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Interferon regulatory factors 1 and 4 in T cell mediated immune modulation

Interferon regulatorische Faktoren 1 und 4 in der T Zell-vermittelten Immunantwort

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Die Quelle unseres Jammers ist,
dass wir mehr von den Laboratorien als von den Oratorien leben.
Albert Einstein (1879-1955)

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ABBREVIATIONS

°C	degree Celsius
µF	micro Farad
µl	micro liter (10^{-9}l)
µM	micro molar
%	procent
Ag	antigen
Ab	antibody
AKP	alkaline phosphatase
APC	antigten presenting cell
Aqua dest.	destilled water
ATP	adenosine tri-phosphate
BAC	bacterial artificial chromosome
BAL	brochoalveolar lavage
bp	base pairs
BCR	B cell receptor
BSA	serum albumin bovine
BSS	Hank's balanced salt solution
CD	cluster of differentiation
DMSO	dimethyl sulfoxide
DNA	desoxribonucleic acid
DNase	desoxyribonuklease
dNTP	2´-Desoxyribonukleosid-5´-triphosphat
DTT	dithiothreitrol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene diamine tetra acetic acid
e.g.	example given
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
E.coli	Escherichia *coli*
Et al.	Et alii
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescin isothiocyanate
g	gramm
G418	Geneticin
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseraddish peroxidase
IA	inner arm
IFNγ	interferon gamma

Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
IRF1	interferon regulatory factor 1
IRF4	interferon regulator factor 4
Irf1$^{-/-}$	deficient in *Irf1* gene
Irf4$^{-/-}$	deficient in *Irf4* gene
ISRE	interferon stimulatory response element
kb	kilobases
KO	knockout
l	liter
LB-medium	Luria-Bertani-Medium
L. *major*	Leishmania *major*
LN	lymph node
M	Molar
mAB	monoclonal antibody
MACS	magnetic activated sell sorter
ME	mercaptoetrhanol
MEF	murine embryonic fibroblast
mg	milligramm
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mM	millimolar
ng	nanogramm
nM	nanomolar
NFκB-	
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PMA	phorbol 12-myristate 13-acetate
pg	picogramm
R	receptor
rm	recombinant murine
RNA	ribonucleic acid
RNase	ribonuclease
RORα	RAR related orphan receptor α
RORγt	RAR related orphan receptor γt
RT	room temperature
SA3’	short arm 3’
SA5’	short arm 5’
sec	second

SDS	sodium dedecyl sulfate
SN	supernatant
SSC	sodium-chloride-sodium-citrate buffer
STAT	signal transducer and activator of transcription
TAE	Tris-Acetate-EDTA-buffer
T-bet	T-box transcription factor, T-box21
TCR	T cell receptor
γδ TCR	gamma delta T cell receptor
TGF-β	tumor growth factor beta
T_H	T helper
TRIS	2-amino-2-hydroxymethyl-propane-1,3-diol
U	Unit
v/v	volume per volume
WT	wildtype
w/v	weight per volume

SUMMARY

The transcription factor interferon regulatory factor (IRF) 1 is essential for T helper cell 1 differentiation. Hereby, the respective *Irf1* knockout mouse displays a severe immune defect and has an Th2 directed immune status *per se*. This effect is measurable by increased production of interleukin-4, a Th2 associated cytokine, and increased levels of immunoglobulin E. By this, the mouse is incapable to cope with intracellular pathogens and eventually dies from infection (e.g. *Leishmania major* infection). Allergies are also mediated by a Th2 driven immune response, therefore the question raised, whether this knockout mouse would react stronger in case of an induced allergy. To address this topic, the murine OVA model for acute asthma was used. Interestingly and despite wide-ranging analyses, it could be shown in this thesis work that the IRF1 deficiency did not result in a more severe asthma pathology. The herein presented data urgently suggest to reconsider the long-standing paradigm of asthma as only being a Th2-diven disease. Newly dicovered Th cell subsets support this opinion.

The *Irf1* knockout mouse not only has a defect in Th cell differentiation, but also clearly shows the requirement of IRF1 in CD8 development. The numbers of $CD8^+$ cells in the periphery is extremely reduced, mainly due to a thymic misregulation of MHC class I. In order to reveal cell-specific functions of IRF1, a conditionally targeted mouse for *Irf1* was successfully generated in the framework of this thesis. This mouse will help to address a variety of questions regarding IRF1 function in a given setting.

Beside IRF1, another factor of the IRF family, namely IRF4 is important for Th differentiation. IRF4 has functions in Th2, Th9 and Th17 cell development, and the IRF4 deficient mouse is completely resistant to murine experimental autoimmune encephalomyelitis, a model for the human disease multiple sclerosis, due to the total incapacity to generate Th17 cells producing IL-17. Surprising data are shown in this thesis, in which γδ T cells from *Irf4* knockout mice are nevertheless totally capable of producing IL-17. The underlying mechanism was closer investigated, and although it was not possible to reveal the actual pathway (which is apparently different from the one triggering IL-17 production in Th17 cells), the possibilities were narrowed down by excluding many other apparently obvious pathways.

Zusammenfassung

Der Transkriptionsfaktor inteferon regulatory factor (IRF) 1 ist essentiell für die T Helfer (Th)-Zell-Differenzierung in Richtung Th1. Die entsprechende Knockout-Maus hat diesbezüglich einen so schwerwiegenden Defekt, daß sie *per se* eine Th2 gerichtete Immunantwort zeigt. Diese zeigt sich in einem erhöhten Level am Th2-Zytokin Interleukin (IL)-4 und einem erhöhten Immunglobulin E-Spiegel. Damit einhergehend ist diese Maus auch anfällig für intrazelluläre Pathogene, denen sie gegebenenfalls erliegt (z.B. eine *Leishmania major*-Infektion). Allergien zeigen ebenfalls eine Th2-gerichtete Immunantwort, daher lag die Vermutung nahe, daß die IRF1-defiziente Maus einen schweren Allergieverlauf aufweisen könnte. Im akuten murinen Asthmamodell, bei dem eine allergische Reaktion mittels Ovalbumin (OVA) provoziert wird, sollte diese Frage untersucht werden. Trotz breitgefächerter Analysen konnte in dieser Arbeit gezeigt werden, daß die IRF1 Maus erstaunlicherweise keine stärkere Pathologie entwickelt. Dieses Ergebnis macht besonders deutlich, daß die seit langem vorherrschende Meinung, daß Allergien, insbesondere Asthma, eine Th2-vermittelte Erkrankung sind, neu überdacht werden muß. Zur Zeit werden immer neue T helfer Subtypen beschrieben, die die hier gezeigten Ergebnisse weiter untermauern.

Die IRF1 defiziente Maus weist auch einen Entwicklungsdefekt von $CD8^+$ T Zellen auf, der deutlich macht, daß IRF1 auch andere Funktionen hat, die sich unabhängig von der T Helfer Zell-Differenzierung abspielen. In der Peripherie sind erheblich weniger CD8 positive Zellen zu finden, hauptsächlich weil im Thymus die MHCI-Präsentation fehlgeleitet ist. Um zellspezifische Funktionen von IRF1 aufzuklären, wurde im Rahmen dieser Arbeit eine für IRF1 konditionell getargetete Maus generiert, die sicherlich im Rahmen vielfältigster Fragestellungen hilfreich sein wird.

Neben IRF1 hat IRF4 eine äquivalente Funktion in der Th2 Zelldifferenzierung, zusätzlich ist es aber ebenso wichtig für Th9 und Th17 Immunantworten. Im Fall der Th17 Zellen ist dieser Effekt derart drastisch, daß in der *Irf4* Knockoutmaus keine Th17-Differenzierung stattfindet und die Maus im murinen Modell für Multiple Sklerose, der experimentellen autoimmunen Enzephalomylitis, für diese Krankheit vollständig resistent ist. Daher überraschte die in dieser Arbeit gezeigte IL-17 Produktion von IRF4 defizienten γδ T-Zellen. Obwohl der zugrundeliegende Mechanismus noch nicht aufgedeckt werden werden konnte, ist es dennoch möglich gewesen, im Rahmen dieser Arbeit verschiedene, naheliegende Signalwege auszuschließen und damit die Möglichkeiten stark einzugrenzen.

1. INTRODUCTION

1.1 ADAPTIVE IMMUNITY

The immune system is comprised of biological structures and mechanisms to protect and defend the organism against foreign pathogens such as bacteria, viruses or parasites or to prevent self-destruction of the organism. Two major parts can be distinguished: the innate immune system and the adaptive immune system.

The innate immune system is able to react immediately and in an unspecific way by few evolutionary conserved mechanisms after encountering a foreign structure. The recognition is triggered by so-called pathogen pattern recognition receptors that recognise widespread microbial components. Examples of cells of the innate immune system are macrophages, natural killer cells and granulocytes.

In contrast, the evolutionary younger adaptive immune system allows a highly specific and efficient response to selected pathogenic structures. This intervention needs training and a tight regulation to prevent unnecessary damage. In case of reinfection this training enables a much faster and more efficient strike. The function of the adaptive immune system is mainly based on T- and B-cell interactions. They are able to specifically recognise a variety of pathogens via highly selective receptors on their surfaces, which are generated by somatic hyper-mutation.

1.1.1 T HELPER CELLS

T helper (Th) cells represent a sub-group of lymphocytes and play an important role in establishing and maximising the capabilities of the adaptive immune system, as well as in activating and directing other immune cells during the course of an immune response. All Th cells are characterised by cluster of differentiation molecule (CD) 4 and develop from lymphoid progenitors and finally maturate in the thymus just like cytotoxic T cells, which can be identified by CD8. During their thymic development, peptide loaded major histocompatibility complexes (MHC) test correct engagement of the T cell receptors (TCRs) in a process called positive selection. To avoid autoreactivity, MHC-

molecules of the thymic epithelium present self-antigens to the developing T cells. A strong avidity leads to elimination of the reactive cell. This mechanism illustrates the phenomenon of central tolerance.

With the discovery that T cell-derived cytokines regulate immunoglobulin (Ig) E production by B cells, T helper cell subsets were first described in mice (MOSMANN *ET AL.* 1986, MOSMANN AND COFFMAN 1989) and later in humans (DEL PRETE *ET AL.* 1991). Among these, Th1 cells produce mainly interferon-gamma (IFN-γ) and tumor necrosis factor-beta (TNF-β), thereby increasing the killing efficiency of macrophages and cytotoxic T cells in response to intracellular pathogens. Lymphotoxin (LT) and TNF enhance this mechanism by activating neutrophils and also B cells to produce opsonising antibodies, which in turn increase microbe elimination. In addition, Th1 cells have been correlated with autoimmune diseases such as type1 diabetes (SKURKOVICH AND SKURKORVICH 2003).

Although more and more cell types and T helper cell subpopulations come into play, according to the current model Th2 cells seem to direct the major response in allergic reactions to common environmental allergens. Th2 cells trigger the recruitment and involvement of other cell types with their production of soluble factors that in the sum result in an inflammatory cascade of an unequalled complexity. Th2 cells are able to directly recognise allergen peptide via their T cell receptor and account for combined involvement of immunoglobulin (Ig) E-producing B cells by secreting interleukin (IL)-4 and IL-13, of mast cells via IL-10 in conjuction with IL-4 (THOMPSON-SNIPES *ET AL.* 1991) and of eosinophils as well as granulocytes by recruiting them via IL-5. It is of note that the secretion of these cytokines is the same reaction Th2 cells give in response to helminth infection! In the industrialised world, a Th2 setting is correlated with the development of allergic diseases such as asthma. Observations in a murine model sustain a major Th2 participation in the development of allergic diseases such as asthma, because transferred Th2 cells induced airway hyperresponsiveness, eosinophilia, and mucus hypersecretion in the recipients (COHN *ET AL.* 1997, COHN *ET AL.* 1998). Similar observations were made in transgenic mice that overexpress Th2 cytokines in the airway epithelium (RANKIN *ET AL.* 1996, LEE *ET AL.* 1997). These results are backed up by experiments in mice unable to mount a Th2 response (CORRY *ET AL.* 1998). Moreover, studies in humans also clearly demonstrated a Th2 response after allergen exposure in atopic patients and revealed accumulation of Th2 cells in target organs

(WIERENGA *ET AL.* 1999). In addition, successful immunotherapy redirected allergen-specific response from Th2 to Th1 (SECRIST *ET AL.* 1993, MCHUGH *ET AL.* 1995).
With respect to the different T cell subsets possibly involved in diseases like asthma, it is of note that the long lasting Th1/Th2 paradigm has to be reviewed in terms of new T helper cell subgroups (*Figure (Fig.) 1.1*) that have recently been described (BRUESTLE *ET AL.* 2007, STAUDT *ET AL.* 2010) and which scientists all over the world are now eagerly working on to identify.

Any new subset is defined by a very specific function (as for Th9 cells) and/or by a subset-specific transcription factor which is usually induced by subset-specific cytokines. Cells of a given subset differentiate from naive T cells which are defined by expression of CD62L on their surface and the absence of activation markers such as CD25 or CD69 (CARTER *ET AL.* 1998, CHEN *ET AL.* 1995, NATARAJAN *ET AL.* 2000). After first engagement of the TCR with its antigen bound to MHCII molecule the naive T cell differentiates to an effector cell of a particular subtype depending on the presence of lineage-inducing cytokine(s). Macrophages, monocytes and dendritic cells are the main producers of IL-12, which induces the differentiation into Th1 cells by a feedback loop to IFN-γ production, whereas IL-4 (initially produced by mast cells) drives the differentiation into Th2 direction. Both of these cytokines also block the differentiation of respective the other one, thereby enhancing the own signaling additionally (ABBAS *ET AL.* 1996, MOSMAN AND SAD 1996, MURPHY AND REINER 2002). IL-12 activates the transcription factors T box expressed in T cells (Tbet) and interferon regulatory factor (IRF1) 1 in a signal transducer and activator of transcription (STAT) 1-dependent manner, thereby promoting the transcription of cytokines (e.g. IFN-γ) that support the differentiation into Th1 cells. IL-4 blocks this mechanism and promote the differentiation towards Th2 by activation of the transcription factor GATA binding protein (GATA) 3 via STAT6 and IRF4 (LOHOFF *ET AL.* 2000, LOHOFF AND MAK 2005, LIEW 2002, MURPHY AND REINER 2002, Mosmann AND COFFMAN 1989 AND 1989 A). Similar pathways are found in all T helper subsets discovered so far. An overview of inducing cytokines and activated transcription factors are depicted in *Fig. 1.1.*
Th2 cells differentiate from naive $CD4^+$ T cells in a lineage-specific cytokine environment and are characterised by the expression of IL-4, IL-5 and IL-13, thereby attracting other cells such as mast cells and eosinophils to the site of inflammation and inducing IgE class switch in B cells.

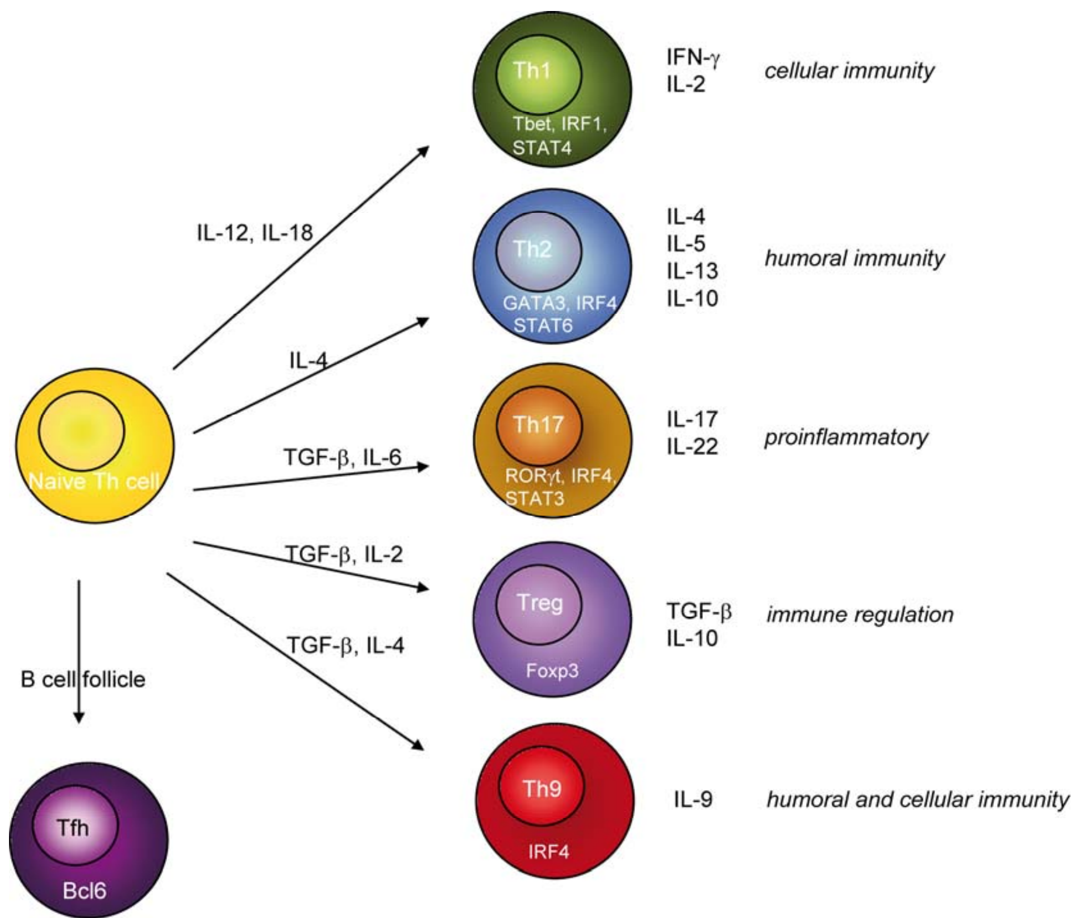

Figure 1.1: ***Differentiation of T helper cell subsets*** *with differentiating cytokines (above arrows), involved transcription factors (within the cells) and subset function including produced cytokines (right part). Fully developed thymocytes are released from the thymus as naïve Th cells. Upon antigen recognition in a given cytokine milieu which induces the involvement of a lineage-specific transcription factor, a naïve Th cell differentiates into a specialised T helper cell subset that is related to an also specialised immunological function. The differentiation of follicular T helper (Tfh) cells takes place in the B cell follicle, but the circumstances and the specific cytokine milieu that lead to their differentiation are still controversally discussed.*

