FcεRII (CD23). FcεRI is the major surface molecule involved in induction of allergic diseases (Kinet, 1999). Initially it was believed that the sole role of FcεRI was to induce immediate-type hypersensitivity reactions through allergen/IgE-mediated crosslinkage on the surface of mast cells and basophils. However, in the last decade evidence has accumulated that in humans FcεRI is expressed not only on mast cells and basophils but also on Langerhans’ cells (Bieber et al., 1992; Wang et al., 1992), monocytes (Maurer et al., 1994), eosinophils (Gounni et al., 1994), peripheral blood dendritic cells (Maurer et al., 1996), and platelets (Joseph et al., 1997). More recent investigations have shown that even in the absence of specific antigen, binding of
monomeric IgE to FcεRI can induce signals leading to FcεRI upregulation (Yamaguchi et al., 1997) and mast cell survival (Asai et al., 2001; Kalesnikoff et al., 2001). These novel findings have shed new light on the functions of FcεRI and increased the interest in this receptor.

In order to investigate surface expression of FcεRI in humans, several monoclonal antibodies (mAbs) against the human α chain of the receptor have been generated (Rigby et al., 2000; Riske et al., 1991; Wang et al., 1992). However, the lack of a mAb against the murine FcεRIα makes it difficult to study specific FcεRIα expression in mouse models. Binding of labeled IgE has traditionally been used to study expression of the muFcεRI, but since IgE binds also to the low affinity receptor, CD23 (Delespesse et al., 1992), and an additional receptor, Mac-2 (Cherayil et al., 1989; Frigeri and Liu, 1992), specific muFcεRI binding cannot be observed. Therefore, the objective of the present study was to generate a mAb against the murine α chain of the high affinity IgE receptor (muFcεRIα). As a first step, we generated a fusion protein composed of the extracellular domain of muFcεRIα and the human IgG1 Fc domain (huIgG1Fc). HuIgG1Fc allowed isolation of the fusion protein via a protein A column. In this report we describe the successful production and isolation of the muFcεRIα-huIgG1Fc fusion protein as well as the results obtained from immunizations of FcεRIα−/− mice with the muFcεRIα-huIgG1Fc fusion protein.
4.3 Materials and Methods

Bone marrow-derived mast cell (BMMC) culture

Femur and tibia bones were collected from C57BL/6 mice and bone marrow cells were flushed out with BSS. Cells were washed and resuspended at an initial concentration of 10^6 cells/ml in IMDM containing 10% FCS, 50 μM 2-Mercaptoethanol, 20 ng/ml recombinant mouse stem cell factor (SCF, R&D Systems), and 5% X63-IL-3 cell-conditioned medium corresponding to about 5 ng/ml IL-3. Cells were maintained in tissue culture petri dishes at 20 ml/dish in a humidified incubator at 37°C with 5% CO₂. Non-adherent cells were initially transferred to new dishes every other day, later once a week and subsequently expanded as necessary. Fresh SCF and IL-3 were provided weekly. 6 week cultures contained >80% mast cells confirmed by FACS analysis of CD117 expression.

Generation of muFceRIα-huIgGiFc fusion protein

Total RNA was isolated from bone marrow-derived mast cells using TRIzol reagent (Invitrogen Life Technologies). First-strand cDNA synthesis from 1 μg total RNA was performed using random hexamers and SuperScript II reverse transcriptase (Invitrogen Life Technologies). A 631 bp fragment encoding the extracellular domain of the muFceRIα gene was amplified using primers FceRIαNhe 5’-CTAGCTAGCATGGTCACTGGAAGGTCTG-3’ and FceRIαXba 5’-GCTCTAGAAGTTGTAGCCAATAATACTTGC-3’ (94°5’; [94°1’; 54°30”; 72°1’]x35; 72°10’; 10°). These primers introduce Nhe I and Xba I restriction sites respectively, both of which were utilized for the cloning strategy. The PCR-product was purified from a 1% agarose gel using the QIAquick gel extraction kit 250 (Qiagen). The isolated fragment was subcloned into pGEM-T (Promega) and transformed into XL-1 blue competent bacteria. Plasmid DNA was isolated from single colonies grown on agar-LB/ampicillin plates using the QIAprep Spin Miniprep kit (Qiagen). Restriction digestion with Nhe I and Xba I (Roche) was performed to select for clones containing the FceRIα DNA insert. Positive clones were sequenced and checked for mutations. Sequences were compared with the mRNA sequence of the murine FceRIα (NCBI: NM_010184) (Ra et al., 1989). The FceRIα DNA with the correct sequence was then excised from plasmids by Nhe I and Xba I restriction digestion and ligated into the pCMVKana^RFcB7H expression vector (kindly
provided by J. Weber, Zürich) containing the sequence coding for the Fc portion of the human IgG. Prior to ligation, the pCMVKanaR-FcB7H vector was also digested with Nhe I and Xba I in order to replace the B7H fragment with the FcsRIα gene. The resulting pCMV-KanaR-muFcsRIα-huIgG1Fc vector was then transformed into XL-1 blue competent bacteria. Plasmid DNA was isolated from colonies grown on agar-LB/kanamycin plates and restriction-digested with Nhe I and Xba I as described above. Positive colonies were confirmed by sequencing. Three samples of pCMV-KanaR-muFcsRIα-huIgG1Fc plasmid DNA were selected for transfection. Unless stated otherwise, all procedures were performed according to the manufacturer’s instructions.

**Transfection**

HEK293T human embryonic kidney cells were cultured in DMEM containing 10% FCS and 50 μM 2-Mercaptoethanol. For transfection, 3-4x10^6 cells were incubated in 25 μM Chloroquine (7-Chloro-4-[4-diethylamino-1-Methyl-butylamino] Quinoline) (Sigma) for 1 hour at 37°C. DNA (1 μg GFP/hygR and 10 μg pCMV-KanaR-muFcsRIα-huIgG1Fc expression vectors) was prepared in 1 ml 250 mM CaCl₂, added dropwise to 1 ml 2xBBS (BES-buffered solution) and incubated 2-5 min. Cells were supplemented with 10 ml fresh medium and mixed gently with the DNA solution. After 8 hours of incubation at 37°C medium was exchanged to remove the CaCl₂ DNA solution. For stable transfection, cells were cultured in presence of 200 μg/ml Hygromycin B.

**ELISA**

ELISA plates were coated with 1.6 μg/ml goat anti-human IgG (Jackson ImmunoResearch Laboratories) in 0.1 M NaHCO₃ (pH 9.6) overnight at 4°C and then blocked with 5% (w/v) bovine serum albumin (BSA). For detection of the muFcsRIα-huIgG1Fc fusion protein in supernatant of transfected HEK293T cells, serially diluted supernatant was added and incubated for 2 hours. Detection of bound muFcsRIα-huIgG1Fc was performed by adding horseradish peroxidase (HRPO)-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) at 0.8 μg/ml for 1 hour. For detection of anti-muFcsRIα-huIgG1Fc antibodies in serum of immunized mice or in supernatant of hybridoma, the muFcsRIα-huIgG1Fc fusion protein or a control hulgG1Fc fusion protein at 10 μg/ml was added to plates coated with goat anti-human IgG and incubated for 1 hour. Thereafter,
serially diluted sera or hybridoma supernatants were incubated for 2 hours, followed by incubation of HRPO-conjugated goat anti-mouse IgG (Sigma) at 1 μg/ml. ABTS peroxidase solution (2,2’-azino-bis-[3-ethylbenzthiazidine-6-sulfonic acid] 0.1 mg/ml, 0.05% H₂O₂, and 0.1 M NaH₂PO₄ pH 4.0) was used for the peroxidase-catalyzed color reaction. Color reaction was read at 405 nm in a Bio-Rad microplate reader.

**Western blot**

Transfected HEK293T cells were grown in serum-free InVitrus medium (R&D Laboratory) for 3 days and supernatant from a confluent T25 flask was collected by centrifugation (15 min, 4000 RPM). 25 μl protein A sepharose CL-4B (Pharmacia Biotech) was added per 1 ml supernatant and incubated on a rotor at 4°C overnight. Sepharose beads were then washed twice with PBS and samples denaturated by incubating for 5 min at 95°C in SDS reducing loading buffer. After centrifugation (3 min, 13000 RPM) proteins were separated on 10% SDS-polyacrylamide Tris gels (Bio-Rad Laboratories) and then electrotransferred to Protran nitrocellulose membranes (Schleicher&Schuell) using a semi-dry transfer unit (SemiPhor Hoefer). Membranes were blocked in 4%milk/PBS overnight at 4°C and probed with HRPO-conjugated goat anti-human IgG antibody (Caltag Laboratories) at 0.7 μg/ml for 2 hours. Revelation was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions.

**Flow cytometry**

To analyze the presence of anti-muFceRIα antibodies in sera of immunized mice or supernatants of generated hybridoma, BMMC were incubated with 1:2 diluted sera or supernatants for 20 min on ice. Thereafter cells were surface-stained with anti-mouse IgG-FITC (2.5 μg/ml, Caltag Laboratories) and anti-mouse CD117-PE (0.7 μg/ml, BD Biosciences). To test for expression of FceRIα on BMMC, cells were incubated with mouse IgE (clone RIE4) at 50 μg/ml and stained thereafter with anti-mouse IgE-FITC (20 μg/ml, Southern Biotechnology). Cells were aquired in a FACS Calibur (BD) and analyzed using FlowJo software.
Mice

C57BL/6 mice were bred and maintained at the Institute of Laboratory Animal Science of the University of Zurich. FcεRIα−/− mice (Dombrowicz et al., 1993) were kindly provided by Jean-Pierre Kinet, Boston, USA.

Immunization

muFcεRIα-huIgG1Fc fusion protein injections:

FcεRIα−/− mice were tolerized against human IgG1 by an i.v. injection with 500 μg of human IgG1κ (Sigma). Two weeks later an initial immunization with 20 μg muFcεRIα-huIgG1Fc fusion protein in complete Freund’s adjuvant (CFA) s.c. was administrated followed by five booster immunizations at 2-week intervals: four with 20 μg muFcεRIα-huIgG1Fc fusion protein in incomplete Freund’s adjuvant (IFA) s.c. and one with 50 μg muFcεRIα-huIgG1Fc fusion protein in IFA i.p.. A final immunization with 50 μg muFcεRIα-huIgG1Fc fusion protein in PBS i.p. was administrated 1 day after the last boost.

Mast cells injections:

FcεRIα−/− mice were immunized five times with 10⁶-10⁷ BMMC in PBS i.p. at 2-week intervals. A final immunization with 10⁶ BMMC in PBS i.v. was administrated 1 day after the last boost.

For both immunization protocols, splenocyte fusion to myeloma cells was performed 3 days after the final immunization.

Hybridoma production

Spleens of immunized animals were isolated and single cell suspensions prepared. 2x10⁷ splenocytes and 2x10⁷ cells of the mouse myeloma cell line X63AG8.653 were mixed. Fusion was induced by adding 1% PEG 4000 (Polyethylenglycol, Merck) in BSS to the cell pellet at 37°C. After fusion, cells were resuspended in pre-warmed HAT-IMDM medium (Hypoxanthine-Aminopterine-Thymidine, Gibco) and distributed into 96-well plates containing peritoneal macrophages isolated from C57BL/6 mice and plated one day before fusion. Hybridoma colonies were detectable 7-10 days after fusion and screening of supernatant was performed once medium had turned orange.
4.4 Results