1.1.2 γδ T CELLS

The TCR is a heterodimer and consists of two protein chains. The majority of T cells carry the alpha-beta (αβ) TCR, whereas low percentages express a TCR with gamma-delta (γδ) chains. Together with αβ T cells and B cells, γδ T cells are found in all vertebrates so far examined. This unconventional T cell subtype shares many cell-surface and effector functions with αβ T cells, such as cytokine production or cytotoxicity. Just like αβ TCRs, the γδ-chains undergo rearrangements for clonal specificity, but the number of variable (V), diversity (D) and joining (J) gene segments which are available for generating different TCRs, are low for γδ TCRs. However, additional mechanisms secure a large repertoire also in this T cell subset. An immense junctional diversity in particular is achieved by N-nucleotide insertion or removal at up to three V/D/J joining sites. Within the γδ-chains, these form the complementary determining region 3 (CDR3) as a major binding site of the γδ TCR for antigens (ROCK *ET AL.* 1994, SCHILD *ET AL.* 1994).
γδ T cells represent only a small proportion, less than 10 % of T cells in the periphery. In mice, γδ T cells have a strong tropism for epithelial surfaces, such as those of liver and skin, and mucosae of respiratory, digestive and reproductive organs. Here, they are a major T cell population (SIM 1995). It is not surprising that mucosal epithelia need an unique immune system, since they serve as an important barrier between inside and outside to protect the organism from pathogenic invasion. At the same time they promote the entry of solid, liquid or gaseous nutrients. γδ T cells are enriched in mucosal tissues compared to peripheral blood or lymphoid organs due to their non-redundant role, which relies on a specific combination of peculiar antigen specificities, tissue distribution and functional properties rather than any of these features individually. Although theoretically 10^{16} varying T cell receptors can be produced for γδ T cells, only a limited set of receptors has been detected so far. The tissue distribution correlates with a predominant combination of particular γ and δ receptor chains (REVIEWED IN BONNEVILLE *ET AL.* 2010). At the population level, γδ T cells are capable of mediating many different functions, but for a particular cell, function is determined by a given tissue. For example after injury, intraepidermal Vγ5Vδ1^{+} T cells and intestinal epidermal Vγ7+ T cells display cytoprotective and immunomodulatory functions by secreting epithelial cell trophic factors such as insulin-like growth factor-1 (IGF1),

inflammatory cytokines such as IFN-γ and IL-2 and have a stronger cytotoxic activity (JAMESON *ET AL.* 2007). On the contrary, Vγ6Vδ1$^+$ T cells mainly produce IL-17 during lung inflammation (SIMONIAN *ET AL.* 2009), Vγ1Vδ6.3$^+$ T cells in the liver secrete IL-4 and IFN-γ, whereas Vγ4$^+$ T cells were shown to produce IFN-γ and IL-17 depending on the system they have been investigated in (HUBER *ET AL.* 2002, LAHN *ET AL.* 2002, ROARK *ET AL.* 2007). The same is true in the human system: Vδ1$^+$ γδ T cells produce a quite broad set of cytokines including IL-4 and IL-17, whereas the Vδ2$^+$ subset displays high cytotoxic activity and these cells produce IFN-γ and TNF (MORITA *ET AL* 1991, FENOGLIO *ET AL.* 2009).
With their spectacular capability to produce very rapidly IL-17 and IFN-γ, γδ T cells are considered to be an essential component of the innate immune system (ROARK *ET AL.* 2007 AND 2008). This early cytokine production by γδ T cells has drawn more and more attention to this special T cell subset. Further, these cells have been shown to play a crucial role for anti-bacterial immune responses in several infectious diseases (LOCKHART *ET AL.* 2006, SHIBATA *ET AL.* 2007). At steady state, γδ T cells express high levels of IL-23 receptor (IL-23R) and also contribute to autoimmunity by hampering regulatory T cell responses (PETERMANN *ET AL.* 2010).
The differentiation of Th17 cells requires several days and the involvement of IL-6 and transforming growth factor (TGF)-β (VELDHOEN *ET AL.* 2006 AND OTHERS). In contrast, γδ T cells are capable of IL-17 production already in the thymus (JENSEN *ET AL.* 2008, SHIBATA *ET AL.* 2008)! Apparently, this IL-17 production occurs independently of any co-stimulation with other cytokines such as IL-6 and also of TCR involvement (LOCHNER *ET AL.* 2008). The mechanism of this pre-setting is still enigmatic. However, a recent study introduced (v-rel reticuloendotheliosis viral oncogene homolog A (avian)) RelA and RelB as crucial factors that control IL-17 production of γδ T cells (POWOLNY-BUDNICKA *ET AL.* 2011).

1.1.3 INTERFERON REGULATORY FACTORS

It has been more than 50 years since the antiviral interferon (IFN) protein family, now termed type I interferon family, was first recognised (ISAACS *ET AL.* 1957, REVIEWED BY PESTKA 2007). The expression of type I interferon genes is strongly regulated, and interferon synthesis, which is induced by viral infections is generally transient.

The mammalian IRFs are a family of transcription factors which comprises 9 members termed IRF1-IRF9. They were first and best described as well as characterised as transcriptional regulators of type I interferons and interferon-inducible genes (MIYAMOTO *ET AL*. 1989, HARADA *ET AL*. 1989 AND1990). In addition, IRF10 has been identified in chickens, but is absent in humans and mice (NEHYBA *ET AL*. 2002).

All IRFs show characteristic structural homologies, although the functions of the individual members of this family differ. IRFs possess an amino (N)-terminal DNA binding domain (DBD) that is characterised by a series of five well-conserved tryptophan-rich repeats. This DBD forms a helix-turn-helix structure and recognises DNA similar in sequence to the IFN-stimulated response element (ISRE) which is present in genes regulated by interferon signaling. In particular, the core sequence GAAA of this element is part of every IRF binding sequence. The less conserved C-terminus of IRFs contains an IRF association domain (IAD) which mediates heterodimerisation. It also bears a regulatory element which divides the IRFs into two groups: IRF3, IRF5, IRF6, IRF7, and IRF9 which have activating functions whereas IRF1, IRF2, IRF4, and IRF8 can either activate or repress gene transcription. Their functions depend on the target gene and also on the partner they interact with. IRFs interact with each other and with other families of transcription factors modifying both their sequence-specific binding capacity and the formation of transcription initiation complexes (TANIGUCHI *ET AL*. 2001; TAMURA *ET AL*. 2008). In addition, posttranslational modifications, such as phosphorylation are known. For example, STAT3 mediates sumoylation of IRF1 via protein of inactivated STAT3 (PIAS3) and thereby represses its activity (NAKAGAWA AND YOKOSAWA 2002). The capacity to interact with multiple partners and posttranslational modifications account for the remarkable variety in IRF functions.

IRFs participate in early host responses to pathogens, tumor suppression, and in hematopoietic cell differentiation. Here, IRF1, IRF2 and IRF8 are involved in myeloid cell differentiation. The expression of IRF1 in immature myeloid bone marrow cells is strongly upregulated during granulocyte differentiation of both human and murine myeloid progenitors in response to granulocytes colony-stimulating factor (G-CSF) (ABDOLLAHI *ET AL*. 1991, TESTA *ET AL*. 2004). The important role for IRF1 in allowing precursor cells to complete their differentiation is confirmed by the observation that inhibition of IRF1 by a functional antagonist such as IRF2 completely inhibits terminal

differentiation. At the molecular level, IRF2 prevents the effects of IRF1 on the late differentiation marker lactoferrin (COCCIA *ET AL.* 2001).

Whereas IRF1 is widely expressed in almost all cell types, expression of IRF8 is restricted to cells of the immune sytem. Knockout studies revealed key functions for IRF8 in myeloid cell lineage selection, macrophage maturation and in control of genes involved in cell growth and apoptosis (TAMURA *ET AL.* 2005). Other knockout studies reported central roles for IRF4 and IRF8 in B cell development (LU *ET AL.* 2008).

Several IRFs are recruited in Myeloid differentiation primary response gene 88 (MyD88)- and Toll/Interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling of Toll-like receptors (TLRs) and are therefore essential for the recognition of a variety of pathogen-associated molecular patterns (PAMPs) (HONDA 2006, AKIRA 2006).

Altogether, this whole family of transcription factors is now recognised as playing a pivotal role in the regulation of innate and adaptive immune responses.

1.1.3.1 INTERFERON REGULATORY FACTOR 1

The main focus of this thesis lies on interferon regulatory factor 1 (IRF1).

IRF1 was originally described as the transcriptional activator of interferon-β (IFN-β) transcription in virus-infected fibroblasts (MIYAMOTO *ET AL* 1988). However, the research focus has meanwhile been redirected to mediating IFN-γ responses, since gene-targeting studies have revealed an IRF1-independent mechanism of IFN-β induction. IRF1 is expressed in a variety of cell types such as T cells, natural killer cells, macrophages, alveolar epithelial cells, endothelial cells or hematopoietic stem cells (TANIGUCHI *ET AL* 2001, LOHOFF AND MAK 2005). Its transcription is induced by STAT1 (COCCIA *ET AL.* 2000; PINE *ET AL*. 1994, REMOLI *ET AL*. 2002). IRF1 possesses a C-terminal transactivation domain with several tyrosine residues. The phosphorylation of theses residues by casein kinase II increases its activity (MERANO *ET AL* 1999). Via IAD 2 IRF1 can interact with other IRFs such as IRF8 (MERARO *ET AL.* 1999). IRF1 regulates the induction of nitric-oxidase and induction of the interleukin-12p35 subunit (IL-12p35) in macrophages, binds to the IL-4-promoter in T cells and thereby blocks Th2 development and promotes Th1 differentiation (COCCIA *ET AL.* 2000, ELSER 2002). Not surprisingly, *Irf1*$^{-/-}$ mice exhibit a

strongly reduced IFN-γ production during *Leishmania major* infection in a gene dose-dependent manner. Instead, higher amounts of Th2 cytokines such as IL-4 and IL-10 are produced, meaning that the T cell response shifts towards Th2 (LOHOFF *ET AL.* 1997). This Th2 type immune response also results in higher amounts of IgE isotype in serum.

Furthermore, IRF1 is associated with autoimmune diseases (GORDON 2003; LIU *ET AL.* 2004), tumor suppression and apoptosis (HARADA *ET AL.* 1998; ROMEO *ET AL.* 2002; TANAKA *ET AL.* 1994; BOUKER *ET AL.* 2005): cells from IRF1-deficient mice are resistant to UV-light and drug-induced apoptosis (REIS *ET AL.* 1994). IRF1 and IRF2 share a binding domain in the *IFN-β*-gene that is also recognised by Blimp-1, which in turn has an important role in the late stages of B cell development (KUO AND CALAME 2004). It would be of interest to know whether and to what extent IRF1 participates in B cell differentiation. However, IRF1-deficient mice do not show any altered B cell populations in spleen, peripheral blood, lymph nodes, bone marrow, and peritoneal exudate cells (MATSUYAMA 1993). Instead, the number of TCRαβ$CD4^-CD8^+$ T cells is dramatically reduced, resulting in a higher susceptibility of these animals to intracellular pathogens. Several years before generation of the IRF1 deficient mouse, it was reported that the development of TCRαβ$CD4^-CD8^+$ T cells is dependent on MHC I expression (ZIJLSTRA *ET AL.* 1990; KOLLER *ET AL.* 1990; VAN KAER *ET AL.* 1992 AND OTHERS) and that its promoter responds to IRF1 (KORBER *ET AL.* 1988; MIYAMOTO *ET AL.* 1988 AND OTHERS). Admittedly, there was no other obvious defect detectable in the *Irf1*$^{-/-}$ mice. Later, it was convincingly demonstrated that IRF1 controls MHC class I expression in the thymus and hence, is responsible for the misregulated $CD8^+$ T cell selection. IRF1 binds also to the promoters of the peptide transporters TAP1 (transporter associated with antigen processing-1) and LMP2 (latent infection membrane protein-2) (WHITE *ET AL.* 1996) which are required for peptide presentation by MHC class I molecules. Expression of these molecules in thymic stromal cells and mature thymocytes was impaired in IRF1-deficient mice (PENNINGER *ET AL.* 1997).

IRF1 IN ASTHMA

Since allergic diseases are considered to be driven by a Th2-mediated immune response, the role of IRF1 has come more into focus, since it drives the T cell differentiation towards Th1 by acting in many cell types, not only T cells.

Type I and type II interferons are induced by environmental factors, such as microbial exposure and may suppress atopic reactions. In this context, IRF1 is of special interest, since it mediates the effects of interferons intracellularly and is induced by viruses, lipopolysaccharide (LPS) and IL-1.

The development of asthma is influenced by poorly understood environmental and genetic factors (COOKSON 2004, OBER AND HOFFJAN 2006). Genome wide screening analyses of single nucleotide polymorphisms (SNPs) of several genes revealed their implication in childhood asthma (MOFFATT *ET AL.* 2007). The *Irf1* gene is located in a cytokine cluster on chromosome 5q 31 that has been shown to be associated with the development of asthma (XU *ET AL.* 2000, and MANSUR *ET AL.* 1998). Sequence studies have revealed that *Irf1* SNPs influence IgE regulation and atopy (SCHEDEL *ET AL* 2008). In humans, polymorphisms of *Irf1* have been associated with a predisposition to develop asthma in the pediatric population (WANG *ET AL*. 2006) .

A dominant feature of asthma pathology is a high level of IgE. The *Irf1*$^{-/-}$ mouse is prone to develop an up-regulated Th2 response, which correlates with secretion of higher amounts of IgE and IgG1, while a Th1 response would lead to production of IgG2a (IgG2c in C57BL/6 mice) (SNAPPER AND PAUL 1987). Taken together, the lack of IRF1 or abnormalities in its expression, including gene variations like SNPs, may contribute to the development and/or severity of allergic asthma.

1.1.3.2 INTERFERON REGULATORY FACTOR 4

The expression of IRF4 is restricted to the immune system. IRF4 as well as IRF8 are unique members of the IRF-family in that they are highly homologous to each other rather than to other IRFs. Both factors have evolved to be critical in mediating the development of lymphoid, myeloid and dendritic cells (OZATO *ET AL.* 2007, PERNIS 2002; LU 2008, KANNO *ET AL*. 2005, TAMURA *ET AL*. 2008).

Unlike IRF1, IRF4 expression is not induced by interferon, but rather by diverse mitogenic stimuli, such as antigen receptor engagement, LPS or CD40 signaling (MITTRÜCKER *ET AL.* 1997, GUPTA *ET AL.* 1999, GRUMONT & GERONDAKIS 2000). All of these stimuli activate the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) pathway, which in turn leads to *Irf4* promoter activation by NFκB heterodimers (GUPTA *ET AL.* 1999, GRUMONT & GERONDAKIS 2000, SHAFFER *ET AL.* 2006, SAITO *ET AL.* 2007). Additionally, IL-4 can activate *Irf4* transcription via STAT6 signaling (GUPTA *ET AL.* 1999, GRUMONT & GERONDAKIS 2000). IRF4 can act as a counterregulator of other IRFs especially of IRF1 and 2. For example, IRF4 is able to repress the expression of some interferon-inducible genes by binding to the ISREs in their promoters, displacing IRF1 and IRF2 (BRASS *ET AL.* 1996, YAMAGATA *ET AL.* 1996). The mechanism how IRF4 represses other genes, such as B cell lymphoma 6 protein (BCL6) is yet undefined. By using its C-terminal transactivation domain, IRF4 can function as a positive transcriptional regulator, but DNA binding of IRF4 alone is weak (BRASS *ET AL.* 1996). However, in conjunction with the ETS-family member PU.1 IRF4 can bind to the 3' enhancers of both κ and λ immunoglobulin light chains (PONGUBALA *ET AL.* 1992, EISENBEIS *ET AL.* 1995, SU *ET AL.* 1996) with high avidity. Due to its structural high similarity IRF8 interacts as nice with PU.1, while other IRF family members cannot (ESCALANTE *ET AL.* 2002, MARECKI *ET AL.* 1999). The composite DNA binding element of the IRF4 and PU.1 binding motifs on this enhancer is termed the ETS/ISRE-consensus element (EICE). Similar domains mediate cooperative binding on other genes including TLRs (REHLI *ET AL.* 2000; VAN DER STOEP *ET AL.* 2004), leading to decreased IL-12 production.

The most striking discovery of IRF4 function was its involvement in B cell development. The finding that IRF4 and IRF8 bind to an EICE motif in the immunoglobulin enhancer region provided early evidence for an overlapping role of both factors in pre-B-cell development (SZABO *ET AL.* 2000, OUYANG *ET AL.* 1998). Studies in IRF4 and IRF8 compound mutant mice (*Irf4/8*$^{-/-}$) revealed both to be individually sufficient to rescue pre-B-cell development. Interestingly, *Irf4,8*$^{-/-}$ pre-B-cells are hyperproliferative, suggesting a negative regulation by IRF4 (and IRF8) of pre-B cell development (MA *ET AL.* 2006). More recent studies demonstrated a role of IRF4 and 8 for the transition from large pre-B-cells to small pre-B-cells by induction of the transcription factors Ikaros and Aioles (LAUERENCE *ET AL.* 2007).

Moreover, IRF4 has a critical role in the adaptive immune response of mature B cells. In ageing *Irf4* knockout mice, mature B cells accumulate in spleen and lymph nodes and

a quantitative defect in the percentages of IgM^{hi}, IgD^{lo} and CD23^{lo} populations has been described (MITTRÜCKER *ET AL.* 1997). In addition, serum immunoglobulins of all isotypes are exceedingly low, because these mice completely lack Ig-secreting plasma cells (KLEIN *ET AL.* 2006).

Apart from its effects in B cells, IRF4 has crucial impact on Th2 and Th17 cell differentiation by controlling cytokine expression and apoptosis (RENGARAJAN *ET AL.* 2002, LOHOFF *ET AL.* 2004, HONMA *ET AL.* 2008, HU *ET AL.* 2002, BRÜSTLE *ET AL.* 2007). As a consequence IRF4 deficient mice have a decreased capability to raise Th2 and Th17 immune responses. In wildtype cells, IRF4 cooperates with nuclear factor of activated T cells (NFAT)1 and/or 2 (TOMINAGA *ET AL.* 2003, RENGARAJAN *ET AL.* 2002) to directly bind to the *IL-4* promoter and activate the IL-4 transcription with implications for Th2 differentiation. Additionally, *Irf4* knockout mice also show a decrease expression of the Th17 lineage-specific transcription factor RORγt, suggesting the involvement of IRF4 in its regulation. This is in line with the incapacity of these mice to develop experimental autoimmune encephalomyolitis (EAE, mouse model for multiple sclerosis) due to the lack of Th17 cells. Transfer of wildtype congenic CD4^{+} T cells from Ly5.1 mice completely rescued disease development (BRÜSTLE *ET AL.* 2007) and confirmed that exactly the transferred cells were the producers of IL-17. Additional data support the importance of IRF4 in the context of Th17 immune regulation, by elucidating the role of IRF4 in orchestrating the transcription factors RAR-related orphan receptor (ROR) α, RORγt and also forkhead box (Fox) p3 (HUBER *ET AL.* 2008), a lineage-specific transcription factor for regulatory T cells (Treg). The expansion of Tregs and Th17 cells seems to be reciprocally regulated (BETTELLI *ET AL.* 2006, MANGAN *ET AL.* 2006, VELDHOEN *ET AL.* 2006, LAURENCE *ET AL.* 2007) and any misregulation (e.g. by lack of IRF4) therefore may have severe implications in autoimmune disease. TGF-β plays a central role in inducing and maintaining Tregs (REVIEWED BY GORELIK AND FLAVELL 2002A, CHEN *ET AL.* 2003, ZHOU *ET AL.* 2008, KULKARNI *ET AL.* 1995, POWRIE *ET AL.* 1996, LI *ET AL.* 2007), and the TGF-β produced by Tregs is suggested to be the main source of this cytokine in Th17 differentiation (LI *ET AL* 2007); *vice versa* IL-21 and IL-23 produced by Th17 cells suppress Treg generation (FANTINI *ET AL*, 2007, IZCUE *ET AL*, 2008)

Altogether therefore, IRF4 plays an important role in differentiation of several Th subsets and thereby has a central position in immune regulation.

1.2 ASTHMA BRONCHIALE

Asthma (Greek for “shortness of breath”) is a serious, chronic inflammatory disease. If left untreated it is potentially life threatening and affects about 7 % of the population in the United States of America, 3-5 % of adults and approximately 10 % of children in Germany and a total of 300 million people worldwide. Reported incidence is increasing globally, causing an increasing burden on health services in both industrialised and developing countries.
(FROM AMERICAN LUNG ASSOCIATION, WWW.LUNG.USA.ORG AND GERMAN SOCIETY OF PNEUMOLOGY AND RESPIRATION MEDICINE, WWW.PNEUMOLOGIE.DE)

1.2.1 THE IMMUNOLOGY OF ASTHMA

Like food allergies, asthma is initiated at mucosal surfaces where environmental allergens contact epithelia. These allergens may possess a variety of properties such as protease activity, stability, or molecular mimicry which gives them the capacity to penetrate mucosal surfaces and induce immune responses. One of the earliest steps in allergic sensitisation is the generation of antigen-specific T cell responses, which is initiated by antigen-presenting dendritic cells (DC) (MELLMANN 2001). Dendritic cells survey the lungs for invading pathogens and inhaled antigens in a network located under the airway epithelium. The epithelia may provide adjuvant signals in response to antigens that direct the migration and maturation state of DCs and T cells (HUH *ET AL.* 2003, HOLT *ET AL.*1990). The DCs then migrate to the draining lymph node to present antigen and costimulatory signals to the T cells, which then in turn migrate to the airways to secrete cytokines and other mediators. These mediators direct the asthmatic response in the lung, for example IL-5 promotes airway eosinophilia, whereas IL-9 recruits mast cells to the lung. The magnitude of both acute and chronic asthma attacks correleates with the number of eosinophils present in the lung (BRADLEY *ET AL.* 1991) and with an increase in bronchial hyperresponsiveness (CARTIER *ET AL.* 1982; COCKROFT *ET AL.* 1987). From biopsy studies, it is known that infiltrating eosinophils degranulate at subepithelial sites, but also deeper in the interstitium (AALBERS *ET AL.* 1993). Presumably,

toxic eosinophil-derived products such as eosinophilic cationic protein and major basic protein are able to induce shedding of epithelials cells, as seen in human adults with asthma, where sloughing of the epithelium has occurred. (BOUSQUET *ET AL.* 1990). Mast cells are well established effectors in the context of allergic diseases and asthma: inhaled antigen crosslinks FcεRI (which is the high affinity receptor for the Fc region of IgE)-bound immunoglobulin IgE, thereby triggering rapid degranulation and release of preformed mediators, such as histamine and certain proteases. IL-13 seems to be involved in goblet cell metaplasia and mucus production. (REVIEWED IN HOLGATE AND POLOSA 2008, BARRETT AND AUSTEN 2009). In addition, long-lived memory T cells are also generated, which take over the monitoring role.

1.2.1.1 PATHOLOGY

Atopy (from the Greek **'atopos'** meaning "out of place") describes a tendency to be "hyperallergic". This encompasses the classical allergic diseases such as atopic dermatitis ("neurodermatitis"), allergic rhinitis ("hay fever") and asthma, which often occurs as a result of untreated or mistreated allergic rhinitis. Although atopy has various definitions, most consistently it is defined by the presence of highly elevated serum levels of IgE antibodies, leading to a positive skin-prick test to common and otherwise harmless allergens (e.g. food, dander, pollen and insect venoms) (KAY 2001). Instead, non-atopic individuals react with equally high-affinity IgG1 levels. Asthma and atopy have been noted to cluster in family and twin studies, all of which suggest a significant genetic component for predisposition (BLUMENTHAL AND BLUMENTHAL 2001).

Asthma is a chronic obstructive condition of the lung that is usually not considered to be part of chronic obstructive pulmonary disease (COPD) (REVIEWED IN BARNES 2008) as this term refers to a combination of bronchiectasia, chronic bronchitis, and emphysema. Instead, asthma is characterised by occasional reversible airway obstruction, bronchial inflammation, bronchial smooth muscle cell hyperreactivity to bronchoconstrictors, and is often associated with excessive mucus production, as illustrated in *Fig. 1.2*. However, if left untreated asthma can result in permanent inflammation and irreversible airway obstruction. Acute attacks may be triggered by exposure to environmental stimulants (allergens), cold air, exercise or emotional stress. In children, viral illnesses are the most

common triggers (ZHAO *ET AL.* 2002). Moreover, genetic predisposition has been demonstrated to play a role in the pathogenesis of asthma. Certain SNPs have been associated with the development of asthma in childhood (BOUZIGON *ET AL.* 2008). Genome-wide searches have highlighted several distinct loci that are linked to asthma, for example the cytokine gene cluster on chromosome 5q, whereas others do not have obvious links to asthma (MOFFATT *ET AL.* 2007, COOKSON 2004, ALLEN *ET AL.* 2003, MANSUR *ET AL.* 1998).

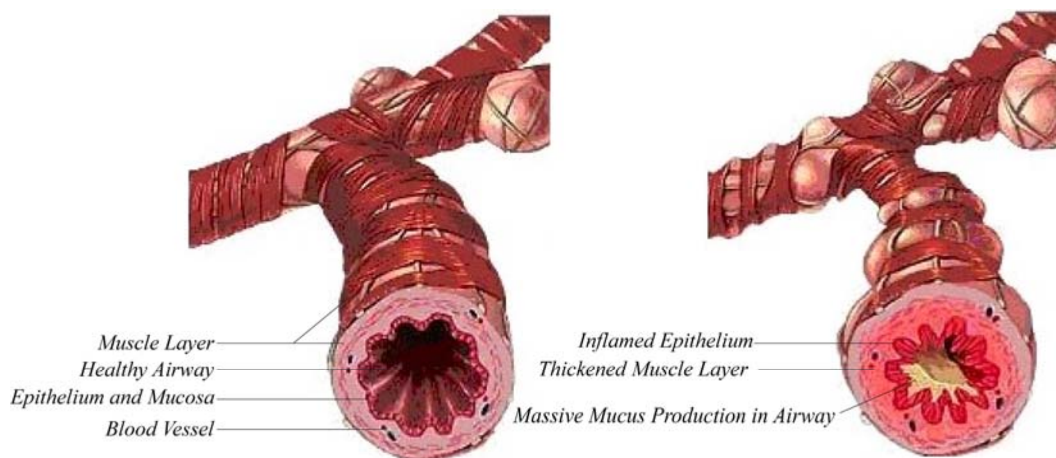

Figure 1.2: ***Left: healthy bronchus with clear airway; Right: inflamed, mucus-filled brochus*.**
(Modified from http://cjcotton.files.wordpress.com/2009/03/asthma.jpg)

It is agreed that atopy is a risk factor for asthma, but it remains controversial to what extent atopy accounts for asthma. The International Study of Asthma and Allergies in Childhood (ISAAC) revealed great variations of the prevalence of asthma and atopic diseases such as allergic rhinoconjunctivitis and atopic dermatitis and raised the question whether the same factors are responsible for disease development in different areas of the world (BEASELY 2003, ISAAC STUDY 1998). Still, asthma and atopy show complex correlation. Depending on both the type and amount of sensitisation, preexisting atopy can increase the risk of developing asthma by 10-20 fold (HOLGATE 1999).

1.2.1.2 ATOPIC AND NON-ATOPIC ASTHMA

By early 20th century, the disease of asthma has been classified in "intrinsic" (non-atopic) asthma and "extrinsic" (atopic asthma) (RACKEMANN 1921). Atopic asthma is the most common form in patients. This is indicated by a positive skin-prick test and the presence of IgE to common allergens such as house dust mite. These patients have allergic inflammation of airways, higher epithelial damage and an increase in basal membrane thickness (AMIN *ET AL.* 2000).

Patients suffering from intrinsic asthma show a negative skin-prick test result. This form of the disease occurs rarely, but tends to be more severe. Although the airway inflammation shows similarities to atopic asthma inflammation and to some extent incorporates the same effector cells, it is mediated by local rather than by systemic IgE production (WENZEL 2006). In contrast to atopic asthma, the non-atopic asthma implies the absence of sensitisation (AMIN *ET AL.* 2000).

Approximately 5 % of patients have severe atopic or non-atopic asthma, which is more difficult to control, even with maximal inhaler therapy. For these people in particular, new therapeutic approaches are desperately needed.

1.2.1.3 EOSINOPHILIC AND NON-EOSINOPHILIC ASTHMA

In eosinophilic asthma, bronchial biopsies, bronchoalveolar lavage fluid and sputum reveal infiltration by eosinophils. The magnitude of both acute and chronic asthmatic reactions correlates with the number of eosinophils present in the lung (BRADLEY *ET AL.* 1991). Activated eosinophils in particular play a major effector role in the asthma pathogenesis by releasing proinflammatory lipid mediators, cytokines, cytotoxic cationic proteins such as eosinophil peroxidase (EPO) and fibrogenic growth factors such as TGFβ1 (LEASK AND ABRAHAM 2004). These mediators promote airway inflammation, tissue injury and lead to mucus overproduction. Eosinophil chemotactic factors, such as CC-chemokine ligand 11 (CCL11, also known as eotaxin-1) and related chemokines are mainly secreted by airway epithelial cells (KRANEVELD *ET AL.* 1997). On the whole, several studies report a strong correlation between clinical severity and the degree of airway eosinophilia (WARDLAW *ET AL.* 1988, BOUSQUET *ET AL.* 1990, JAYRAM *ET AL.* 2006), whereas eosinophilia and airway hyperresponsiveness appear to be independent

components of the asthma phenotype (BRIGHTLING *ET AL.* 2002); but these findings are controversial (CARTIER *ET AL.* 1982; CROCKROFT *ET AL.* 1987).

The development of simple methods to assess airway inflammation non-invasively using induced sputum which are applicable to a wide variety of patients has renewed interest in investigation of the pathological diversity of asthma. It was detected that, in contrast to eosinophilic asthma, non-eosinophilic asthma is characterised by neutrophil infiltration and in this shows similarity to COPD. In addition, several studies have clearly shown that eosinophils are not omnipresent in asthma (TURNER *ET AL.* 1995, FAHY *ET AL.* 1995). Instead, neutrophil infiltration has been noted in some patients with severe asthma (JATAKANON *ET AL.* 1999, WENZEL *ET AL.* 1999) and in those who died suddenly and unexpectedly of asthma (SUR *ET AL.* 1993).

Non-eosinophilic asthma is of particular interest for a few reasons. Several uncontrolled studies suggested that there is a correlation between non-eosinophilic pathology with a faint short-term response to inhaled steroid therapy (PAVORD *ET AL.* 1997, TURNER *ET AL.* 1995, MEIJER *ET AL.* 2002, BACCIO *ET AL.* 2006). This has been confirmed in a double blind controlled placebo study (BERRY *ET AL.* 2005). Subsequently, non-eosinophilic asthma has been shown to be present in 25 % of adult patients and in about 50 % of patients treated with high dose of inhaled corticosteroids (GIBSON *ET AL.* 2001). These findings are also supported by Wenzel and coworkers (1999), who described a subgroup of patients with refractory asthma that had a distinct non-eosinophilic pathology with normal basal membrane thickness on bronchial biopsy. However, it remains unclear whether and to what extent (strong) corticosteroid treatment is particularly associated with more severe disease. Potentially pathogenic bacteria have been identified in 43 % of patients with neutrophilic asthma, a finding that is in keeping with Rackemann's identification and classification (1921 AND 1927, PAVORD 2007).

This pathology of non-eosinophilic asthma makes clear that the concept of a mainly Th2-driven disease has to be reconsidered and the involvement of the innate immune system must not be underestimated (DOUWES *ET AL.* 2002). However, there is evidence for T helper 2 cells in animal studies and human mediating allergic airway inflammation. Links may exist to other condition of neutrophilic airway inflammation such as in response to a wide variety of stimuli instancing viral and bacterial infection or inhaled irritants such as saline or cigarette smoke and other pollutants (ANEES *ET AL.* 2002, CLAPP *ET AL.* 1994).

1.2.1.4 T HELPER CELLS AND ASTHMA

The long standing paradigm of asthma as a Th2-mediated disease loses more and more its plausibility. For instance, although mucus production is mediated via the Th2 product IL-13 (WHITTAKER *ET AL*. 2002, STEENWINCKEL *ET AL*. 2007), both *in vivo* and *in vitro* studies have demonstrated that the cytokine IL-9 induces metaplasia directly in the absence of inflammation (READER *ET AL*. 2003; VERMEER *ET AL*. 2003). Large amounts of IL-9 are produced by Th9 cells which have recently been distinguished from Th2 cells as a new T helper cell subset (VELDHOEN *ET AL*. 2008, DARDALHON *ET AL*. 2008). In IL-9 transgenic mice, subepithelial fibrosis was observed and seems to be involved in airway remodelling (VAN DEN BRÛLE *ET AL*. 2007, DOHERTY *ET AL*. 2009). However, their role in the human disease is still speculative and one has to keep in mind that IL-9 can be produced by several cell types including mast cells (VOCK *ET AL*. 2010)

The identification of Th17 cells a few years ago lead to the development of a new immunological hot spot. The participation of Th17 cells in asthma has been first introduced in 2001 in reports that present high amounts of their product IL-17 in plasma samples (WONG *ET AL*. 2001) and increased IL-17 mRNA levels in tissue samples of asthmatic patients (MOLET *ET AL*. 2001). But not until 2008, this new T helper cell subset was identified as disease-mediating cells. The study by Pène *et al*. demonstrated highly activated Th17 cells in biopsies from patients with severe asthma. In fact, these cells represented 20 % of all infiltrating lymphocytes (PÈNE *ET AL*. 2008). These findings suggest that Th17 cells play a crucial role in the development of a severe asthma phenotype and are further supported by data that show glucocorticoid resistance of these cells in the setting of a murine asthma model (MCKINLEY *ET AL*. 2008). Taken together Th17 cells are significantly involved in cellular responses during development of airway disease and resistance to common treatment strategies with steroids.

1.2.2 EPIDEMIOLOGY

In developed countries, asthma and allergic inflammation, including food allergies have increased over the past 50 years and are now the most prevalent chronic diseases of childhood. Between 1980 and mid-1990s, childhood asthma prevalence has more than doubled in the United States of America (LOCKSLEY 2010), following similar trends for other allergic disorders (BACH 2002). The increase in relatively short time periods implicates environmental parameters as overwhelming factors of disease risk.

The reunification in Germany provided the unique opportunity to monitor the effects of different lifestyles and changing environmental factors on allergic diseases. Results of the Federal Health Survey in 1998 showed that 40-43 % of adults have had an allergy. Regarding allergic rhinitis, 17 % of adults living in Western Germany and only 11 % of those living in Eastern Germany claimed in interviews of the study that they were allergic. When comparing this study to earlier studies, the prevalence of allergic rhinitis increased over 70 % during less than one decade. The relative increase was higher in East than West Germany. In general, women suffer more frequently from an allergic disease than men. The increase of prevalence of allergy is supported by three studies of the national health survey of the German Cardiovascular Prevention Study (DHP) from 1984-1991 (HERRMANN-KUNZ 2000, WIESNER *ET AL.* 1994) and the german Health Survey East from 1990/91 (HOFFMEISTER AND BELLACH 1995).

Results from several pediatric studies conducted after the fall of the iron curtain showed a decrease in positive skin-prick tests from west to east, from Sweden to the Baltic states, and as far east as Uzbekiztan. This can be interpreted as confirmation that Western lifestyle and living conditions induce or promote the development of allergic sensitisation, at least in children (BJÖRKSTEN 1996, NICOLAI *ET AL.* 1997).

It is of note that in Germany, children with an immigration background and children from a lower socio-economic status are less affected by an atopic disease as determined by the Children and Youngsters Health Survey (KINDER-UND JUGENDGESUNDHEITSSURVEY KIGGS, SCHLAUD 2007). This is in contrast to a British study, where the most deprived had higher incidences and lifetime prevalence of asthma and more histories of being prescribed an asthma-related drug (SIMPSON 2010).

It should be kept in mind that prevalence rates depend on the phenotypic definition applied at the time of a study and that some decrease or plateau-phases in industrialised countries during the last years are a result of redesigned questions. The National Health Intelligence Service (NHIS) findings in the UK are a good example of this phenomenon. In contrast to the previous NHIS asthma survey that produced asthma period estimates, the new-designed questions in 1997-2000 produced an estimate of asthma attack prevalence, which is a more specific definition and lowered the asthma prevalence statistics compared to pre-1997 (AKINBAMI *ET AL.* 2003).

When evaluating literature on a multinational level over time (EDER *ET AL.* 2006), the diversity of asthma prevalence is remarkable, and reflects the complex character of the disease. In general, in developed countries asthma prevalence is rising, but considerable difficulty remains in comparing the data of different countries, not only due to the study design, but also because of the lack of studies for comparable types of patients in the same space of time. In Germany the most prevailing data for children come from the KiGGS in 2007, whereas the most recent data concerning adults are from the 1990s!

The rise of asthma is paralled by a rise of atopy, as measured by a positive skin-prick test, but only part of the rise in asthma can be associated and explained by atopy (PEARCE *ET AL.* 1999). Triggers for both include exposure to tobacco smoke, allergens and air pollution, whereas living on a farm, especially during early childhood, seem to play a protective role (LEYNAERT *ET AL.* 2001, BRAUN-FAHRLANDER *ET AL.* 2002). Several factors such as breast feeding and early-childhood infections, access to day care and the number of siblings remain controversially discussed (WEISS 1997, NICOLAI *ET AL.* 1997).

The change to a modern, Western life style comes along with high hygiene standards and reduced exposure to microbes. These living conditions seem to increase the risk of developing atopy. In the more affluent, industrialised world asthma has become the most common chronic disease in children. One possible explanation is what has been summarised as and is now known as the hygiene hypothesis.

1.2.2.1 Hygiene Hypothesis

The hygiene hypothesis was originally postulated by David Strachan in 1989. He studied the epidemiology of a national sample of 17.414 British children born during one week in March 1958 and followed them up to the age of 23. The most striking observation was that at the age of 11 (when it was assumed most families were complete), the incidence of hay fever was inversely related to the number of children in the household, excema in the first year of life was related independently of hay fever to the number of older children in the household. He reckoned that allergic diseases could be "prevented by infection in early childhood transmitted by unhygienic contact with older siblings, or acquired prenatally from a mother infected by contact with her other children." (Strachan 1989). The idea of the beneficial impact of infection for atopic illnesses had been proposed much earlier. Strachan based his hypothesis on epidemiological association, namely by statistical relation of two variables, while the interrelation of infection and atopy had been noted in several studies suggesting the beneficial effects of parasite infection (Godfrey 1975, Preston 1970) and microbe-rich environment (Gerrard 1976).

1.3 Generation of a conditional gene knockout mouse

1.3.1 In the beginning, there was the mouse (apologies to the bible)

For almost an entire century, the mouse has been used in genetic analyses to reveal the mechanism of cancer biology and immunological processes. The mouse is the most widely used model in medical research, for very simple reasons. It is relatively small, it occupies only a small amount of space in animal facilities and is very reproductive. Also, the costs are reasonably low for mice compared with larger animals such as rats.
The progress, scientists have made over the past decades in molecular biological techniques have also resulted in genetically modified animals, especially mice. Since it has become possible to genetically modify and even target single genes in mice, a vast surge of interest in almost all facets of mammalian biology using mouse models, has spread over the scientific community.