Cloning of the fusion gene muFceRIα-huIgG₁Fc

The initial objective of the present study was to generate a fusion protein composed of the extracellular domain of the murine α chain of the high affinity IgE receptor (muFceRIα) and the Fc portion of the human IgG₁ (huIgG₁Fc). The huIgG₁Fc was added to the muFceRIα protein to enable isolation of the expressed protein via binding to a protein A column. To achieve expression of the muFceRIα-huIgG₁Fc fusion protein, the sequences coding for both the muFceRIα extracellular domain and the huIgG₁Fc fragment were cloned together into an expression vector (pCMV-KanaR). To obtain the muFceRIα sequence, bone marrow-derived mast cells (BMMC) from C57BL/6 mice were prepared and total RNA was isolated from the cultured cells, which had been tested for purity by CD117 expression analysis and of which FceRIα expression was confirmed by IgE binding (Figure 18A). The extracellular domain of the murine α chain of the high affinity IgE receptor was then amplified using specific primers, which also introduced restriction sites for the enzymes Nhe I and Xba I. As shown in Figure 18B, the primers aligned in exon 1 and exon 5 of the muFceRIα gene yielding a PCR-product of 631 bp. The PCR-product was then subcloned into the pGEM-T vector and transformed into XL-1 blue competent bacteria. Single clones containing the insert were identified by restriction digestion using Xba I and Nhe I and then sequenced. Three samples (4c, 5b, 8a) with the correct sequence of muFceRIα were then cloned into the pCMV-KanaR-huIgG₁Fc expression vector containing the huIgG₁Fc (Figure 18C).
Figure 18. Amplification of the muFceRIα sequence and cloning of the pCMV-muFceRIα-hulgG1Fc expression vector. BMMC were analyzed for purity (CD117 expression) and expression of FceRI (binding of IgE) (A). The percentage of the cells present in each quadrant is indicated. A primer set aligning within exon 1 and exon 5 of the muFceRIα sequence and containing restriction sites for Nhe I and Xba I was used in RT-PCR on total RNA isolated from BMMC (B). The upper line indicates the protein domains of FceRIα (TM=transmembrane, CP=cytoplasmic). The resulting 631 bp PCR-product coding for the extracellular domain of muFceRIα was cloned into the pCMV-KanaR-hulgG1Fc vector (C). Kanamycin resistance was used for amplification of the vector.
Transfection and screening for cells expressing the muFceRIα-huIgG1Fc fusion protein

The human embryonic kidney cell line HEK293T was transfected with the pCMV-muFceRIα-huIgG1Fc vector to achieve expression of the muFceRIα-huIgG1Fc fusion protein. To allow selection of transfected cells, a vector expressing the green fluorescence protein (GFP) and Hygromycin resistance (HygR) was co-transfected into HEK293T cells together with the pCMV-muFceRIα-huIgG1Fc vector. Transfected cells were identified by GFP expression, using a fluorescent microscope. All three transfections obtained from the constructs 4c, 5b, and 8a showed positive cells based on the presence of green fluorescence (Figure 19A). At different time points after transfection, presence of the muFceRIα-huIgG1Fc fusion protein in the supernatant of transfected cells was determined by ELISA (Figure 19B). Cells transfected with the construct 4c produced the highest concentration of protein, however, cells transfected with constructs 5b and 8a also showed substantial fusion protein expression. As all three transfected cell lines produced the fusion protein, all three were subcloned in order to obtain clonal cell lines. All cell lines derived from single cells were tested by ELISA to determine if fusion protein expression was maintained. Over 300 clones were tested (data not shown) of which 31 with highest protein expression were chosen to be expanded and tested again by ELISA. Three clones, labeled 8a3B, 5b4B, and 4c1A, with the highest protein expression were selected for further investigation. Western blot analysis revealed that clone 8a3B appeared to produce the highest amounts of the muFceRIα-huIgG1Fc fusion protein (Figure 19C). This clone was therefore selected for further expansion and subsequent isolation of the fusion protein.
Results Part III

Figure 19. Transfection of HEK293T cells with three constructs coding for the muFcsRIα-hulgG1Fc fusion protein. pCMV-muFcsRIα-hulgG1Fc vectors were co-transfected into HEK293T cells together with a vector coding for GFP and HygR. After transfection cells were grown in presence of Hygromycin and tested for GFP expression. Cells transfected with three selected constructs of pCMV-muFcsRIα-hulgG1Fc (4c, 5b, and 8a) are depicted 16 days after transfection (A). ELISA analysis for presence of hulgG1Fc in supernatants of transfected cells 19 days post transfection (B). A 3-fold serial dilution was performed on initially undiluted supernatants. Background of the ELISA was 0.16. Western blot analysis for presence of hulgG1Fc in the supernatants of three clonal transfected cell lines (8a3B, 5b4B, and 4c1A) (C). A control hulgG1Fc fusion protein was used as reference for the results. ELISA and Western blot results are representative of three separate experiments.

Isolation of the muFcsRIα-hulgG1Fc fusion protein

In order to purify the muFcsRIα-hulgG1Fc fusion protein, four liters of supernatant of the clone 8a3B were concentrated down to 100 ml and the fusion protein was isolated by binding to a protein A column. Bound protein was eluted from the column and collected in 4 ml fractions. As shown in Figure 20A, fractions 5 to 11 contained high amounts of protein with the protein peak eluting in fraction 6. The presence of the muFcsRIα-
huIgG_{1}Fc fusion protein in fractions 5 through 11 was then tested by specific ELISA with all fractions revealing the presence of high amounts of fusion protein (Figure 20B). Fractions 5 to 11 were therefore pooled and the total amount of protein determined. The final yield of the isolated muFcsRI\alpha-huIgG_{1}Fc fusion protein was 6 grams at a concentration of 0.1 mg/ml.