1.3.1.1 Gene targeting

Until it was possible to create planned gene alterations at a specific locus in the genome, a long way around had to be taken. The first gene targeting studies were performed with yeast (Hinnen *et al.* 1978) and homologous recombination was introduced by Orr-Weaver and colleagues (1981). Orr-Weaver claimed that free ends of DNA appeared to be “recombinogenic” and insertion was much more frequent when a double strand break was present in both the host DNA and the incoming DNA in the region of homology. In the early 1980s, several laboratories began model experiments to investigate homologous recombination following the introduction of exogenous DNA into mammalian cells (reviewed in Bollag *et al.* 1989). The most striking study was reported by Smithies and colleagues in 1985 in which they demonstrated homologous recombination in the human β-globin locus in a planned manner (1985). Several principles for directed gene targeting could be derived from their experiments, such as, and probably most importantly that native gene targeting is possible. Using a joined marker such as a G418 resistance allows selection and enrichment of positive events and the targeting of a gene can be achieved without introduction of foreign or additional

sequences into other sites within the genome. Parameters that affect the targeting frequency, including length of homology between locus and incoming DNA (THOMAS AND CAPECCHI 1989), the size of the DNA fragment to be inserted (MANSOUR *ET AL.* 1990 AND MOMBAERTS *ET AL.* 1991), and the transcriptional state of the selected gene (NICKOLOFF AND REYNOLDS 1990) have all been widely examined.

The "progenitors" of present day stem cells were pluripotent embryonal carcinoma cells derived from transplantable teratomas. More than 40 years ago, Stevens and Little observed the spontaneous occurrence of these kind of teratomas in mouse strain 129 (STEVEN AND LITTLE 1954). The pluripotency of these cells was illustrated by generation of a variety of different tissues all derived from a single cell (KLEINSMITH AND PIERCE 1964). However, the majority of these teratomas were karyotypically abnormal *in vivo* and were not applicable in germline transmission experiments.

The embryonal stem cells (ES cells) that are now used and referred to as EK cells, were isolated by **E**vans and **K**aufman in 1981 from the inner cell mass of a 129 SvE mouse blastocyst (1981); independently, similar cells were isolated by Martin from **I**mprinting **C**ontrol **R**egion mice (1981). The Sv129-derived ES cells proved to permit germline transmission allowing the routine construction of chimeric mice, which are capable of transmitting the targeted gene to their offspring (BRADLEY *ET AL.* 1984). The first germline-competent chimeras were obtained in 1986 by two different groups using different methods. Robertson and colleages infected ES cells retrovirally with a neomycin resistance gene (ROBERTSON *ET AL.* 1986), whereas Gossler used calcium phosphate DNA precipitation to introduce the DNA into the stem cells (1986). The germline-competent animals were obtained after returning G418 resistant cells into a blastocyst.

In culture, usually on primary embryonic fibroblasts (GOSSLER *ET AL.* 1986) or embryonic fibroblast cell lines (BRADLEY *ET AL.* 1984), it is important that ES cells are kept in an undifferentiated status. Leukemia inhibiting factor (LIF) is capable of inhibiting ES cell differentiation (SMITH *ET AL.* 1988, WILLIAMS *ET AL.* 1988). However, different ES cell lines vary in their capability of germline transmission. Frequencies can range from 0 to 70 % (MCMAHON AND BRADLEY 1990). Cultured ES cells may accumulate mutations or epigenetic changes such as methylations, which may affect their pluripotency in a given culture. It is possible that culture conditions and handling impact on the quality of the ES cells. It is also of note that germline transmission is affected by the choice of the blastocyst donor mouse strain. C57BL/6 mice have been shown to be the fittest blastocyst donors in their capability of germline transmission (KOLLER *ET AL.* 1989, SCHWARTZBERG *ET AL.* 1989).

With this background, several groups have isolated cell lines derived from C57BL/6 mice (SCHMITT *ET AL.* 1991) and Birgit Ledermann and colleagues have found that C57BL/6-derived stem cells are germline-competent when injected into BALB/c blastocysts (personal communication with Dr. Birgit Ledermann).
Several studies have been carried out to develop methods for the differentiation of ES cells into cells of selected tissues (REVIEWED IN DONOVAN AND GEARHART 2001). In 1998, human ES cells were established, raising great hope for human ES cell donors in tissue transplantation therapies (THOMSON *ET AL.* 1998). However, allograft rejection and ethical concerns regarding early sacrifice of human embryos are the major problems for this application. Therefore, a lot of effort has been dedicated to the generation of inducible human pluripotent stem cells, which are induced from somatic cells (TAKAHASHI *ET AL.* 2007; YU *ET AL.* 2007). To date, the generation of knockout animals by gene-targeting, which involves the use of stem cells, is limited to mice. Attemps in other animals including rats have been unsuccessful so far. However, cloned animals can be produced in many species by nuclear transfer technologies (DENNING AND PRIDDLE 2003). Recently, a new method using spermatogonial stem cells has been introduced, which offers potential application in species other than the mouse (TAKEHASHI *ET AL.* 2010).

1.3.1.2 INDUCIBLE GENE TARGETING - THE CRE-LOX SYSTEM

Although modern molecular techniques allow very precise gene alterations *in vivo*, these approaches may result in major complications. First and most important, the complete loss of a given gene product can cause embryonal lethality, which makes it impossible to analyse its function at later developmental stages. Second, an organism may be capable of compensating for certain functions that do not appear in the wildtype state. Another possibility is that the organism reacts to the mutation with a complex, secondary phenotype.
Solutions to these problems could be provided by new strategies of inducible gene targeting (KÜHN *ET AL.* 1995). Several site-specific recombinases have been described in yeast and bacteria. These enzymes efficiently catalyse DNA recombination at recognition sequences 25-150 bp in size, which is large enough to not be expected to be found in the mammalian genome. In 1987/88 Brian Sauer introduced the Cre-loxP site specific recombination in yeast and mammalian cells (1987, 1988). The Cre-recombinase

(**c**yclisation **re**combination) of the phage P1 appeared to be exceptionally useful, because of its simplicity and efficiency. In the phage, Cre has two major roles: after infection, it circularises the plasmid DNA, but also stabilises the plasmid by recombination (integration) into the host genome during the lysogenic cycle (SEGEV AND COHEN 1981, HOCHMAN *ET AL.* 1983). By that it promotes survival of the phage during bacterial division. In addition, dimeric plasmids are separated so they can be distributed equally, thereby preventing the disappearance of the vector by dilution (AUSTIN *ET AL.* 1981). This happens, if the 2 daughter plasmids perform crossover and stick together. If this bond is not resolved at the time the cell divides, one of the daughter cells will not recieve a copy of the plasmid.

P1 derived Cre is a 38-kDa protein and recognises an inverted repeat 34 bp in size, a site called loxP (from: locus of X-over of P1) (STERNBERG 1978, STERNBERG *ET AL.* 1986, HOESS AND ABREMSKI 1990).

The loxP site consists of two 13 bp inverted repeats that flank an 8 bp non-palindromic core sequence (spacer), which gives the whole loxP site a direction (*Fig 1.3*). The recombination between two loxP sites of the same direction results in excision of the interjacent DNA. Inverted orientation of the two loxP sites results in inversion of the flanked DNA rather than excision. The breaking and religation of the DNA is restricted to the core region and procedes one strand after the other. This is mediated by a transient phosphotyrosine DNA-protein bonding with the Cre. Unlike other recombinases, Cre does not require help from host factors or special DNA conformation for efficient functionality. These properties define the enormous usefulness of Cre (SAUER 1987). Hence, Cre-loxP-strategies have become standard for *in vivo* loss of function studies (KÜHN *ET AL.* 1995, MARTH 1996).

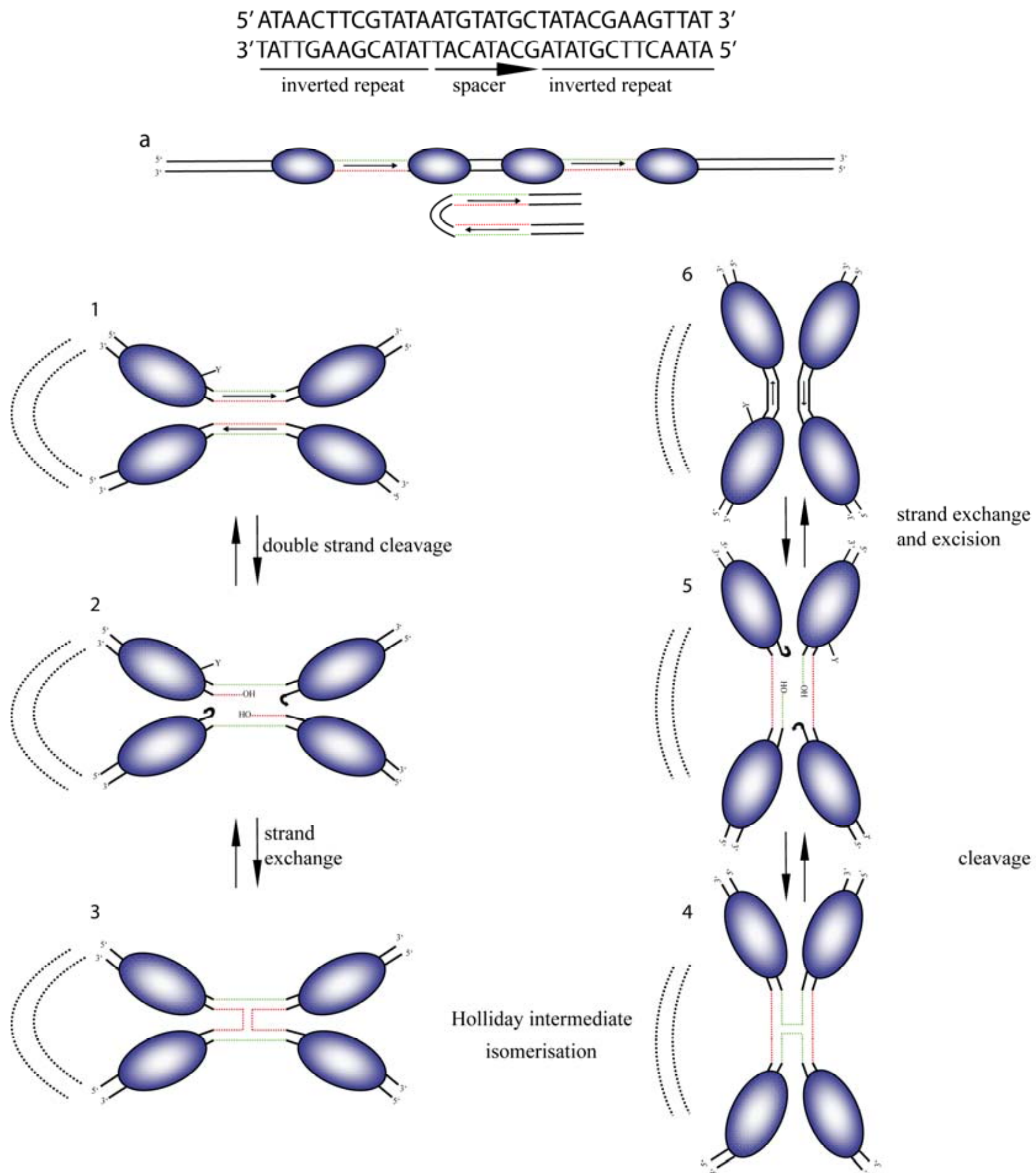

Figure 1.3: Sequence of a loxP site and scheme of Cre-mediated DNA modification based on the models concerning strand-swapping *(NUNES-DÜBY ET AL. 1995) and Cre-loxP structure (GUO ET AL. 1997). 4 molecules of Cre-recombinase bind to the target DNA (a and 1). Conserved tyrosine (y) residues of the recombinase cleave the backbone of the DNA (2). The released 5' ends undergo intermolecular nucleophilic attack of the partner phosphotyrosine to complete the exchange of the first strand and form a Holliday intermediate (3,4), before second strand cleavage (5)and finally excision (6) of the sequence between the loxP sites.*

Tissue-specific gene alteration using flanking loxP sites is an attractive method that was introduced by Gu (GU 1994). Exogenous DNA can be incorporated into a host genome by pronuclear injection into a fertilized zygote. This method is used to produce transgenic animals as „gain-of-function" mutants, which are designed to either express a novel gene or to misexpress a normal gene. Without gene homologies between the gene of interest and the host genome, the foreign DNA will integrate at a non-predictable site. In contrast, for precise gene alteration exogenous DNA integrates into the host DNA by homologous recombination. This mechanism is used to insert the necessary loxP sites at the same time together with the target sequence of the gene of interest.

Animals created with a specifically targeted locus are then mated to Cre-transgenic mice to use Cre-mediated gene alteration in a given tissue. For this purpose the Cre gene has been inserted in a way that it is expressed under a tissue-specific promoter to ensure a specified deletion of the floxed (flanked by loxP sites) gene in the offspring. That this extraordinary mechanism is functional was originally demonstrated by T cell-specific inactivation by 40 % of the DNA polymerase β gene (*pol β*) while there was none in any other tissue at the same time (GU *ET AL.* 1993). The reason for the incomplete gene inactivation can be traced back to the fact that Gu *et al*. used the wildtype Cre. Modern enzymes carry a Kozak-fragment, which allows more efficient translation in mammalian cells due to by this mediated ribosomal binding in eucaryotic cells (KOZAK 1986, SAUER AND HENDERSON 1990).

In order to be able to verify this specific event, the *neo* resistance gene is usually used as a marker for positive selection of the targeted locus and is introduced into the stem cells together with the gene-of-interest-sequence. Unfortunately, *neo* itself can negatively affect gene expression of neighboring genes (ARTELT *ET AL.* 1991, RIJI *ET AL.* 1994, FIERING *ET AL.* 1993): the bacterial *neo* gene comprises cryptic splice sites that may interfere with eucaryotic mRNA splicing (JACKS *ET AL.*1994, MEYERS *ET AL.* 1998, NAGY *ET AL.* 1998) and subsequently reduce the effective mRNA levels of the targeted gene, potentially resulting in a so-called hypomorphic allele. Such an attenuated allele in the germline may cause a knock-down effect (LIU *ET AL.* 1998, YAKAR *ET AL.* 1999, HOLZENBERGER *ET AL.* 2000). Hence, it might be important to remove the marker from the targeted locus in an adjacent step. This is usually accomplished by additional loxP sites that flank the *neo* resistance gene and by transient Cre-expression in the targeted stem cells. To do this, an expression vector that encodes for the Cre-recombinase is transduced into the positively targeted stem cells to address the floxed *neo* gene, but of course also the floxed gene of

interest. However, on a statistical basis, cells will be detectable in which only the *neo* gene, but not the gene of interest was removed. Of importance for this limited activity of Cre is the fact that the Cre-expressing plasmid does not replicate and will eventually vanish from the cell culture by dilution, when the cells proliferate. Finally, cell clones with deleted *neo*, but conserved floxed gene of interest have to be identified by Southern blotting.

The most convenient feature of this conditional knockout strategy is that the same loxP-tagged mouse can be used for every tissue for which a transgene for Cre is available, simply by mating the animals for a desired gene disruption *(Fig 1.4).* This gives the possibility of answering a variety of questions related to a given specific gene expression and its function. The choice of the Cre-expressing mouse is a crucial step in the generation of the final conditional knockout animal in order to avoid mosaic expression of Cre or additional deletion apart from the desired tissue. It is important to analyse the Cre activity and desired tissue-specific expression prior to mating the animals by Western blotting.

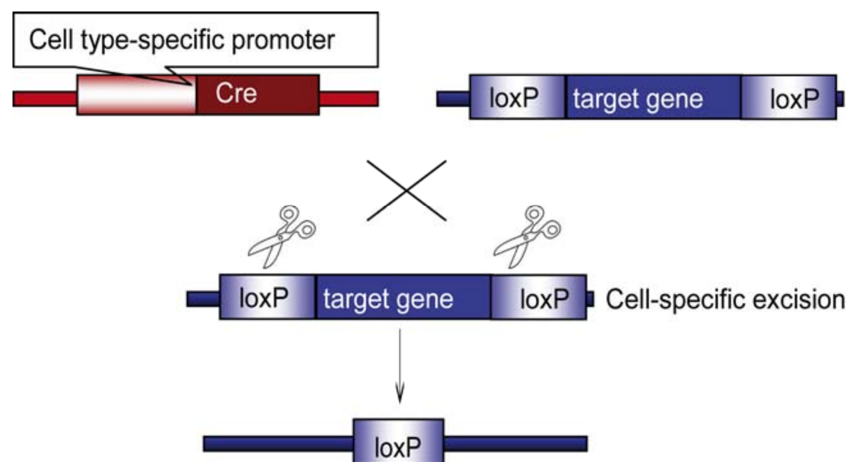

Figure 1.4: ***Cell-specific Cre-mediated excision of the desired target gene.*** *By mating a targeted animal with a partner that expresses Cre under a specific promoter, the gene of interest is deleted in the offspring.*

All together, Cre-mediated gene alteration provides a very successful tool to study selected genes *in vivo* in the context of a many different question.

1.4 OBJECTIVE

In the absence of IRF1, Th cells have a severe defect in the differentiation into the Th1 lineage. Along with this phenomenon, the respective complete knockout mouse cannot raise an efficient immune response towards intracellular pathogens and shows signs of allergy without sensitisation. Hence, this mouse was to be investigated in the settings of allergic asthma.

With regard to the many functions of IRF1 and in order to be able to further specify its functions in a given cell type, a conditional knockout mouse for IRF1 was to be generated as the main task of this thesis work.

Beside IRF1, also IRF4 has strong impact on T helper cell differentiation. IRF4 is essential for lineage decisions towards Th2, Th17and Th9. Parts of this thesis work concentrated on the function of IRF4 in γδT cell cytokine expression.

2. MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 ANTIBODIES

2.1.1.1 UNCONJUGATED

α-CD3	protein A purified, 145-2C11
α-CD28	protein A purified, 37.51
α-pSTAT1 (Tyr 701)	#9171, Cell Signaling
α-pSTAT3 (Tyr705)	#9131, Cell Signaling
α-STAT1	#9172, Cell Signaling
α-STAT3	124H6; #9139,Cell Signaling
α-IFN-y, ELISA capture and detection	R4-6A2, BD Pharmingen
α-IL-4	BVD4-1D11, BD Pharmingen
α-IL-5	TRFK5, BD Pharmingen
α-IL-10	JES5-2A5, BD Pharmingen
α-APC micro beads	Miltenyi
Dynabeads® Mouse T-Activator CD3/CD28	Invitrogen
α-OVA IgE	BD Pharmingen
α-OVA IgG1	BD Pharmingen

2.1.1.2 CONJUGATED

α-CD4-Phycoerythrin (PE)	GK 1.5, eBioscience
α-CD8-Fluorescein isothiocyanate (FITC)	53-6.7, eBioscience
α-IL-4 Alexa 486, α-IL-4 PE	11B11BD, Pharmingen
α-IFN-γ-FITC	XMG1.2, ebioscience
α-IFN-γ (biotinylated)	XMG1.2, BD Pharmingen
α-IFN-γ-PerCP-Cy5.5	XMG1.2, Biolegend
α-γδ TCR-APC	ebioGL.3, eBioscience
α-IL-17-PE/FITC	ebio17B7, eBioscience
α-rat IgG2a-PE (isotype control)	eBioscience
α-IgG1-FITC (isotype control)	eBioscience
α-rat-IgG1-PerCP-Cy5.5 (isotype control)	RTK 2071, Biolegend
Streptavidin-AKP	554065, BD Pharmingen
α-mouse IgE (biotinylated)	BD Pharmingen
α-mouse IgG1 (biotinylated)	BD Pharmingen
α-mouse IgG (peroxidase-coupled)	Sigma-Aldrich
α-goat IgG-HRP	eBioscience

α-beta actin	AC-15, Sigma-Aldrich
α-OVA IgE	BD Pharmingen
α-OVA IgG1	BD Pharmingen

2.1.2 Cytokines

recombinant (r) mouse (m) IL-23	R&D Systems
rm IL-1β	PeproTech
rm IFN-γ	PeproTech
rm IL-6	PeproTech

2.1.3 Chemicals

Aprotinin	Biomol
β-Mercaptoethanol (ME)	Sigma-Aldrich
2-Amino-2-hydroxymethyl-propane1,3-diol (TRIS)	Roth
3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma-Aldrich
4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES)	Roth/Sigma-Aldrich
Acrylamide Mix Rotiphorese® 30 Gel	Roth
Agarose	SeaKem
Albumin bovine (BSA)	Sigma-Aldrich
Alpha dCTP p^{32} radionukleotide	Hartmann Analytik
Ammonium chloride	Roth
Ammonium persulfate (APS)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Aqua. dest	Braun
BM Blue POD Substrate	Roche
Brefeldin A	Sigma-Aldrich
Bromephenol blue	Sigma-Aldrich
BSA Albumin Fraktion V	Sigma-Aldrich
Ca_2Cl	Sigma-Aldrich
Chymostatin	Sigma-Aldrich
Clicks RPMI	Biochrom
Collagenase D	Roche
Dextransulfate	Sigma-Aldrich
DiffQuick®	Dade-Behring
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma-Aldrich
DMEM (Dulbecco's modified Eagle medium)	PAA
DNA loading dye 6x	Fermentas
Epigallocatechin-3-gallate (EGCG), STAT1 inhibitor	Sigma-Aldrich
Eosin G	Merck
Erythrocyte lysis buffer	Sigma-Aldrich
Ethanol, absolute	Merck
Ethidiumbromid 1 % (1 mg/ml)	Roth
Etylene diamine tetraacetic acid (EDTA), pH8	Sigma-Aldrich

Etylene diamine tetraacetic acid, powder (EDTA)	Sigma-Aldrich
Fetal Calf Serum (FCS)	Greiner
Ficoll (Typ 400-DL)	Sigma-Aldrich
Ficoll powder (Typ 400)	Sigma-Aldrich
Folic acid	Sigma-Aldrich
Formaldehyde 37 %	Sigma-Aldrich
G418	Sigma-Aldrich
Ganciclovir	Sigma-Aldrich
Gelatine	Sigma-Aldrich
Glacial actetic acid	Roth
Glucose	Fluka
Glycerol 98 % pure	Roth
Glycine	Roth
Halt™ Protease inhibitor Single use cocktail 100x	Thermo Scientific
Hämatoxilin II, (Gill)	Merck
Hank's Balanced Salt Solution (BSS)	Biochrom
Herring sperm DNA	Promega
Hydrochloric acid (HCl)	Roth
Inject® Alum ($Al(OH)_3$)	Pierce
Ionomycin	Sigma-Aldrich
KCl	Roth
K actetate	Sigma-Aldrich
Ketamin (10 mg/ml)	Inresa
L-Arginine	Sigma-Aldrich
L-Asparagine	Sigma-Aldrich
LB Agar	Roth
LB Medium powder	Roth
Leupeptin	Biomol
L-Glutamine Solution	Biochrom
LIF /Leucemic inhibitory factor)	Cell culture, AG Pfeffer
Mangan-chloride	Sigma-Aldrich
MEM NON-essential amio acids	Invitrogen
Methanol p.a.	Roth
Milk powder	Roth
Mitomycin C	Sigma-Aldrich
Monensin	eBioscience
N,N,N',N'-Tetramethylethylendiamin	Sigma-Aldrich
Na acetate	Sigma-Aldrich
Nacitrate	Sigma-Aldrich
NaCl	Sigma-Aldrich
NaHCO3	Sigma-Aldrich
NaOH pellets	Sigma-Aldrich
NaOH solution	Roth
NaP	Sigma-Aldrich
Na pyruvate	Roth
NH_4Cl	Sigma-Aldrich
Ovalbumin Grade V	Sigma-Aldrich
Ovalbumin Grade VI	SigmaAldrich
PBS without Mg^{2+} and Ca^{2+} for ES cells	Biochrom
Penicillin G	Biochrom
Penicillin G/Streptomycin 100x solution	Gibco
Pepstatin A	Biomol
Phenylmethanesulphonylfluoride (PMSF)	Sigma-Aldrich
Percoll, Biocoll	Biochrom
Phenol/Chloroform	Roth

Phorbol 12-myristate 13-actetate (PMA)	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	Biochrom
p-Nitrophenyl Phosphate (PNPP)	Sigma-Aldrich
RNase A	Biozym Scientific GmbH
Rompun 2%	Bayer
Roti Clear (Xylol)	Roth
RPMI Roswell Park Memorial Institute Medium	Sigma-Aldrich
PS1145, NFκB inhibiotor	Sigma-Aldrich
Rubidium cloride	Roth
Saccharose	Sigma-Aldrich
Saponin	Sigma-Aldrich
Sarcosyl	Sigma-Aldrich
Schiff's Reagent	Roth
Sodium dodecyl sulfat (SDS)	Sigma-Aldrich
Stattic V, STAT3 inhibitor	Calbiochem
SYBR Gold nucleic acid gel stain	Invitrogen
TRIS-HCl	Roth
Tryphan blue	Gibco
Trypsin/EDTA (0.05 % (w/v) /0.02 % (w/v))	Gibco
Tween20	Roth
Vitamin solution	Sigma-Aldrich

2.1.4 KITS

Dual-Luciferase Reporter Assay System	Promega
NucleoSpin® Plasmid Mini Kit	Macherey&Nagel
NucleoSpin® Plasmid Midi Kit	Macherey&Nagel
PCR Core Kit	Qiagen
NucleoSpin® Extract II-Kit	Macherey&Nagel
NucleoBond BAC 100	Macherey&Nagel
Megaprime DNA labeling system (for alpha-dCTP)	GE healthcare
Miltenyi $\gamma\delta^{+}$ T cell isolation Kit	Miltenyi Biotech
IL-22 ELISA: READY-SET-GO! Mouse Interleukin-22 (IL-22)	ebioscience
IL-17 ELISA: Duo Set ELISA mouse IL-17	R&D Systems
"RNeasy Micro Kit"	Qiagen
cDNA Maxima First Strand Systhesis Kit for RT-qPCR	Fermentas
qPCR Core kit for Sybr green I	Eurogentec
qPCR™ Core Kit	Eurogentec
BCA protein quantification Kit	Pierce
Super Signal Femto maximum sensitivity substrate	Thermo Scientific

2.1.5 Laboratory equipment and consumables

Item	Supplier
Cell culture dish (150x20 mm, 100x20 mm, 50x20 mm, 35x20 mm)	Becton Dickinson
Cell culture plate (96-, 48-, 24-well flat bottom, 96-well round bottom)	Greiner/Costar
Cuvettes, Gene Pulser, 0.4cm (Nr. 165-2088)	BioRad
Electroporator Gene Pulser	Biorad
ELISA plates	Dynex
ELISA reader	Dynex
AriaIII Cell Sorter	Becton Dickinson
Casy® Cell Counter	Schärfe Systems
Fluorescence activated cell sorting FACS Calibur	Becton Dickinson
Films, Kodak MS and MP	Sigma-Aldrich
Film exposure cassette with 2 amplifying screens	Sigma-Aldrich
Gel gadget for agarose gel electrophoresis	Made inWorkshops at Philipps University Marburg and Heinrich Heine University Düsseldorf
Incubator	Heraeus
Hamilton Pipette	Hamilton
Heating block TCR 200	Roth
Hybridisation oven	Heraeus
Megafuge1.0 R / Biofuge Pico (Rotors #2704/#3328), 1700 rpm equals 470 g	Heraeu
Microscope BX51	Olympus
Microwave	Sharp
Membrane Hybond-N	GE Healthcare
Miltenyi LD, LS, MS colums and magnets	Miltenyi
Nanodrop ND-1000 Spectrophotometer	PeqLab
Nylon mesh	GE Healthcare
Object slide cover slip	Menzel
Object slides	Menzel
Pasteur pipette	Brand
PCR cycler	Biometra
Power supply	Consort, Scie-Plas
Reaction cups, 1.5 and 2 ml	Eppendorf
Reaction tubes, 15 and 50 ml	Greine
Shaker	IKA
Sterile filter	Sarstedt
Sterile filter, 24 mm	Costar
Sephadex colums	GE Healthcare
Scintillation counter	Life technologies
Syringes and needles of different sizes	BD Microlance
Heating block	Roth
Waterbath	Memmer
Whatman paper	Schleicher+Schüll
Western Blot gadget	Peqlab
Western Blotting membrane PVDF	Roche

2.1.6 MEDIA, BUFFERS AND SOLUTIONS

Acrylamide gels:
Separation gel (8 %): 2.3 ml H_2O, 0.33 ml acrylamide mix (30 %), 0.25 ml 1 M Tris (pH 8.8), 0.02 ml SDS (10 %), 0,02 ml ammonium persulfate (APS, 10 %), 0.002 ml TEMED
Separation gel (10 %): 1,9 ml H_2O, 1,7 ml acrylamide mix (30 %), 1,3 ml 1 M Tris (pH 8.8), 0.05 ml SDS (10 %), 0,05 ml ammonium persulfate (APS, 10 %), 0.002 ml TEMED
Stacking gel (5 %): 1.4 ml H_2O, 1.3 ml acrylamide mix (30 %), 1.3 ml 1,5 M Tris (pH 6.8), 0.05 ml SDS (10 %), 0.05 ml ammonium persulfate (APS, 10 %), 0.003 ml TEMED

Aerosol solution
1 % OVA Grade VI in PBS.

Ammonium Chloride solution (NH_4Cl):
9.1 g NH_4Cl dissolved in aqua dest, 20 ml 1M HEPES solution added and filtered sterile.

BSS:
BSS-powder for 10 l, 0.6 g penicillin G, 1g streptomycin sulfate, 14.25 g $NaHCO_3$ and 23.83 g HEPES dissolved in 10 l aqua dest., pH-value adjusted to 7.2 and filtered sterile.

Cell lysis buffer per $5x10^7$ cells or 1 tail tip:
500 µl TNE (see below), 7.5 µl proteinase K (10 mg/ml), 25 µl pronase E (10 mg/ml) and 50 µl SDS solution (10 %), freshly prepared.

Clicks Roswell Park Memorial Institute Medium (Clicks RPMI):
Instant Clicks powder dissolved in aqua dest. plus 60 mg/l penicillinG, 100 mg/l streptomycin sulfate, 14.25 $NaHCO_3$ and 23.83 g HEPES added, pH-value adjusted to 7.2 and filtered sterile.
Add before using: 1 % l-glutamine solution (volumen/volumen (v/v)), 10 % FCS (v/v), 0.5 % ME-Stock (see below) solution (v/v).

Clone Medium:
500 ml Dulbecco's Modified Eagle Medium (DMEM) high glucose, supplemented with L-Glutamine (2 mM final), β-Mercapto-Ethanol (50 µM final), penicillin/streptomycin solution (100 U/ml final); additions from bought solutions: 5 ml non-essential amino acids, 5 ml folic acid, 7.5 ml sodium pyruvate, 5 ml L-arginine/L-asparagine, 5 ml vitamin solution, 50 ml fetal calf serum.

Cracking solution:
100 µl 2 N NaOH, 50 µl SDS solution (10 %), 0.2 g saccharose. Adjust H_2O to final volume of 1 ml (approx. 850 µl).

Denaturating buffer:
0,5 M NaOH and 1.5 M NaCl dissolved in aqua dest, freshly prepared before use.

50x Denhardt's:
10 g Ficoll 400, 10 g polyvinylpyrrolidone and 10 g BSA dissolved in aqua dest., adjust to a final volume of 1 l.

Dextran sulfate solution:
50 % (weight/volume =w/v) dextran sulfate in sterile aqua dest., stirred overnight at RT, **do not autoclave**! (heat sensitive).

Diethanol amine buffer:
400 mg $MgCl_2 \cdot 6H_20$ dissolved in approx. 450 ml aqua. dest., 48.5 ml diethanol amine added and stirred for approx. 1h. pH-value adjusted to 9.8 filled up to 500 ml. Stored at 4 °C in the dark.

1 M DTT:
Dissolve 3.085 g 1,4-dithio-DL-threitol (DTT, MW=154.25) in 20 ml 10 mM sodium acetate (pH 5.2). Divide in aliquots of 1 ml and sterilise by filtration.

ELISA blocking solution:
1x PBS with 1% BSA.

Coating buffer:
0.1M $NaHCO_3$ pH 8.2 ($NaHCO_3$ (MW = 84.01g/mol).

0,5 M EDTA:
Dissolve 186.12 g disodium ethylenediaminetetraacetate–H_2O (Na_2 EDTA–$2H_2O$, MW =372.24g/mol) in 800 ml H_2O; adjust pH value to 8.0 and adjust volume to 1 l. Sterilize by autoclaving. Store at room temperature.

ES cell culture medium:
DMEM, supplemented with 1 % L-glutamine solution, penicillin/streptomycin solution (penicillin G sodium 100x), 0.2 U/ml **L**eukemia **I**nhibitory **F**actor (LIF), 500 µl of ME-Stock-solution and 15 % FCS.

EM cell freeze medium:
80 % feeder cell medium, 10 % FCS, 10 % DMSO.

ES cell freeze medium:
70 % complete culture medium, 20 % fresh FCS and 10 % DMSO, filtered sterile.

ES cell lysis buffer:
10 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5 % sarcosyl, 0.4 mg/ml proteinase K (freshly added)
Mastermix for one plate: 5 ml lysisbuffer + 200 µl proteinase K (10 mg/ml).

FCS:
Thawed serum inactivated by heating at 56 °C for 40 min, cooled to room temperature (RT) and filtered sterile.

Feeder cell culture medium:
DMEM supplemented with 1 % L-glutamine solution, 5 ml penicillin/streptomycin (penicillin G sodium solution 100x), 500 µl of ME stock solution and 5 % FCS.

0,25 N HCl:
dilute 21 ml of a 37 % HCl-solution in 1 l of aqua dest..

HEPES solution (1M):
238.3 g HEPES dissolved in 500 ml aqua dest., stirred for 2 h at 37 °C and pH-value adjusted to 7.3.

Herring sperm DNA: 10 mg/ml
Original stock solution diluted to 10 mg/ml in aqua dest., aliquoted and frozen for longtime storage.

Immunisation solution
10 mg OVA GradeVI in 200µl PBS per animal.

Lysis buffer for Luciferase Assay
D-luciferin 470 µM, CoenzymeA 279 µM, DTT 33.3 mM, ATP 530 µM, $(MgCO_3)4Mg(OH)2x5H_2O$ 1.07 mM, $MgSO_4$ 2.67 mM, Tricin 20 mM, EDTA 0.1 mM, pH7.8.

Lysis buffer for Western Blotting:
40 mM Tris pH 8.0, 100 µM PMSF, 1 µg/ml chymostatin, 5 µg/ml pepstatin A, 5 µg/ml aprotinin, 5 µg/ml leupeptin.

M2 Medium for blastocyst transfer
M2 medium, supplemented with penicillin/streptomycin (penicillinG sodium solution, 100x).

MACS buffer:
PBS with 0.5 % BSA (w/v) and 2 mM EDTA pH 8.0.

ME stock solution (10 mM):
0.7 ml 2-mercaptoethanol dissolved in 1 l PBS and filtered sterile.

Narcosis-solution for anesthesia, 5x:
10 ml Ketamin (50 mg/ml)/3.125 ml Rompun in PBS.

NaP:
1 M sodium phosphate dissolved in aqua dest., pH value adjusted to 6.4. Autoclaved for longtime storage.

Neutralisation buffer:
0.5 M Tris-HCl dissolved in aqua dest., pH value adjusted to 7.4 and 0.5 M NaCl added and autoclaved for longtime storage.

PBS:
PBS dry substance for 10 l dissolved in aqua dest., pH value adjusted to 7.2 and autoclaved.

RF1 buffer for competent cells:
Dissolve 12 g rubidium chloride (RbCl), 9.9 g mangan chloride ($MnCl_2$x 4 H_2O), 30 ml potassium acetate 1 M, pH 7.5 (Ka-acetate), 1.5 g calcium chloride ($CaCl_2$x 2H_2O) and 150 g glycerol in 1000 ml H_2O and adjust pH to 5.8 with glacial acetic acid. Filtered sterile and stored at 4 ºC.

RF2 buffer for competent cells:
Dissolve 1.2 g RbCl, 11 g $CaCl_2$ x2 H_2O, 150 g glycerol and 20 ml 0.5 M 3-(N-morpholino) propanesulfonic acid, pH 6.8 (MOPS) in 1000 ml H_2O. Filtered sterile and stored at 4 ºC.

Roswell Park Memorial Institute Medium (RPMI)
500 ml medium supplemented with 10 % FCS, 5 ml non-essential amino acids, with L-glutamine (2 mM final), β-mercapto-ethanol (50 µM final), penicillin/streptomycin solution (100 U/ml final).

Sample buffer for Western blotting:
150 mM Tris (pH 6.8)/ 1.2 %SDS/ 3.3 % glycerin/ 1.8 % bromphenol-blue.

Saponin buffer:
0.3 % saponin (w/v) dissolved in PBS and 2 % FCS (v/v) added. For storage filtered sterile.

20x SSC:
3 M NaCl and 300 mM Na-Citrat x 2 H_2O dissolved in aqua dest., pH value adjusted to 7.0 and autoclaved for longtime storage.

Southern blot washing buffer 1:
20x SSC diluted to 2x SSC in sterile aqua dest., SDS added to final concentration of 0.1 %.

Southern blot washing buffer 2:
20x SSC diluted to 0.1x SSC, SDS added to final concentration of 0.1 %.

50 x TAE:
Dissolve 242 g Tris in 500 ml aqua dest., add 100 ml EDTA (pH 8) and 57.1 ml glacial actetic acid, adjust volume to 1 l with aqua dest.. Store at room temperature.

10/1 TE buffer:
Tris dissolved to final concentration of 10 mM and EDTA to final concentration of 1 mM in H_2O.

TNE solution:
10 ml 1 M Tris (pH 8.0), 20 ml 5 M NaCl, 2 ml 0.5 M EDTA diluted ad 1000 ml aqua dest. and autoclaved.

Western blot transfer buffer:
200 mM glycin, 25 mM Tris-HCL pH = 8.8, substituted with 20 % methanol (v/v) before use.

Wash buffer for ELISA:
PBS with 0.05 % tween (g/v).

Wash buffer Western blot:
100 mM NaCl, 10 mM Tris-HCl pH 7.5 and 0.05 % tween (v/v).

Western Blot electrophoresis buffer
30 g Tris-Base/ 10% SDS/ 250 g glycin ad 1 l dH_2O.