![Graph A](image)

**Figure 20. Elution and analysis of the muFcsRI\alpha-huIgG_{1}Fc fusion protein.** Four liters of supernatant was collected from transfected clone 8a3B and concentrated to 100 ml. Fusion protein was purified by binding to a protein A column and bound protein eluted in 4 ml fractions. The OD (280 nm) of the eluent was measured (A). Fractions with a high OD are depicted in yellow. Presence of huIgG_{1}Fc in each of the fractions 5-11 was measured by ELISA (B). Samples were pre-diluted 1:10 and then a 3-fold serial dilution was performed. Background of the ELISA was 0.14.
Immunization of FcεRIα−/− mice with the muFcεRIα-huIgG1Fc fusion protein

After successful generation of the muFcεRIα-huIgG1Fc fusion protein, the second aim of this study was to generate monoclonal anti-muFcεRIα antibodies. To obtain such antibodies, mice deficient in FcεRIα (Dombrowicz et al., 1993) were chosen for immunization with the fusion protein as these mice should recognize FcεRIα as a foreign protein and therefore produce anti-FcεRIα antibodies. Prior to immunization with the fusion protein, FcεRIα−/− mice were tolerized against human IgG1 by injection of a high amount of human IgG1. Tolerance against human IgG1 was induced in an attempt to prevent production of antibodies against the huIgG1Fc portion of the fusion protein. To generate an anti-FcεRIα antibody response, FcεRIα−/− mice were then immunized with the muFcεRIα-huIgG1Fc fusion protein six times at two-week intervals. Blood was collected prior to each immunization in order to investigate the presence of anti-muFcεRIα antibodies in the serum. The immunization protocol was performed three times with 3-4 mice per group. Representative results obtained from one immunization regime are shown in Figure 21 (FACS) and Figure 22 (ELISA).

The presence of anti-muFcεRIα antibodies in serum of immunized FcεRIα−/− mice was analyzed by FACS using BMMC. These BMMC were CD117 positive and expressed high amounts of FcεRIα as visualized by staining with IgE and anti-IgE (Figure 21A). Thus, in order to detect anti-muFcεRIα antibodies, serum was incubated with BMMC and anti-muFcεRIα binding was subsequently detected using fluorochrome-labeled anti-mouse IgG antibody, as illustrated in Figure 21B. None of the immunized FcεRIα−/− mice had detectable amounts of anti-muFcεRIα antibodies in their serum, although serum from mouse #3 may have contained small amounts of antibody as a small increase in mast cell binding was observed (Figure 21C).
Figure 21. FACS analysis of sera of FcεRIα−/− mice immunized with the muFcεRIα-huIgG1Fc fusion protein. BMMC (CD117⁺) expressing high levels of FcεRI (binding to IgE) were used for FACS analysis (A). To detect anti-muFcεRIα antibodies in blood of immunized mice, serum was incubated with BMMC and binding to FcεRIα was subsequently detected by fluorochrome-labeled anti-mouse IgG antibodies (B). Analysis of serum of four FcεRIα−/− mice collected before the third, fourth and fifth boost with the muFcεRIα-huIgG1Fc fusion protein is shown (C). The percentage of the cells present in each quadrant is indicated.

Serum samples were also analyzed by ELISA for the presence of antibodies binding to the muFcεRIα-huIgG1Fc fusion protein. To confirm that antibodies present in the sera of immunized mice were binding to the muFcεRIα portion of the fusion protein and not to the huIgG1Fc fragment, a control huIgG1Fc fusion protein was included in the analysis, as illustrated in Figure 22A. Results of ELISA analysis using the muFcεRIα-huIgG1Fc fusion
protein for antibody binding detection showed high levels of protein binding in sera collected before the third, fourth, and fifth boost with the muFcεRIα-huIgG1Fc fusion protein (Figure 22B, orange symbols). However, sera collected prior to the fifth boost also revealed high protein binding to a control huIgG1Fc fusion protein (Figure 22B, green squares). Moreover, analysis of serum collected prior to the first immunization with the muFcεRIα-huIgG1Fc fusion protein also revealed binding to the control huIgG1Fc fusion protein (Figure 22B, green circles). These data indicated the presence of anti-huIgG1 antibodies in the sera of immunized FcεRIα−/− mice. Such antibodies were most likely generated after injection of the human IgG1 used for tolerization and could be detected in the ELISA but not in the FACS analysis due to the different experimental setup.

Surprisingly, immunization of FcεRIα−/− mice with the muFcεRIα-huIgG1Fc fusion protein caused development of skin lesions, which were not observed in control mice injected with adjuvant alone. Due to the skin lesion development as well as the apparent absence of specific anti-muFcεRIα antibodies, a different strategy of immunization was attempted where FcεRIα−/− mice were immunized with BMMC expressing high levels of FcεRI.
Immunization of FcεRIα−/− mice with bone marrow-derived mast cells.

Immunization of FcεRIα−/− mice with the muFceRIα-huIgG1Fc fusion protein revealed two difficulties. First, rather than generating specific anti-muFceRIα antibodies, immunized mice produced antibodies against the human IgG1. Second, the immunization caused severe skin lesions to a degree that some mice had to be removed from the experiment. This prompted us to develop a new strategy for generation of anti-muFceRIα antibodies. We decided to immunize FcεRIα−/− mice with BMMC, which express high levels of FcεRIα on the cell surface. Blood was collected before each BMMC injection and serum was screened for presence of anti-muFceRIα antibodies using the same FACS and ELISA protocols as described for immunization with the muFceRIα-huIgG1Fc fusion protein (Figures 21B and 22A). The immunization regime was performed twice on 3-4 mice per group and representative results of two mice are depicted in Figure 23. Interestingly, the presence of mouse IgG antibodies binding to BMMC in serum of FcεRIα−/− mice increased upon immunization with BMMC, as monitored by FACS showing a shift of stained BMMC from about 1% to 8% (Figure 23A). This suggested that increasing amounts of BMMC-binding antibodies were induced upon BMMC immunization. ELISA analysis also revealed the presence of antibodies binding to the muFceRIα-huIgG1Fc fusion protein in the sera of FcεRIα−/− mice immunized with BMMC (Figure 23B, closed symbols). No such binding was observed in sera from naïve FcεRIα−/− mice (Figure 23B, open symbols). Importantly, no antibody binding was detected using a control huIgG1Fc fusion protein (data not shown) indicating that antibodies present in serum of immunized mice bound specifically to the muFceRIα portion of the muFceRIα-huIgG1Fc fusion protein.

Thus, both the FACS and ELISA data confirmed induction of antibodies in FcεRIα−/− mice in response to immunization with BMMC. B cells from immunized mice were therefore used to generate hybridoma cell lines producing anti-muFceRIα antibodies. Splenocytes were fused with myeloma cells and supernatants of successfully generated hybridoma single cell clones were tested for the presence of anti-muFceRIα antibodies by specific FACS and ELISA. Over 100 hybridoma clones were generated, however, none of these hybridoma were found to produce antibodies binding to either BMMC by FACS (Figure 24) or to the muFceRIα-huIgG1Fc fusion protein by ELISA (data not shown). Thus, the hybridoma production did not lead to generation of a monoclonal anti-muFceRIα antibody.
Figure 23. Detection of anti-FcεRⅠα antibodies in FcεRⅠα-/- mice immunized with BMMC. FACS (A) and ELISA (B) were performed on sera of immunized mice as described in Figure 21B and 22A, respectively. The top panels show control stains of BMMC with IgE to confirm high levels of FcεRⅠ expression and with anti-mouse IgG used as secondary Ab in the subsequent stains. Representative results of two mice (#2 and #6) are shown. The percentage of the cells present in each quadrant is indicated. For ELISA analysis, serum was pre-diluted 1:10 and then a 3-fold serial dilution was performed. Background of the ELISA was 0.1.
Figure 24. Analysis of hybridoma generated from BMMC-immunized FcεRIα⁻/⁻ mice. Supernatant of generated hybridoma clones was tested by FACS using BMMC as described in Figure 21B. Six representative clones of over 100 tested hybridoma are depicted. The percentage of the cells present in each quadrant is indicated.
4.5 Discussion

The high affinity IgE receptor, FcεRI, regulates signals leading to immediate-type hypersensitivity reactions by binding of IgE on mast cells or basophils. In the presence of specific antigen, the receptor is crosslinked leading to degranulation of the cell and release of allergic mediators (Kinet, 1999). Signaling through FcεRI can also be triggered by monomeric IgE, in the absence of antigen, resulting in FcεRI upregulation and cell survival (Asai et al., 2001; Kalesnikoff et al., 2001; Yamaguchi et al., 1997). In addition to its classical functions on mast cells and basophils, in humans, FcεRI has also been shown to be expressed on Langerhans’ cells (Bieber et al., 1992; Wang et al., 1992), monocytes (Maurer et al., 1994), eosinophils (Gounni et al., 1994), peripheral blood dendritic cells (Maurer et al., 1996), and platelets (Hasegawa et al., 1999).

To allow specific investigation of FcεRI expression and function in mouse models, we undertook to generate a monoclonal anti-mouse FcεRIα antibody. The first aim was to generate a fusion protein composed of the extracellular domain of the murine FcεRIα and the human IgG1 Fc fragment. For this purpose, sequences coding for muFcεRIα and huIgG1Fc were cloned into an expression vector and transfected into a mammalian cell line. Expression of the fusion protein was confirmed by ELISA and Western blot analysis detecting huIgG1Fc in the supernatant of transfected cells. The muFcεRIα-huIgG1Fc fusion protein was isolated from the supernatant using binding properties of huIgG1Fc to protein A.

After successful generation and isolation of a considerable amount of purified fusion protein, the second aim of this project was to use this muFcεRIα-huIgG1Fc fusion protein to immunize FcεRIα−/− mice in an attempt to generate anti-muFcεRIα antibodies. FcεRIα−/− mice are deficient in the α chain of the high affinity IgE receptor, thus immunization with the fusion protein should induce production of antibodies against FcεRIα. However, as the fusion protein was also composed of the huIgG1Fc domain, we were obliged to tolerize the mice with huIgG1 in order to prevent generation of antibodies against the huIgG1Fc portion of the fusion protein. Nevertheless, our results suggest that rather than tolerization, FcεRIα-deficient mice developed anti-huIgG1 antibodies. One possible way to overcome such a problem would be to remove the huIgG1Fc portion of the fusion protein from the muFcεRIα portion prior to immunization. A separation of the two portions would have
been possible by introducing a cleavage site, such as for Factor Xa, into the expression vector between the sequences coding for muFcsRIα and huIgG1Fc.

We encountered an additional difficulty with the muFcsRIα-huIgG1Fc fusion protein when attempting to investigate binding to IgE. Although we could detect binding of muFcsRIα-huIgG1Fc to anti-huIgG antibodies by both ELISA and Western blot, despite numerous protocols used, we were not able to show binding of muFcsRIα-huIgG1Fc to murine IgE. This may indicate that the muFcsRIα portion of the fusion protein was not properly folded such that the three-dimensional structure did not reveal the IgE-binding site. Alternatively, the presence of the huIgG1Fc portion may have prevented binding of IgE, possibly due to steric hindrance. In either case, removal of the huIgG1Fc portion by Factor Xa, as suggested above, might have allowed successful binding of IgE to the muFcsRIα portion.

Surprisingly, FcsRIα−/− mice immunized with the muFcsRIα-huIgG1Fc fusion protein responded with unexpected skin lesions. At present the mechanism behind induction of these skin lesions is unclear, even if the response appeared to be specific for the fusion protein, as adjuvant alone had no effect. Although the formation of these lesions may be immunologically interesting, they nevertheless prevented us from pursuing the immunization protocol using the muFcsRIα-huIgG1Fc fusion protein. As an alternative we developed a different immunization method based on injections of FcsRIα−/− mice with BMMC expressing high levels of FcsRI. Analysis of serum of FcsRIα−/− mice immunized with BMMC revealed the presence of antibodies binding to BMMC, however, generation of hybridoma clones from splenocytes of these mice did not result in production of anti-muFcsRIα antibodies. The reason for unsuccessful generation of hybridoma producing anti-muFcsRIα antibodies is presently unclear. It is possible that generation of additional hybridoma would eventually yield specific antibody production. However, during the completion of this study a commercial anti-muFcsRIα antibody became available and the project was therefore terminated.