2.1.7 MICE AND CELLS

Mice were the only laboratory animals used for asthma and infection experiments or for organ extraction. Mice were kept under specific pathogen free conditions (SPF) in individual ventilated cages (IVC) at 20 °C and artificial light-dark-cycles (12 hours each). Food and water were provided *ad libitum*. The following inbred mouse strains, cell lines and bacteria were used:

Mice	
C57BL/6 mice, wildtype	Harlan Winkelmann
BALB/c mice, wildtype	Harlan Winkelmann
CD1 mice	Harlan Winkelmann
Irf1$^{-/-}$ mice, C57BL/6	own breeding
Irf1$^{-/-}$ mice, BALB/c	self backcrossed to the F8 generation, own breeding
Irf4$^{-/-}$ mice, C57BL/6	own breeding
Stat4$^{-/-}$ mice, BALB/c	kindly provided by Prof. Dr. Max Löhning
Cell lines	
E14.1 embryonic stem (ES) cells from Sv J129/Ola mice	kindly provided by Prof. Dr. Klaus Pfeffer
C57BL/6 blastocysts	kindly provided by Prof. Dr. Klaus Pfeffer
293-mTLR9-luc	kindly provided by Prof. Dr. Stefan Bauer
Bacteria	
Escherichia. coli DH10B strain	Invitrogen
Escherichia. coli Top10	Invitrogen
Streptococcus pneumoniae PN36	kindly provided by Prof. Dr. Robert Bals

2.1.8 PLASMIDS

pEasyFlox	Target vector for genomic manipulation for generation of a knockout mouse. 3 flox sites, Amp^r, Neomycin^r included, kindly provided by Prof. Dr. Klaus Pfeffer, Düsseldorf NCBI ACCESSION: CQ918568 VERSION: CQ918568.1 GI:56208490
pICre	low copy plasmid, carrying the gene for Cre-recombinase. Used to excise the neomycin gene cassette within the targeted stem cell, kindly provided by Prof. Dr, Klaus Pfeffer, Düsseldorf
pBluescript	Fermentas GmbH
IRF1 BACclone	CHORI, Children's Hospital Oakland Research Institute „RP23-303F24“Oakland, USA

2.1.9 Enzymes

Antarctic Phosphatase	New England Biolab
AvrII/XmaJI	Fermentas GmbH
BamHI	Fermentas GmbH
ClaI	Fermentas GmbH
DNase I	Invitrogen
EcoRI	Fermentas GmbH
EcoRV	Fermentas GmbH
HindIII	Fermentas GmbH
KOD Hot start DNA polymerase	Novagen
NotI	Fermentas GmbH
Pfu-DNA polymerase	Fermentas GmbH
Pronase E	Sigma-Aldrich
Proteinase K	Qiagen
RNase	Biozym Scientific GmbH
SalI	Roche
SpeI	Fermentas GmbH
T4-Ligase	Fermentas GmbH
XhoI	Fermentas GmbH

2.1.10 Oligonucleotides

CpG-ODN 1668 TCCATGACGTTCCTGATGCT

Primers for *Irf1* gene targeting

<u>5’ flanking probe</u>	
5’fp for:	5’ GAGCAGGAGCCTATCTATGTAG 3’
5’fp rev:	5’ CCCTGTCTCTCCTCTGCC 3’
Short Arm 5’	
SA5’ for: XhoI/NotI	5’CCGCTCGAGGCGGCCGCGAGACATACACTACCATCAG 3’
SA5’ rev: HindIII	5’ CCCAAGCTTGGCTTAGACATCCAGCAGACTG 3’
Short Arm3’	
SA3’ for: BamHI	5’ CGGGATCCCCTGTAGTTACACAAGTAGCC 3’
SA3’ rev: ClaI	5’ GTCAATCGATGCGACCTGGGCCCTGCTGGC 3’
Inner Arm5’	
IA5’ for: SalI	5’ ACGCGTCGACCTCTGGGCATGTGAGAAGCCCC3’
IA5’rev:SalI	5’ TTCCGCGGCCGCTATGGCCGACGTCGA...
	...CCAGCACCTGCCCAAAGCGACC 3’

Primers for quantitative realtime PCR:

rorc:

RORγt forward	5’ TTTGGAACTGGCTTTCCATC 3’
RORγt reverse	5’ AAGATCTGCAGCTTTTCCACA 3’

hprt:

HPRT forward	5’ CTGGTGAAAAGGACCTCTCG 3’
HPRT reverse	5’ TGAAGTACTCATTATAGTCAAGGGCA 3’

Tbx21:

Tbet forward	5’ GTGGATGTGGTCTTGGTGGAC 3’
Tbet reverse	5’ ACATATAAGCGGTTCCCTGGC 3’

rora:

RORα forward	5’ TCTCCCTGCGCTCTCCGCCGCAC 3’
RORα reverse	5’ TCCACAGATCTTGACTGGA 3’

Primers for genotyping of the conventional *Irf1* knockout mice:

Irf1:

IRF-1 F:	5’ TTCCAGATTCCATGGAAGCACGC 3’
IRF-1 R	5’ ATTCGCCAATGACAAGACGCTGG 3’

2.1.11 SOFTWARE

GraphPadPrism4	GraphPrism
Cell Quest Pro	Becton Dickinson
FlowJo	Tree Star
Cell[F]-Imaging Software	Olympus
Relevation™	Dynex
XPlasMap	Freeware

2.1.12 STATISTICAL ANALYSIS

Statistical calculation for asthma experiments was performed with 1 way ANOVA analysis including the Tukey's Multiple Comparison Test. Associated data points were analysed by Grubb's test to exclude significant outliers. (http://www.graphpad.com/quickcalcs/Grubbs1.cfm)

2.1.13 SERVICES

All sequencing has been conducted at Seqlab Sequencing Laboratories Göttingen (www.seqlab.de)

2.1.14 DATABASES AND ONLINE TOOLS

BLAST:	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Ensembl mouse:	http://www.ensembl.org
Primer design and analysis:	http://frodo.wi.mit.edu/primer3/
Invitrogen's Vector NTI:	http://www.invitrogen.com
Online Vector drawing tool:	http://www.infosake.com/plasmid/plasmidFrame.php
ApE – Plasmid editor:	http://www.biology.utah.edu/jorgensen/wayned/ape/

2.2 METHODS

2.2.1 GENERATION OF A CONDITIONAL KNOCKOUT MOUSE LINE FOR IRF1

2.2.1.1 PREPARATION OF GENOMIC DNA FROM MOUSE TISSUE

For genotyping *Irf1*$^{-/-}$ mice, DNA was extracted from mouse ear punches by using the PUREGENE® DNA Purification Kit following the manufacturer's instruction protocol for mouse tails. PCR was performed according to Matsuyama *et al*. (1993). In analogy to this mouse the targeting stategy was developed as described in 2.2.1.6.

2.2.1.2 STANDARD POLYMERASE CHAIN REACTION (PCR)

PCR is used to amplify a single or a few copies of a particular DNA fragment by repeated cycles of denaturation, primer annealing and generation of the complementary strand, thereby leading to exponential amplification of a selected part of the DNA template.

Here, PCR was used to amplify all DNA fragments of the murine *Irf1* gene needed for the construction of the target vector to manipulate the *Irf1* gene for the generation of the conditional gene knockout. For amplification of all *Irf1*-fragments, the **B**acterial **A**rtificial **C**hromosome **BAC**clone **RP23-303F24 IRF1** was used as template DNA. Specific primers were used as indicated in the primer list. The primer sequences (see section 2.1.10) included sequences for specific restriction sites (see section 2.1.10) to allow cloning into the target vector pEasyFlox.

The primers for the short arm (SA) on the 3' end of the targeting location were combined with restriction sites for ClaI and BamHI, SA5' for HindIII and XhoI; in addition, the inner arm (IA) primers received the restriction site for SalI on both sides.

OPTIMIZING PCR CONDITIONS

Each PCR needed optimised conditions as indicated in the following table (*Tab. 2.1).* For amplification, high fidelity polymerases with proofreading activity were used. All PCRs constisted of a 3 step PCR programm with 30 cycles and were conducted in a Biometra Gradient Cycler.

To develop a functional PCR protocol, several considerations were made:

a) First, the amount of target DNA should not exceed certain limits in order to allow molecule amplification, rather than to catch too many primers which would prevent amplification.

b) For genomic DNA, 100 ng are sufficient, plasmid DNA can be used to a lesser extent, mainly due to its having fewer secondary structures. Although a BAC clone carries the whole gene but is still a plasmid, 60 ng were considered and proven to be sufficient.

c) The CG-content of primers should be between 40-60 % for optimal annealing.

d) The calculated melting temperarture T_m of both primers used in the reaction should not differ by more than 5 °C.

e) Annealing temperature is usually chosen 5 °C lower than calculated T_m, but was tested empirically for each reation.

f) Complementary sequences between both primers and secondary structures such as hair pins should be avoided. Using primer design programme primer3 and BLAST search help to avoid secondary structures and improves primer specificity.

Table 2.1: PCR conditions and protocols for amplification of the required gene fragments of the Irf1 genomic locus *(SA3', SA5', 5'fp, and IA) to be cloned into the target vector and for the probe, as required for Southern blot detection. The fragment sizes refer to their genomic lengths. Below, the cycler conditions are listed.*

Gene fragments	SA3', 575 bp	SA5', 541 bp	5'fp, 532 bp	IA, 1662 bp
10x PCR Buffer according to polymerase	5 µ buffer without $MgSO_4$	5 µ buffer with 15 mM $MgSO_4$	5 µl KOD buffer	5 µl buffer with 15 mM $MgSO_4$
$MgSO_4$ (25 mM)	5 µl	5 µl	5 µl	5 µl
dNTPs 10 mM ea	1 µl	1 µl	1 µl	1 µl
forward primer 10 µM	0.5 µl	0.5 µl	0.5 µl	0.5 µl
reverse primer 10 µM	0.5 µl	0,5 µl	0,5 µl	0.5 µl
polymerase	0.5 µl Pfu-Pol (2,5 U/µl)	0,5 µl Pfu-Pol (2.5 U/µl)	KOD-Pol (1 U/µl)	0.5 µl Pfu-Pol (2.5 U/µl)
template DNA, 60 ng/µl	1 µl	1 µl	1 µl	1 µl
aqua dest. ad 50 µl	36.5 µl	36.5 µl	40 µl	36.5 µl
Initiation	94 °C, 3 mins	94 °C, 3 mins	94 °C, 2 mins	94 °C, 3 mins
Denaturation	94 °C, 1 min	94 °C, 1 min	94 °C, 15 sec	94 °C, 1 min
Annealing	60 °C, 1,5 mins	62 °C, 1,5 mins	55 °C, 30 sec	65 °C, 1,5 mins
Elongation	72 °C, 2,5 mins	72 °C 2,5 mins	68 °C, 1 min	72 °C 2,5 mins
terminal elongation	72 °C, 7 mins	72 °C 7 mins	68 °C, 3 mins	72 °C 7 mins

Subsequently, the PCR reactions were analysed by agarose gel electrophoresis.

2.2.1.3 Agarose Gel electrophoresis

Gel electrophoresis includes a group of techniques to separate and characterise molecules in terms of their physical properties, such as size, shape or charge.
The simplest method to detect and analyse nucleic acid is agarose gel electrophoresis. A PCR-product, digestion fragment or genomic DNA can be separated and visualised in the agarose via ethidiumbromide staining. Depending on the fragment size, the densitiy of agarose was chosen from 1.5 % for smaller PCR-products, 1 % for larger PCR-products and restriction reactions as well as 0.8 % for genomic DNA samples.

2.2.1.4 Gel clean-up

To extract and dissolve single DNA fragments from the gel matix, the NucleoSpin® Extract II-kit by Macherey & Nagel was used according to the manufacturer's instructions. In brief, the DNA containing gel piece was redissolved in buffer and loaded onto a silica membrane that catches the DNA. The purified DNA fragment was then eluted in distilled, sterile water. All subcloned *Irf1* DNA fragments designated for cloning into the target vector were excised from the agarose gel and purified as described above.

2.2.1.5 PCR clean up

DNA fragments that did not need to be separated from a mixture of DNA fragments of different sizes such as PCR products, were also purified by using the NucleoSpin® Extract II-kit by Macherey & Nagel. Here, the main point was buffer exchange and removal of unbound nucleotides and unused primers. DNA fragments were eluted in distilled sterile water. All *Irf1* PCR fragments appointed for subcloning into the pBluescript vector and the 5 prime probe for Southern blot detection were purified as just described.

2.2.1.6 CLONING OF THE TARGET VECTOR

The targeting strategy was based on the Cre-lox system (see 1.3.1.2). For this purpose the plasmid pEasyFlox, which contains 3 loxP sites was used. The fragment for the inner arm (IA) was cloned into this target vector between two loxP sites. The vector also carries a *neomycin* gene cassette for selection of positively targeted ES cell clones (see 2.2.1.16), which is also flanked by loxP sites.These loxP sites served to remove the neomycin gene cassette later on. The process of site-directed recombination from the vector into the ES cell requires flanking genomic regions, the so called short arms (herein termed SA3' and SA5'), which were cloned on both sides of the floxed region. In the following, this cloning is described in detail.

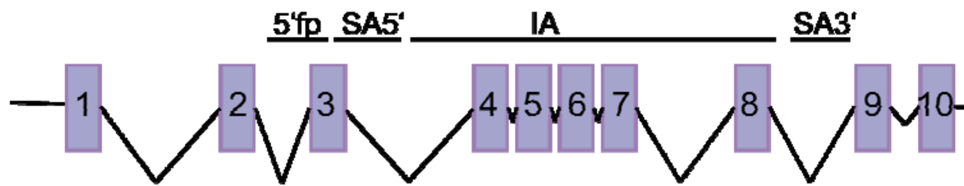

Figure 2.1: Homologous recombination and targeting strategy for the conditional gene targeting of the Irf1 locus *with indicated exons 1-10. The exons 4-8 (inner arm=IA) will be deleted. The short arms (SA5' and SA3') are designated for homologous recombination. The 5'probe (5'fp) is located in the untouched adjacent region to detect all manipulations.*

According to the targeted disruption of the *Irf1* gene in the conventional knockout mouse that has been generated by Tak Wah Mak and colleagues (MATSUYAMA *ET AL.* 1993), the same genomic location for the conditional knockout was chosen, with one exception: Whereas Mak and colleagues eliminated exons 3-7, we decided to disrupt exons 4-8, because this way we did also not stay in the reading frame, and because the initially intended strategy implied the use of a genomic Sfi-restriction site, which was also available in the target vector. This Sfi restriction site is located at the 3' end of the intron sequence (SA3') adjacent to exon 8 and therefore the area downstream of exon 8 rather than exon 7 was used for homologous recombination. After the first attempts of the construction of the target vector the advantage of this Sfi restriction site was revised

and a closely located ClaI restriction site was finally used. However, by exchanging the restriction site, the overall strategy was still applicable and not changed.

Staying out of the reading frame was of great importance for our strategy. The starting point of translation is at the beginning of the second exon. Together with the third exon, this region encompasses less than 200 nucelotides. By destroying the reading frame as a result of the Cre-mediated recombination, any transcript of the exons behind the targeted exons will (together with the short product of exons 2 and 3) encode for a product that has no IRF1-related function, because it most likely forms no functional-related 3-dimensional structure. Alternatively, the knock-out may be able to influence splicing of the hypothetical transcript of exons 9 and 10 so that splicing is no longer possible. In this situation, only exons 2 and 3 will result in a very small product which most likely will be degraded by nonsense mediated decay processes (personal comunication with Dr. Sandra Beer-Hammer, AG Prof. Dr. Pfeffer). The conventional knockout mouse only transcribes a short mRNA, which is not yet translated (MATSUYAMA *ET AL.* 1993). This small RNA is detectable by Nouthern blot. In analogy, if our conditional knockout mouse (after crossing with a Cre-deleter mouse) would transcribe a similar small RNA, it should also be detectable by Northern blot.

Sequences for flanking probes that are needed for genomic Southern blotting were chosen in adjacent regions which are not to be manipulated to ensure exact genomic positioning of the recombined *Irf1* exons.

In a first step, specific *Irf1* PCR products were subcloned into pBluescript (Fermentas) and were sequenced by the Sequence Laboratory Seqlab. By using Blast alignment, the sequences were verified. The plasmids were transformed into *E.coli* strain DH10B by heat shock transformation. To do this, chemically competent (refer to section 2.2.1.9) bacteria were mixed with ligated vector and incubated at 42 °C for 2 mins, followed by 30 mins rest on ice and finally plated on LB agar dishes, supplemented with ampicillin (refer to section 2.2.1.10).

From the pBluescript vector, the *Irf1* gene fragments were then cloned into the target vector pEasyFlox. This vector was constructed by Klaus Rajewskys Lab and was kindly provided by Prof. Dr. Klaus Pfeffers Lab in Düsseldorf.

After completion, the target vector was re-sequenced (see appendix) and several control digestions were performed to verify correctness of the insertions.

2.2.1.7 Digestion of DNA

Plasmid (usually 500 ng or up to 1 µg) or genomic DNA (for Southern blotting up to 10 µg) was digested according to the manufacturer's instruction for the individual enzyme. In general, 5 units of a given enzyme were used for plasmid digestion and 20-30 units were used for genomic DNA (see also section 2.2.1.19).

2.2.1.8 Ligation of DNA

Ligation of vector with insert DNA was performed according to the manufacturer's recommendation for T4-ligase (Fermentas).

2.2.1.9 Preparation of chemically competent cells

Competent cells are bacteria in a physiological state which allows DNA uptake from the surrounding medium. By manipulation with rubidium chloride, this state can be experimentally induced.

A fresh LB-medium culture was inoculated with 500 µl of a 3 ml overnight bacterial culture and was incubated on a shaker at 37 ºC until an optical density OD_{600} of 0.6-0.7 was reached. This is equivalent to 3.3 Macfarlane (McF). The bacteria were pelleted at 3000 rpm for 8 mins at 4 ºC. The supernatant was discarded, the pellet was resuspended in 33 ml RF1 buffer and incubated on ice for 15 mins.
Again, the suspension was centrifuged at 3000 rpm for 8 mins at 4 ºC, followed by resuspension of the pellet in 5 ml RF2 buffer and 15 mins incubation on ice. After repeated resuspension, the solution was shock frozen at 100 µl aliquots and stored at -80 °C.

2.2.1.10 Heat shock transformation

Each single aliquot of chemically competent bacteria was supplemented with 1-5 µl of ligation mix from an overnight ligation reaction and incubated on ice for 30 mins. Subsequently, the mixture was heat shocked at 42 ºC for 2 mins, immediately cooled on ice for 5 mins before being transferred into 1 ml of pre-warmed LB-Medium and shaken for 45-60 mins at 37 ºC. Subsequently, the bacteria were pelleted in a centrifuge at 1500 rpm. The pellet was resuspended in 100 µl LB-Medium, 10 µl and 90 µl were plated seperately on selection agar plates supplemented with antibiotics and incubated overnight at 37 ºC.

2.2.1.11 Plasmid cracking

E.coli cells can be disrupted in alkaline solution containing detergent.
This method is a quick test for the presence of the desired insert in plasmids, as long as the insert is at least approximately 1000 bp in size. The lysate of one large colony contains a sufficient amount of DNA to be detected in a single lane of an agarose gel.
Cell material from a single colony was transferred into a reaction tube containing 25 µl cracking mix and 25 µl 10 mM EDTA. The mixture was incubated for 5 mins at 70 ºC, followed by cooling on ice. Vigorous vortexing may help to break genomic DNA into fragments of 20-30 kb in size, although this step is not mandatory. The samples were completed by adding 3 µl of loading dye, consisting of 50 % (v/v) 4 M KCl and normal 6x gel loading dye. This mixture was incubated on ice for 5 mins followed by a centrifugation step for 5 mins at 15000 rpm at room temperature. The supernatant was loaded onto a 1 % agarose gel. The empty vector served as control standard instead of a DNA ladder.

2.2.1.12 Plasmid purification

Plasmids needed to be extracted from bacterial cultures and were purified by using the NucleoSpin® Plasmid-kit according to the manufacturer's instructions. Plasmid DNA was eluted in destilled sterile water.

Since a BAC clone is an extremely large plasmid, it needs specialised buffer conditions and a size-adapted silica membrane. Hence, the NucleoBond® BAC 100 Kit was used to purify the *Irf1* BAC clone since it provided optimal purification conditions for BAC clones.

2.2.1.13 Preparation of embryonic fibroblasts

All embryonic stem (ES) cell culture work was conducted in the lab of Prof. Dr. Klaus Pfeffer (Düsseldorf) with the support of Karin Buchholz.