In conclusion, a muFcsRIα-huIgG1Fc fusion protein was successfully generated, expressed in the HEK293T cell line, and substantial amounts of protein were purified. The fusion protein was shown to bind specifically to anti-huIgG but not to IgE. Immunization of FcsRIα−/− mice with the muFcsRIα-huIgG1Fc fusion protein or with BMMC did not result
in generation of anti-muFceR1α antibodies, however, the fusion protein is available in large amounts and can be used in future if needed.
5 DISCUSSION

5.1 The functions of IgE

IgE, formerly referred to as “reagin”, was first recognized as a new immunoglobulin class in 1967 (Ishizaka and Ishizaka, 1967; Johansson and Bennich, 1967). However, initial description of reagin was performed much earlier, when Cooke and Vander-Veer published a first report on the genetic predisposition to allergy in 1916 (Cooke, 1916). The role of reagin in hypersensitivity was further confirmed by Prausnitz and Küstner who demonstrated in 1921 that this serum factor was responsible for allergic reactions (Prausnitz, 1921). Thus, the first identified function of IgE was related to its involvement in the development of allergy. However, it seems rather astonishing that IgE would evolve to induce harmful immune responses suggesting that there must also be a beneficial function of IgE in immunity that has led to the evolution of this antibody. Indeed, in 1964 Bridget Ogilvie proposed that IgE (at that time still called reaginic antibody) may play an important role in immunity to parasitic helminth infections (Ogilvie, 1964). Since then, a large body of literature has accumulated to demonstrate the host-protective function of IgE in parasite infections, as reviewed by Hagan (Hagan, 1993). Although high IgE levels are associated with most parasite infections and a protective role has been assigned to IgE against parasites such as Schistosoma mansoni (Capron et al., 1987) and Trichinella spiralis (Dessein et al., 1981), the involvement of IgE in host-defense mechanisms remains controversial for many parasite species. This suggests that IgE participation in parasite infections may strongly depend on the characteristics of individual parasites, including different life cycles, host penetration routes, and tissue localizations. Thus, further investigation is required to determine the mechanisms regulating effector elements involved in protection against each individual parasite species.

Taken together, IgE is involved in two types of immune responses. On one hand, IgE is produced in response to parasite infections and may be involved in anti-parasitic host-defense. On the other hand, IgE mediates adverse immune responses resulting in allergic disease. The most prevalent parasite infections (ascariasis, trichiasis, and hookworm infections) are believed to affect nearly two billion people worldwide, mostly in East Asia and Sub-Saharan Africa (de Silva et al., 2003). These numbers are far from declining and
afflict considerable human suffering and economic hardship. Understanding the factors mediating protection against parasites is therefore of high importance. Contrary to nematode infections, allergy mostly affects western countries, which may explain why significantly more research has been done on IgE in the context of allergic disease than on immunity to parasites. Interestingly, both branches of IgE biology, parasite protection and allergy, appear to rely on the same effector mechanisms. Thus, investigation of either of the two “faces” of IgE may provide valuable means to diminish the harm of both allergy and parasite infections.

5.2 The epidemic of allergy

The benefits of a contemporary westernized lifestyle, such as low infant mortality due to improved sanitation and access to drinkable water, strongly correlate with an increased prevalence of allergy. The incidence of allergic disorders has risen greatly in the past decades reaching epidemiological dimensions, particularly in the developed world. Most allergic reactions are not life threatening, however they cause distress for millions. Quality of life is significantly decreased by allergic rhinitis, eczema, and asthma, which interfere with sleep, intellectual functioning, and recreational activities. Food allergies cause constant concern with eating habits and fear of unnoticed ingestion of the respective allergen. More dramatic outcomes of allergy-related pathologies are illustrated with anaphylaxis and severe asthma, which can cause death. Statistical studies of allergic disorders report alarming results. In countries such as the UK, Australia, or New Zealand, around 25% of children under the age of 14 are reported to be affected with asthma and/or eczema (1998). A few years ago the cost of treating asthma in the United States was estimated to be about 6 billion US dollars per year (Smith et al., 1997) and may now be significantly higher. Allergic diseases are a feature of westernized societies and have therefore been suggested to result from reductions in the nature and frequency of childhood infections. To explain the correlation between reduced infectious stress and increased prevalence of allergy, different models have been proposed, the two most prominent being the hygiene hypothesis and the counter-regulatory model (Wills-Karp et al., 2001). However, both of these models have been disputed and additional evidence is required to clearly demonstrate the environmental factors and immunological regulators responsible for the observed increase in allergic disorders.
5.3 Mechanisms of IgE-mediated immune responses

Allergic conditions are believed to share a unique mechanism of disease induction, which involves high levels of IgE production. Indeed, total serum IgE concentrations have been shown to positively correlate with the severity of allergic reactions (Burrows et al., 1989; Laske and Niggemann, 2004). Binding of IgE to its high affinity receptor, FcεRI, expressed most importantly on mast cells, followed by antigen-mediated crosslinking of receptor-bound IgE, induces the release of allergic mediators leading to symptoms of atopic disease (Kay, 2001). The pathology of atopy includes weal and flare eruptions of the skin (hives and urticaria), rhinitis, asthma, and anaphylaxis. Consequently, investigating the factors that control IgE production is essential to understand the pathogenesis of allergy. The regulation of IgE production is based on the activation of Th2 cells and secretion of Th2-type cytokines, most importantly IL-4 and IL-13 (Geha et al., 2003). Understanding the mechanisms regulating the induction of Th2-mediated immune responses is crucial to provide effective means against allergic disease.

5.4 Remaining questions to be answered

Despite much progress in allergy research, many questions concerning the development of the disease remain open and hinder the development of effective therapies. A great number of factors have been shown to influence the outcome of allergic responses, including early events during fetal life and childhood, genetic background, and lifestyle (Cookson, 1999). This illustrates the complexity of the disease and explains the difficulty of successful therapy and prevention.

One of the most basic questions regarding IgE production concerns the polarization of the T helper response. Naïve CD4+ T cells differentiate upon encounter of specific antigens into two functionally distinct effector subtypes, Th1 and Th2 (Abbas et al., 1996). Polarization of T helper cells depends mainly on the cytokine milieu. Thus, induction of Th1 cells requires the presence of IL-12, which is produced by APCs after pathogen-dependent activation of Toll-like receptors (Dabbagh and Lewis, 2003). On the other hand, development of Th2 cells requires the cytokine IL-4, which is also crucial for the production of IgE (Finkelman et al., 1988). However, the molecular basis for Th2 polarization is poorly understood. Recent findings have suggested that thymic stromal lymphopoietin (TSLP) produced by epithelial cells at the site of antigen entry in skin and
mucosa may interact with human dendritic cells. These interactions have been suggested to induce production of Th2-attracting chemokines, such as CCL17 and CCL22, and Th2 differentiation characterized by secretion of IL-4, IL-5, and IL-13 (Soumelis et al., 2002). However, in mice TSLP has no effect on dendritic cells (Leonard, 2002) implying that other mechanisms must be involved in Th2 differentiation in the murine system. Ample research has been performed to investigate the relevant source of IL-4, however, the mechanisms involved in its induction remain unclear (Liew, 2002). Thus, as long as the factors inducing a Th2-type response are not elucidated, the initiation of IgE-mediated responses will remain unresolved. Several cell subsets have been shown to have the capacity of producing IL-4. Mature CD4+ T cells have been reported to be a sufficient source of IL-4 for initial induction of IgE synthesis (Schmitz et al., 1994). Mast cells (Plaut et al., 1989) and basophils (Seder et al., 1991) are known to produce IL-4 upon crosslinkage of high affinity Fce receptors. Interestingly, mast cells and eosinophils have been shown to express IL-4 in bronchial mucosa of asthmatic patients (Ying et al., 1997) and cultured basophils have been demonstrated to produce biologically active IL-4 and express functional CD40L contributing to IgE production by B cells (Yanagihara et al., 1998). Recent studies using IL-4 reporter mice have shown IL-4 synthesis by basophils and eosinophils in response to infection with a Th2-inducing parasite (Min et al., 2004; Voehringer et al., 2004). Furthermore, natural killer (NK) T cells have been suggested to favor the development of Th2 cells by providing a source of IL-4 (Yoshimoto and Paul, 1994). Thus, initial induction of IL-4 might involve several still unresolved mechanisms, which may depend on factors such as the tissue localization of pathogenic infection or the nature of the antigen.

While the cytokine environment can clearly modulate the fate of a naïve CD4+ T cell, other factors have also been described to influence Th development, such as nature, dose, and localization of antigen (Constant and Bottomly, 1997). These characteristics may explain why some pathogens preferentially induce one of the Th subsets, such that intracellular microorganisms induce Th1 responses while extracellular forms of pathogens cause Th2 differentiation. The number of antigenic epitopes available to the precursor Th cell during priming appears to play an important role. This suggests that efficiency of uptake and processing by APCs, which has been shown to be influenced by the initial form of the antigen (Constant et al., 1995), may indirectly influence the development of Th cells.
Controversial data have been reported on the effect of antigen dose on Th cell polarization, as reviewed by Constant and Bottomly (Constant and Bottomly, 1997). While several studies have reported that high doses of immunogen induce Th1 responses and low doses favor Th2 responses (Bancroft et al., 1994), others have shown opposite results (Wang et al., 1996). Thus, the experimental settings of each study need to be evaluated.

Another still intriguing part of IgE-related immunity relates to the function of its high affinity receptor, FcεRI. Although the role of FcεRI in mediating allergic reactions is well established, other physiological functions of FcεRI are less clear. Research from the past decade has greatly improved our knowledge of the biology of FcεRI. The main progress is due to the discovery of major differences between the composition and cellular distribution of human and murine FcεRI. While in mice FcεRI is expressed only on mast cells and basophils with an obligatory tetrameric structure described by the αβγ2 chain composition, the human FcεRI can be found both as a tetramer on mast cells and basophils and additionally as a trimer (αγ2) on Langerhans’ cells, monocytes, eosinophils, peripheral blood dendritic cells, and platelets (Kinet, 1999). These findings revealed an unexpected participation of FcεRI in antigen presentation (Novak et al., 2003a). Furthermore, investigation of the β chain of the high affinity IgE receptor demonstrated that β serves as an amplifier of FcεRI functions by enhancing the signaling cascades transduced through the receptor (Lin et al., 1996) and by increasing FcεRI cell surface expression (Donnadieu et al., 2000). These important functions of FcεRIβ imply distinct physiological roles for the classical tetrameric receptor complex and the trimeric isoform lacking the β chain. Additionally, polymorphism within the FcεRIβ gene has been associated with atopic phenotypes (Hill and Cookson, 1996). Interesting findings have also been reported on the α chain of the receptor. FcεRIα has been proposed to be target for specific anti-FcεRIα autoantibodies able to release histamine from basophils and mast cells leading to development of chronic urticaria (Fiebiger et al., 1995). In particular, an imbalance between FcεRI occupancy and natural anti-FcεRIα antibodies has been suggested to play an important role in the pathogenesis of autoimmune urticaria (Horn et al., 2001). Another remarkable feature of the high affinity IgE receptor is that the surface expression of FcεRI is regulated by its own ligand, IgE. Binding of IgE to FcεRI has been shown to upregulate surface expression of the receptor in mast cells and basophils (Lantz et al., 1997;
Yamaguchi et al., 1997) by receptor stabilization at the membrane (Borkowski et al., 2001). This implies that increased IgE levels may enhance the sensitivity to pathogens or allergens due to an increased number of receptors occupied by IgE with more antigen specificities. Finally, a role in protection against parasites has also been assigned to FcεRI. Increased liver pathology in response to S. mansoni has been demonstrated in FcεRIα-deficient mice (Jankovic et al., 1997) and eosinophil-mediated cytotoxicity against S. mansoni has been shown to involve FcεRI (Gounni et al., 1994). Thus, the high affinity IgE receptor has a very broad range of functions, of which some are not yet completely understood and require further investigation.