CD1 mice were killed on day 14 after a positive plug test. Uteri were extracted and placed in feeder medium. Embryos were dissected and yolk bags, liver and heads were removed. Embryos were sieved, washed and plated on 10 cm culture dishes. When the cells had grown to confluence, they were split on 15 cm culture dishes until the cells had again grown to confluence, approximately after 4-5 days. These murine embryonic fibroblasts (MEFs) were subsequently used as feeder cells in murine stem cell cultures. A confluent 15 cm dish was treated with 5 ml Trypsin/EDTA for 10 mins. The reaction was stopped with 5 ml feeder medium. 50 µl/well of this suspension were used for 96 well plates, 100 µl/well for 48 well plates. Larger culture plates were used when containing a confluent MEF layer to feed the stem cells.

2.2.1.14 Freezing of embryonic fibroblasts

When the embryonic fibroblasts had grown to confluence on 15 cm culture dishes, the plates were washed with PBS and trypsinised with 5 ml trypsin/EDTA for 10 mins. Resuspended cells were washed once in feeder medium and frozen in freezing medium

constisting of 80 % feeder medium, 10 % FCS and 10 % DMSO. One 15 cm dish was sufficient for 4 freezing tubes.

2.2.1.15 Electroporation of embryonic stem cells with the target vector

To prepare the E14.1 embryonic stem cells for targeting, feeder cells (MEFs) had to be thawed 3 days before starting the actual stem cell culture. MEFs were always treated with mitomycin C (10 µg/ml), to prevent proliferation. Each feeder cell culture needed to be renewed after 3-4 days in order to have sufficient numbers of MEFs, since they survive only approximately 4 days.

Embryonic stem cells were cultured for 10 days in ES- cell medium together with the MEFs before electroporation. The ES cells were treated every day by either exchange of media or splitting onto larger MEF containing plates, starting with dishes 5 cm in diameter via 10 cm dishes up to 15 cm in diameter; $5x10^7$ stem cells (usually pooled from 3 15 cm-dishes, resuspended in 7 ml PBS without Mg_2Cl) are sufficient for 10 vials for parallel electroporation with the linearised target vector.

The ES cells were electroporated in 800 µl PBS without salts at 125 µF and 500 V. After a rest of 10 mins on ice, the contents of each cuvette was distributed onto two MEF containing 10 cm dishes for 2 days before selection with Geneticin (G418, 200 µg/ml) was begun. G418 is an antibiotic that can be neutralised by a neomycin resistance gene which was included in the target vector. Cells that survived in G418 supplemented media were successfully transfected. To select against non-homologous recombination events, the nucleosid analogue Gancyclovir® (2 mg/mg) was added to the culture. In the case of random insertion of the thymidine kinase from the target vector into the ES cell DNA as an indication of non-homologous recombination, the cells die. The use of the guanine analogue Gancyclovir® leads to early termination of DNA replication, which results in nonsense gene products.

Positive (="targeted") clones were picked on day 10 and verified by Southern blotting.

2.2.1.16 Screening of ES cell clones after gene targeting

The medium in the 10 cm culture dishes was exchanged by PBS.
Single clones were picked from culture dishes with a microtitre pipette in a maximum volume of 20 µl and placed in individual wells of a 96 well plate. Only colonies round in shape without any obvious signs of differentiation were chosen. Cells of a single colony were separated by treatment with trypsin/EDTA at 37 °C for 3 mins only, since ES cells react extremely sensitive and may die due to prolonged incubation times. The reaction was stopped by adding stem cell medium and fresh feeder cells which were provided for each well.
After 24 hrs the culture medium was replaced by fresh stem cell medium, on day two the clones of one 96 well plate were split onto two 48 well plates for freezing on day 4 (see 2.2.1.15), whereas the 96 well plate was kept cultured until day 7 for screening by Southern blotting. On day 4, the 96 well plate was split again onto another 96 well plate to generate a duplicate as backup for a second Southern blot screening. Every day the culture medium was exchanged, and on day 3 fresh MEFs were provided. For DNA preparation directly from the 96 well plates, refer to the Southern blotting section 2.2.1.19.

2.2.1.17 Freezing of ES cells

First, the culture plates were washed with PBS. To generate a single cell suspension, stem cell colonies were separated by trypsin/EDTA for 3 mins at 37°C, very thouroughly resuspended with stem cell medium to stop the reaction and completed with the same amount of freezing medium. The whole plate was sealed, wrapped with Saran wrap and cooled down at -20 °C for 30 mins and finally stored at -80 °C.

2.2.1.18 CRE-MEDIATED DELETION OF THE NEOMYCIN GENE CASSETTE IN EMBRYONIC STEM CELLS

It is necessary to delete the neomycin gene cassette (*neo*) from the genome to generate a targeted site which is only modified by loxP sites. Therefore, the ES cell clones, in which integration of the targeting vector had been verified by Southern blotting, were transiently transfected by electroporation with 30 µg of the pICre plasmid which encodes for the Cre-recombinase. It expresses low amounts of Cre-recombinase and does not replicate. Finally, it vanishes from the culture by dilution due to cell expansion. The efficiency of *neo* deletion is approximately 1-10 %.
Positively targeted clones (as depicted in *Fig. 2.2*) were thawed and expanded in stem cell medium with MEFs until they formed a confluent layer on a 10 cm culture dish. Approximately $5x10^6$ cells were electroporated as described in paragraph 2.2.1.15. Two days after electroporation, the cells were plated at low densities ($5x10^3$, $1x10^4$, $5x10^4$ cells) on 10 cm dishes with feeder cells. The stem cells were fed daily with fresh medium. On day 8 after electroporation, clones were picked and cultured as before for the targeting and subsequently for screening as described in paragraph 2.2.1.16.

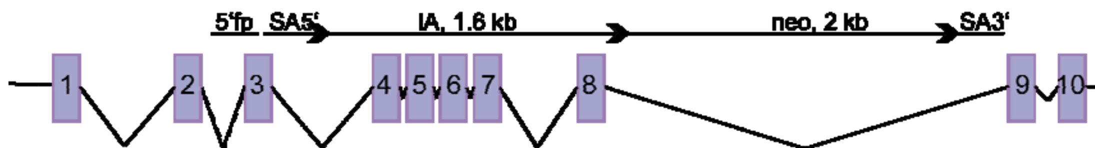

***Figure 2.2: Scheme of the successfully targeted Irf1 gene** with indicated exons 1-10. Arrows indicate the inserted loxP sites. IA=inner arm, SA5'=short arm 5' of IA, SA3'=short arm 3' of IA, neo=neomycin resistance gene cassette.*

2.2.1.19 SOUTHERN BLOTTING

Southern blotting is a combined method for the detection of specific DNA sequences within a DNA sample first separated by agarose gel electrophoresis and transfered to a positively charged nylon membrane, subsequently followed by probe hybridisation. The

gene-specific probe can be labelled either radioactively or with a fluorescent dye. Here, radioactive labelling was used, as it provides higher sensitivity. This method was first introduced by the British biologist Erwin Southern (SOUTHERN 1975).

PREPARATION OF GENOMIC DNA FROM ES CELL CULTURES

96 well culture dishes with grown ES cells were washed with PBS and cell lysis buffer was applied, freshly supplemented with 0.4 mg/ml proteinase K. After overnight incubation in a wet chamber at 56 °C, the DNA was precipitated by 100 % ethanol, followed by two washing steps with 70 % ethanol.

For verification of single positive clones, cells were thawed from 48 well plates, recultured and subsequently split on 6 well plates via 24 well and 12 well plates. The confluent cell culture on the final 5 cm dish was harvested and distributed equally into a precooled cryotube (see section 2.2.1.17 for freezing details) and into a 15 ml tube for lysis. After precipitating with 100 % ethanol, the DNA could be wound up with a pipette tip or glass stick and rinsed by dipping into 70 % ethanol. The DNA was then resolved in distilled water or 10/1-Tris/EDTA buffer.

DIGESTION OF GENOMIC DNA

For largescale screening of ES cell clones, digestion of DNA was performed within the 96 well dishes, and for single clones in Eppendorf reaction tubes. Overnight digestions were performed by mixing end-concentrations of 1 x enzyme-specific reaction buffer with 1 mM DTT, 100 µg/ml BSA, 50 µg RNaseA and 20-30 units of enzyme in distilled water. 20-30 units of enzymes were used per digestion in a 96 well dish or per 15 µg DNA.

For targeting confirmation, digestion with AvrII/XmaJI was used to detect insertion of the neomycin gene cassette into the target site of the *Irf1* gene, since AvrII restriction sites flank this region.

As the neomycin gene introduces a new SpeI restriction site into this genomic region, a digestion with SpeI was performed for additional confirmation.

For neomycin deletion, an additional digestion was performed with BamHI. This restriction site was used to insert SA3' into the target vector and is not located in the wildtype genome. This way it was possible to distinguish between a wildtype allele and a floxed allele after *neo* deletion.

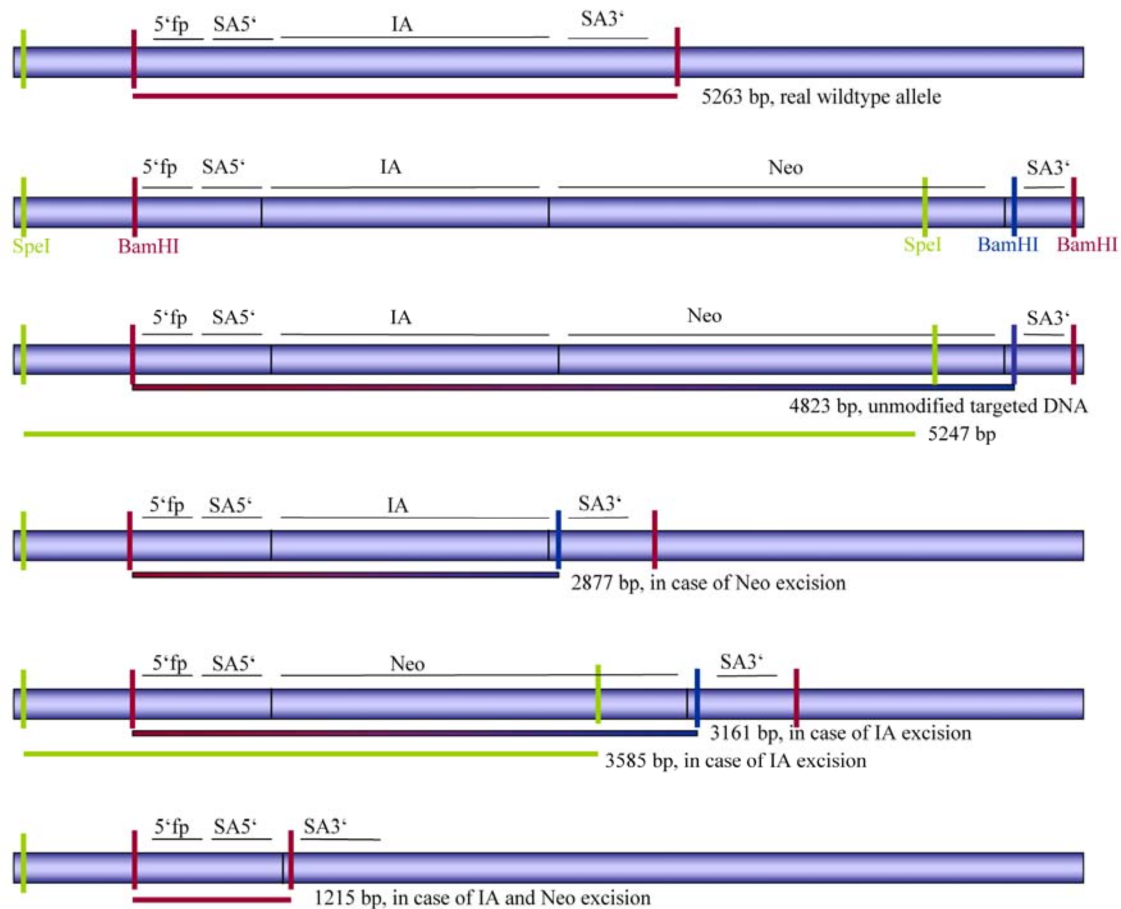

Figure 2.3: Overview of all possible events on the Irf1 gene by intracellular Cre expression *and how to distinguish between these fragments. Restriction analysis with BamHI will result in a fragment of 5263 bp for the wildtype allele, 1215 bp, if both IA and Neo were removed, 2877 bp in case of neo deletion in the floxed allele and 4823 bp in case of inactive Cre in the targeted Irf1 sequence. Additional information will be given by restriction analysis with SpeI to prove neo existence with a fragment of 5247 bp.*

Southern Blotting

Digested DNA was separated by agarose gel electrophoresis with 0.6-0.8 % agarose at 25-30 V overnight. Afterwards, the gel was incubated on a shaker in 0.25 N HCl, followed by denaturating buffer and neutralising buffer, each step for 20-30 mins. Capillary blotting was performed with 10x SSC overnight *(Fig. 2.4).* For fixation of the DNA on the membrane, the membrane was baked for a minimum of 30 mins at 80 °C.

Labelling and Hybridisation

The probe was labelled with ^{32}P by random priming with ^{32}P-α-dCTP nucleotides according to the manufacturer`s instructions. In brief, 20 ng of 5' probe DNA were mixed with reaction buffer, random primers, 50 µCi ^{32}P and Klenow-fragment and incubated for 30 mins at 37 °C, followed by 120 mins incubation at room temperature. Unbound nucleotides were removed by using sephadex columns according to the manufacturer's instructions. For better efficiency, samples were loaded twice. The labelling efficiency was measured in a scintillation counter by Cherenkov radiation measurement. This is electromagnetic radiation emitted when a charged particle such as an electron passes through an insulator at a constant speed which is greater than the speed of light in that medium. The charged particle polarises the surrounding molecules, which then rapidly return to their ground state by emitting light. This is the characteristic blue glow of nuclear reactors in atomic power plants is due to Cherenkov radiation, named after the Russian scientist Pavel Alekseyevich Cherenkov, who received the Nobel Prize in 1958 and was the first to characterise this phenomenon (Cherenkov 1934).

For hybridisation, the large blot membranes were rolled into hybridisation meshes to ensure open access of the labeled probe to the whole membrane. Hybridisation was performed overnight in hybridisation flasks at 42 °C, followed by several washing steps:

2x washing buffer 1 at RT

1x 30 mins at hybridisation temperature 42 °C with washing buffer 1

1x 30 mins at 50 °C with washing buffer 1

followed by several washing steps each for 30 mins with washing buffer 2 and increasing temperature up to a maximum of 65 °C, until the radiation intensity was below 80 Bequerel (Bq).

For autoradiography, each membrane was wrapped with Saran foil before being placed on a film. The film was exposed in an exposure cassette with two intensifying screens overnight or for 3 days at -80 °C to enhance the signal.

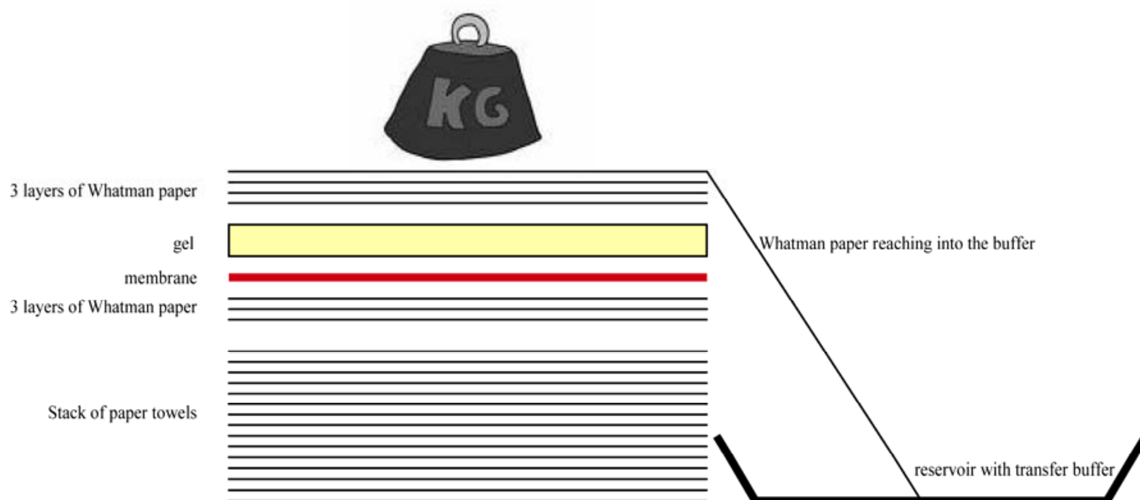

Figure 2.4: Scheme of a Southern blot set up*. The DNA is transferrd from the gel onto the membrane by capillary forces that run through the pile.*

2.2.1.20 BLASTOCYST INJECTION

This work was conducted by Nicole Küpper, technician in Prof. Dr. Klaus Pfeffer's lab, our cooperation partner in Düsseldorf.

12 C57BL/6 females were treated with 5 U pregnant mare's serum (PMS) and two days later with 5 U human chorionic gonadotropin (hCG) to induce super-ovulation before being mated to C57BL/6 males.

Uteri of pregnant C57BL/6 females (day 3.5 post "conception") were extracted and placed into a 35 mm culture dish containing M2 medium. Horns of uteri were flushed with M2 medium using a 0.25 gauge needle to blow out the blastocysts.

Vasectomised males are needed to produce pseudopregnant females which will receive the blastocytes by uterine transfer. After mating with a sterile male, the female reproductive tract becomes receptive for transferred embryos, while the own unfertilised eggs degenerate.

Blastocyst transfer was performed according to standard procedures (HOGAN *ET AL.* 1994). In brief, for blastoycst transfer, the lower back of the anesthesised recipient mouse was shaved and desinfected. The transfer pipette was loaded with blastocysts. The uterus was layed open during surgery and blastocysts were gently transferred. In general the transfer results in 75 % of embryos coming to term.

2.2.2 PROTEIN-BIOCHEMICAL METHODS

2.2.2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a biochemical method of detecting and quantifying antibodies or antigens in a liquid sample which bind to a surface-bound capture antibody. In a second step an antigen-specific detection antibody is bound onto the antigen that receives either another amplifying antibody which carries an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AKP) or is linked to the enzyme itself. The substrate for the enzyme causes a colour change which is detectable by photometric analysis.
Cell culture supernatants and brochoalveolar lavage (BAL) fluid were tested for secreted cytokines such as IL-22, IL-17 and IFN-γ. For IL-17 the DuoSet ELISA®-kit was used whereas for IL-22 Ready-SET-Go! ®-kit was applied.
For quantification of secreted OVA-specific immunoglobulins of the IgG1 and IgG2a (BALB/c mice) IgG2c (C57BL/6 mice) subclasses ELISAs from serum samples were performed. The consecutive procedure, when a non-commercial protocol was applied,

was the following. 96-well flat-bottom plates were coated overnight in a wet chamber at 4 ˚C with a suitable capture antibody or with 20 µg/ml OVA (Grade VI) in 0.1 M $NaHCO_3$ pH 8.3. The next day, the plates were washed and blocked with PBS/1% BSA for 2 hrs at room temperature. After several washings, 40 µl of the samples were loaded and incubated overnight at 4 ˚C in a wet chamber. Cell culture supernatants and BAL were used pure or diluted up to 1:20. For the detection of OVA-specific antibodies sample dilutions of 1:1000 for IgG1 and 1:10 for IgE were used. Secondary antibodies were applied after further washing. Depending on the enzyme coupled to the detection antibody, either p-nitrophenyl phosphate (PNPP) or blue horseradish peroxidase (POD) substrate solution was added. Finally, the absorption at 405 nm or 450 nm was measured in a photospectrometer.