5.5 Questions addressed in this thesis

The aim of this thesis was to address some of the questions regarding the role of IgE in allergic diseases, the mechanisms involved in the regulation of IgE induction, and the interactions of IgE and FcεRI.

Results Part I

Animal models of Th2-type conditions should provide suitable tools for investigation of the early steps of immune responses leading to IgE production and consequently to allergic reactions. The fur mite infestation model, described in the first part of the results, was characterized to determine its value as a tool to investigate mechanisms involved in the induction of IgE and to determine its suitability as a model to study allergy. The immune response induced by a mixed population of murine fur mites, Myocoptes musculinus and Myobia musculi, was therefore characterized. The results showed that high levels of serum IgE induced in mice in response to mite infestation involved the same mechanisms as those known for allergy-inducing IgE in human disease, namely Th2 cell-dependent CD40/CD40L interactions and secretion of IL-4. The kinetics and tissue localization of isotype switching to IgE in B cells was investigated in detail and revealed that mite antigens appear to enter via the skin from where they are transported to skin-draining lymph nodes, the site of T cell activation and B cell switch to IgE.

Furthermore, the IgE repertoire induced in response to mite infestation was analyzed. Preliminary results revealed little mutations in the Vh, Dh, and Jh segments of mite-induced IgE. This suggests that the analyzed IgE had not undergone somatic hypermutation and
therefore may not have gone through a germinal center reaction. It has been postulated that in certain conditions, such as allergic rhinitis, B cells can undergo class switch recombination to IgE at the site of inflammation, i.e. outside secondary lymphoid organs (Takhar et al., 2005). Thus, further characterization of mite-induced IgE is required to determine whether it may result from local B cell activation.

Importantly, antigen-exposure during mite infestation occurs in a fully physiological way. Fur mites are natural murine parasites and mice are infested via direct contact with a previously infested mouse. Thus, no artificial injections are required and mice are exposed to physiological antigen loads. This implies an enormous advantage of the mite infestation model compared with other models used in allergy research, such as immunization with high amounts of purified protein antigens in adjuvants or infection with high doses of nematode parasites. The close resemblance of the mite infestation model to allergen sensitization in humans, both involving exposure to antigen loads corresponding to those present in the natural environment, makes this model a valuable tool to study the mechanisms regulating IgE induction and development of allergic conditions.

Results Part II

The high affinity IgE receptor, FcεRI, is an important player in IgE-mediated immune responses. The functions of FcεRI, in particular the role of its expression on cells such as APCs and eosinophils, are not fully understood. Therefore, the second part of the present thesis was dedicated to provide better knowledge of the biological role of FcεRI by generating a transgenic mouse model. The aim was to generate mice that would mirror the human distribution pattern of FcεRI. If successful, such a model should provide important insight into the role of FcεRI expression on multiple cells in humans on which the receptor is lacking in the murine system, including Langerhans’ cells, monocytes, eosinophils, dendritic cells, and platelets. Comparison of immune responses in WT mice with those in animals expressing FcεRI with a human distribution should provide information on the importance of these cells in IgE-mediated reactions. For example, the presence of FcεRI-positive Langerhans’ cells and eosinophils in a murine model should allow a novel way for investigation of atopic dermatitis and asthma and provide new insight into the role of IgE in these diseases. Generation of hmFcεRIαβ Tg mice was based on the usage of the human FcεRIα promoter to drive expression of the α and β chains of the murine FcεRI with the
cellular distribution pattern found in humans. Although mRNA expression of the
transgenes was detected in the targeted cell types of the generated transgenic mice, no
FceRI protein expression could be shown. The lack of detectable protein expression may be
due to retention and degradation of the receptor complex in the ER or to absence of
transcription of the transgenic mRNA. Further investigation of this transgenic model, such
as confocal microscopy and sequencing of the transgenic receptor chains, is therefore
required to enable its usage for investigation of the role of FceRI expression.

Results Part III

Investigation of FceRI requires tools allowing precise detection of its expression. Specific
monoclonal antibodies are commonly used to analyze the expression of cell surface
molecules. As no anti-mouse FceRIα antibodies were available, we made an attempt to
generate a specific antibody against the α chain of the murine high affinity IgE receptor.
For this purpose a fusion protein composed of the murine FceRIα and the human IgG1 Fc
portion was generated and used to immunize FceRIα-deficient mice. However, the applied
protocol of α chain protein injections into FceRIα-deficient mice did not result in the
expected synthesis of anti-FceRIα antibodies. Moreover, a commercial anti-mouse FceRIα
antibody became available during the completion of this project prompting us to cease
further investigation. Nevertheless, the successfully generated and purified muFceRIα-
huIgG1Fc fusion protein is available in large amounts and can be used at further point if
needed.

5.6 Concluding remarks

Last but not least, applying the knowledge gained from experimental animal models to the
human condition requires much care. Differences between results obtained from rodent
systems and the pathology of human allergic disease have to be evaluated to avoid
misleading conclusions. The finding that IgE may be produced locally, in the nasal mucosa
of patients with allergic rhinitis (Smurthwaite et al., 2001) and the lung mucosa of
asthmatics (Wilson et al., 2002), implies that serum IgE or skin reactivity to common
allergens might underestimate a critical role of IgE in disease. Locally limited IgE, such as
IgE content of mucosal fluids, has to be additionally investigated to fully elucidate the role
of this intriguing antibody in allergy.
6 REFERENCES


REFERENCES


112 REFERENCES


REFERENCES


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