2.2.2.2 Surface staining

Expression of surface molecules was verified by specific fluorescence-labelled antibody staining. Cultured cells were washed in PBS/1% FCS and incubated for 15 mins in the fridge with the desired antibody. Thereafter, unbound antibodies were removed by a following washing step with PBS/1% FCS.

2.2.2.3 Intracellular cytokine staining (ICS)

After surface staining the cells were fixed in 2 % paraformaldehyde for 15 mins at room temperature. A washing step with PBS/1% FCS and one with saponin-buffer was followed by adding of 100 µl of staining solution containing cytokine-specific antibodies in saponin-buffer. The resuspended cells were incubated for 15 mins at 4° C in the dark. Surplus antibodies were washed off with saponin-buffer and subsequently PBS/1% FCS.

Afterwards the cell samples were analysed by flow cytometry using a FACS Calibur machine.

2.2.3 ASTHMA EXPERIMENTS

2.2.3.1 MOUSE MODEL FOR ADJUVANT-FREE ACUTE ALLERGIC ASTHMA

The induction of allergic asthma is based on an inflammatory reaction against an otherwise harmless allergen. For this model 3 subcutaneous injections (s.c.) of 10 mg ovalbumin Grade VI (OVA) in PBS without any adjuvant, such as alum, were applied for sensitisation (day 0, 7 and 14), followed by 20 mins 1 % OVA Grade V aerosol treatment on days 26, 27, 28 *(Fig 2.5).* The inhalation of the allergen causes the infiltration of eosinophils and lymphocytes into the lung tissue. If lung function was measured on day 29, then the mice were killed on day 30 by a final dose of narcosis solution, otherwise the mice were killed on day 29.
In the herein described experiments *Irf1*$^{-/-}$ and control mice (n=8 mice per group) were sensitised as indicated.
These experiments were performed in collaboration with the SFB/TR22 and the group of Prof. Renz in Marburg.

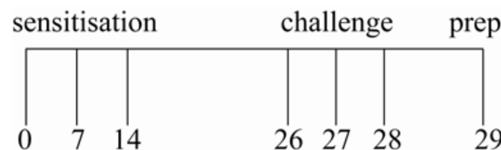

Figure 2.5: Sensitisation protocol for acute murine experimental asthma induction*. Numbers indicate days of treatment.*

2.2.3.2 PREPARATION OF SERUM

Mice were killed with a final dose of 5x narcosis-solution. Blood was drawn from axillary blood vessels for serum antibody analysis. After clotting (45 mins at RT) serum was separated by centrifugation at 4000 rpm for adjacent quantification of immunoglobulins by ELISA as described above. Meanwhile, serum samples were stored at -20 °C.

2.2.3.3 Bronchoalveolar lavage (BAL)

The thorax was opened and lungs of OVA-sensitised or PBS control mice were first rinsed with 1 ml PBS through the trachea with a 20 G needle, followed by additional 4 ml PBS. The BAL contains cells from proximal and distal airways. The cell content of the first millilitre was pelleted and the supernatant was stored at 20 °C for later analysis. The cells were again resuspended in 1 ml of PBS, counted in a Casy® cell counter and further analysed for morphological quantification of invaded cell populations per millilitre using a cytospin. Afterwards, both the residual of the first millilitre and the 4 ml were jointed and the remaining cells were restimulated with PMA and ionomycin (refer to section 2.2.4.7) in the presence of BrefeldinA for cytokine expression analysis by intracellular cytokine staining. Since the amount of cells in the BAL fluid differ between the individual animals, equal numbers of cells, usually $1x10^5$-$2x10^5$ cells/ well on 96 well plates, were restimulated.

2.2.3.4 Cytospin analysis

The BAL cells were pelleted, supernatants were stored for other analyses, and the cells were again resuspended in PBS/1% BSA. 50 µl of this suspension was diluted in 150 µl PBS/1 % BSA. By using a cytospin at 700 rpm for 5 mins the cells were directly adhered onto an object slide. For differential cell type counts the cells needed to be stained. To do this, the DiffQuick® method was used. After air drying of the object slides, the cells were fixed and dipped into eosin staining solution for 1 min, followed by staining with heamatoxyllin solution. Surplus staining solutions were rinsed away with water. At 400 x magnification the cells were microscopally distinguished and populations were counted.

2.2.3.5 Lung embedding

After the BAL, the left lung was disconnected from the rest of the organ close to the bifurcation of the lungs by using a thin thread. This part of the lung was frozen in liquid nitrogen and stored for the possibility of later RNA analysis.
The right lung was then filled and thereby fixed with 6 % paraformaldehyde (PFA). Fixation was completed by storing the lung in 6 % PFA for 24 hrs. Subsequently, the lungs were embedded in 2 % agarose and according to „Systematic Randomised Uniform Sampling“ (SURS) cut into 6 mm thick pieces and embedded in paraffin wax. The embedding of the lungs was conducted in Marburg in the Institute of Pathology of the University Hospital Gießen and Marburg.

2.2.3.6 Preparation of lung slides

The paraffin-embedded lungs were cut into thin slices of 3 µm thickness by using a microtome and placed onto object slides. This work was done in the group of Prof Renz, our cooperation partners within the SFB/TR22.

2.2.3.7 Haematoxylin-Eosin-Staining (HE)-staining

For examination of actual pathophysiological changes and lung damage induced by allergic asthma, the lung slices were stained with haematoxylin and eosin. First the samples were de-paraffinated in xylol (2x 10 mins) before rehydration in descending alcoholic concentrations (each step 2x 5 mins in 100 %-, 5 mins 96 %,- and 5 mins 70 %- ethanol). After rinsing with water, the slides were stained in haematoxylin for 3 mins. A resting step in water for 5 mins secured persistence of the blue staining in the tissue. Next, the samples were stained in eosin for 2 mins and again rinsed with water. Afterwards the tissue was dehydrated in ascending alcoholic solutions (1 min in 96 %-, 2x 3 mins in 100 % ethanol followed by 2x 10 mins xylol). Histomount® was used to cover the samples with cover slides.

2.2.3.8 Periodic-acid Schiff staining, PAS staining

Mucus production by goblet cells can be taken as a characteristic of the asthmatic lung. Because goblet cells are only rarely found in healthy tissue. The periodic-acid Schiff staining (PAS) makes these cells visible. Deparaffinated and rehydrated tissue slices (see section for HE staining) were incubated in 0.5 % periodic acid for 10 mins and washed in water for another 3 mins. The samples were transferred into Schiff's reagent for 15 mins, followed by 1 min in haematoxylin. After a final rinsing step the samples were dehydrated in alcoholic solutions (1 min each 70 %, 96 % and 100 % ethanol). After final 15 mins incubation in xylol, the object slides were covered.

2.2.3.9 Ova-specific restimulation of Lymphocytes

Trigger-dependent cytokine production gives an additional impression on the severity of asthma in line with all other measured parameters. For this purpose spleen and lung lymphnodes from all animals of an experiment were meshed for single cell suspension. The lymphocytes were antigen-specifically restimulated at $1x10^6$/ml with 10 µg/ml OVA in complete medium for 72 hrs on 24 well culture plates. Since the spleen reflects only the systemic immune condition of the organism which does not necessarily comply with the local immune response in the lung, the option of investigating splenocytes was abandoned in later studies.

2.2.4 γδ-T cell Experiments

IRF4 deficient precursor $CD4^+$ T cells were found to be unable to differentiate into Th17 cells (Brüstle *et al.* 2007). The missing transcription factor was responsible for the induction of IL-17 cytokine production. However, γδ T cells are partially considered as cells of the innate immune system and the regulation of their IL-17 production may function differently.

2.2.4.1 Extraction of lymphocytes from liver

Mouse livers were prepared, meshed through a sieve with pores of 70 µm in size, and again filtered through a second sieve. The pelleted homogenate was resuspended in 5 ml 40 % Percoll solution (diluted in RPMI media) and layered on 3 ml 70 % Percoll solution. The gradient centrifugation was performed at 1600 rpm for 20 mins without break and at room temperature. The thin interface contained the lymphocyte population, which was harvested by careful pipetting and washed twice in PBS/1%FCS. Erythrocytes were lysed by incubation with erythrocyte lysis buffer for 3 mins. The procedure was stopped by adding PBS/1 %FCS and cell preparation was finalised by an adjacent washing step.

2.2.4.2 Preparation of lyphocytes from lymphoid organs

Thymi, spleens, peripheral and mesenterial lymphnodes were dissected from sacrificed mice and sieved through a nylon filter of 70 µm pore size. Erythrocyte lysis followed as described before.

2.2.4.3 Preparation of whole lung homogenate

Streptococcus pneumoniae-infected mice were killed by injection of a final dose of 5x narcosis-solution. Lungs were perfused with PBS through the heart-lung-circulation system, before lungs were removed. The organs were cut into small pieces and digested with 1 mg/ml collagenase D in PBS for 30 mins at 37 °C. Tissue pieces were sieved through nylon filters and centrifuged at 1500 rpm for 5 mins. The resulting homogenate was used for restimulation experiments.

2.2.4.4 Preparation of intraepithelial lymphocytes from gut

The faeces was removed from the gut of wildtype and *Irf4* knockout mice after lengthwise opening and the gut was cut into several pieces. After a quick rinse in PBS, the gut pieces were transferred into a 50 ml reaction tube containing 25 ml of complete medium and shaken by gentle agitation at 37 °C for 30 mins. Afterwards, the reaction tube was shaken vigorously by hand to dissolve the cells out of the epithelium. The supernatant was filtered through a sieve, while the gut pieces were shaken again in 10 ml of fresh media. Again, the supernatant was filtered through a sieve with 70 µm pore size. The liquid phase containing the desired cells was centrifuged for 5 mins at 1500 rpm. If only lymphocytes were desired, a Percoll gradient was subsequently conducted as described in 2.2.4.1.

2.2.4.5 Magnetic cell sorting - MACS® of γδ T cells

To purify γδ TCR^+ T cells out of a mixed cell sample such as lymphoid organ homogenate, the MACS® separation technique for positive purification of γδ T cells was used following the manufacturer's instructions. In brief, magnetically labelled γδ T cells in a given suspension were retained in a magnetic field and eluted after removal of the MACS column from the magnetic separator. This method allows a high purity at reasonable or even high recovery of the desired cell population.

2.2.4.6 Stimulation of γδ T cells

Gamma delta T cells were purified as described from peripheral lymphnodes and spleen and cultured in 96 well round bottom plates at $1x10^5$ cells/well in 200 µl volume of clone media in the presence of 25 ng/ml IL-23, 10 ng/ml IL-1β or both. Polyclonal T cell stimulation was induced by adding α-CD3 and α-CD28 dynabeads to the culture at a 2/3 ratio, meaning 2 beads/3 cells. These beads mimic activation by antigen presenting cells by utilising the same surface molecules bound to a three-dimensional bead similar in size to an antigen presenting cell. For the inhibition experiments the NFκB inhibitor

PS1145 was used at 5 µM. The cells were cultured for 3 days at 37 °C. If supernatants were taken after 48 hrs of culture and FACS measurement was intended, the golgi blocker monensin was added to the culture for 4 hrs at a final concentration of 2 µM (usually 1 µl/ml) to enrich the amount of intracellular cytokine and prevent its secretion.

2.2.4.7 Restimulation with PMA and Ionomycin

For *ex vivo* analysis cells or restimulation after 72 hrs of *in vitro* culture, $1x10^5$/well γδ T cells on 96 well plates were restimulated for 4 hrs with 50 ng/ml PMA and 750 ng/ml ionomycin in a total volume of 200 µl. If intracellular cytokine staining was desired, a golgi blocker was added to the cell culture, either 10 µg/ml of brefeldinA or a final concentration of 2 µM (usually 1 µl/ml) monensin, to enrich the amount of intracellular cytokine and prevent secretion.

2.2.4.8 Culture of Streprococcus pneumoniae

For mouse infection experiments, 10 µl of a *Streptococcus (Strep.) pneumoniae* frozen aliquot were plated on Columbia agar plates and incubated at 37 °C overnight. The next day, 20 ml of a 1:1 mixture of LB-medium and FCS were inoculated with 1-3 colonies and placed on a shaker at 37 °C with intermediate rotation until an optical density of 1 McF was reached, taking approximately 8 hours, since *Strep. pneumoniae* is a relatively slow growing bacterium. This density equals 250-500 million cfu/ml.

For infection of mice, the bacteria were pelleted by centrifugation at 4000 rpm for 5 mins and washed 3 times with PBS. After resuspension in 1 ml PBS, the bacterial cells were ready for infection.

2.2.4.9 Infection of mice with *Streptococcus pneumoniae*

C57BL/6 mice and *Irf4*$^{-/-}$ mice were anesthesised with 1x narcosis solution. Their neck slightly overstretched and 20 µl of bacteria-in-PBS-solution was applied intranasally. Mice were killed on day 3 after infection, lungs were removed prepared and analysed as described.

2.2.4.10 Cytokine measurement by quantitative Realtime PCR (qRT-PCR)

This PCR technique is used to amplify and simultanously quantify a specific target DNA by including a fluorescent probe or dye in the reaction.
For analysis of the transcriptional regulation of the IL-23 production by γδ-T cells, a TaqMan®-PCR for *Tbx21, rorc and rora* was performed on an ABI Prism 7500 Sequence Detection System. For RNA sample preparation, cell pellets were washed twice in PBS and resuspendend in 200 µl of PBS, followed by RNA isolation via the „RNeasy Micro Kit®" according to the manufacturer's instructions. The resulting RNA content was measured by photometric analysis; cDNA synthesis was performed using the "cDNA Maxima First Strand Systhesis Kit for RT-qPCR®" with oligo-dT-primers.
The following PCR conditions were used for the detection of *tbet* and *rorc/a* expression: 1x reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 250 nM (*Tbx21*) or 500 nM (*rorc/a*) each primer, SybrGreen 1:2000 dilution, 0.025 U of HotGoldstar polymerase. The temperature profile was the following: 50 °C 2 mins, 95 °C 10 mins, 40 repeats 95 °C 15 seconds 60 °C 1 min. Results were normalised to *hprt* as housekeeping gene by the ΔΔct method as specified by Applied Biosystems.

2.2.4.11 Luciferase Assay

293-mTLR9-luc cell are previously described HEK293 cells, which are stably transfected with a NκFB luciferase reporter plasmid and mTLR9 10^4 cells/well were seeded in 96 well microtiter plates in 100 µl (DMEM high glucose, 7,5% FCS). After 8 hrs, the cells were stimulated with CPGs (1 µM CpG-ODN 1668, TCCATGACGTTCCTGATGCT; kindly provided by Prof. Dr. S. Bauer; Bauer S. *et al.*

2001) and the NFκB inhibitor PS 1145 (Sigma; 5 nM) or medium alone in a total volume of 200 µl overnight. The next day, the supernatants were discarded, the cells were lysed in 50 µl lysis buffer (Dual-Luciferase Reporter Assay System, Promega) and an equal volume of substrate solution (D-luciferin 470 µM, CoenzymeA 279 µM, DTT 33.3 mM, ATP 530 µM, $(MgCO_3)4Mg(OH)2x5H_2O$ 1.07 mM, $MgSO_4$ 2.67 mM, Tricin 20 mM, EDTA 0.1 mM, pH7.8, kindly provided by Prof. Dr. Stefan Bauer) was added for luciferase detection measurement. This assay was used as a positive control for the NFκB inhibitor PS 1145.

3. RESULTS

3.1 GENERATION OF A CONDITIONAL KNOCKOUT MOUSE LINE FOR *IRF1*

Irf1 is expressed in a variety of different cell types. Its functions as tumor suppressor and also as key transcription factor for T helper cell differentiation towards a Th1 immune response are well known in the literature. In order to reveal a cell–specific function of IRF1 and a potential role of a certain IRF1 producing cell type in the course of a given disease or malfunction, the availability of a conditional IRF1 knockout system would be of great benefit. The main task of this thesis project was to generate a conditional knockout mouse for *Irf1*.

Amplification of *Irf1* PCR fragments for conditional gene targeting.

With the indicated primers (see 2.1.10) and the availability of the *Irf1*-BAC clone as template DNA, all PCR protocols for the necessary *Irf1* gene fragments were able to be established *(Fig. 3.1.1, the sizes refer to the genomic lengths)*. The primers comprised also the necessary sythetic restriction sites for the respective subsequent cloning step as indicated in the following paragraph.

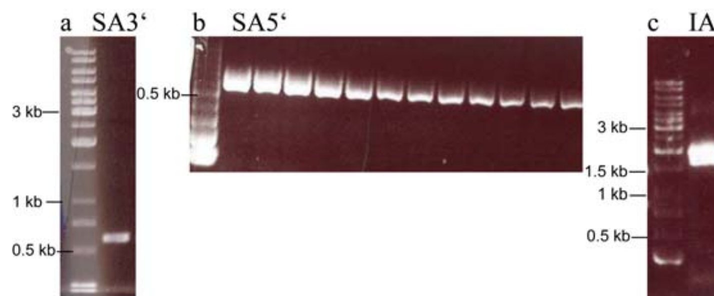

Figure 3.1.1: ***Successful generation of the PCR products for targeting****. a) PCR fragment of the 3' short arm (SA3'), 573 bp in size. b) temperature gradient 60-70 °C for the 5' short arm (SA5'). 62 °C was later used for amplification of the 541 bp long fragment. c) the fragment of the inner arm (IA) was 1662 bp in size and perfectly amplified.*

After PCR amplifications and purifications from the gels, all fragments were consecutively cloned into the target vector pEasyFlox. First, SA3' was inserted into the vector by digestion of vector and PCR product with ClaI and BamHI, followed by ligation. SA5' was added to the resulting intermediate vector, using XhoI and HindIII digestion, thereby also inserting a NotI restriction site into the vector next to the XhoI restriction site for later linearisation. Finally, the inner arm (IA) was cloned to complete the target vector using only SalI. It was of great importance to keep a determined order of insertion for the PCR fragments, since the inner arm sequence came with an internal restriction site for XhoI which would have disturbed cloning of SA5'. Hence, IA had to be cloned last.

To screen for clones which had incorporated the SA3`and subsequently SA5`, plasmid preparations of approximately 50 colonies each of SA3' and SA5' (for each insertion attempt) were prepared and the plasmids were tested by digestion with the enzymes used in the correlative cloning step. Positive clones showed a band of the size of the PCR product in addition to the linearised vector.

The insertion of an insert of at least 1000 bp in size could be detected by plasmid cracking; Usually, all three bands (supercoiled, linearised and nicked) run more slowly in the gel than the bands of an empty vector. This way it was possible to screen litterally hundreds of potential clones for successful insertion of the inner arm. The respective vector without the desired insert was then used as standard instead of a DNA ladder. *Fig. 3.1.2* shows an example of an agarose gel of cracked bacterial DNA with a promising candidate for IA cloning (and therefore of the completed target vector) that was subsequently further analysed.

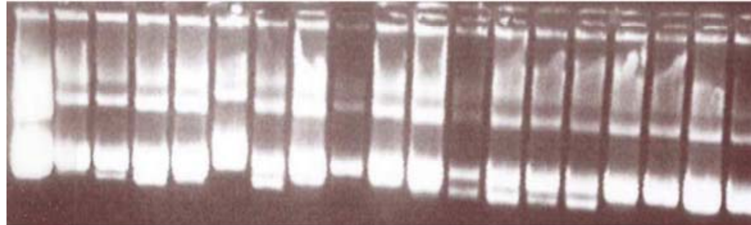

Figure 3.1.2: ***Agarose gel with DNA-containing supernatant of cracked bacterial clones:*** *check for insertion of the inner arm (IA). The left lane contains the empty vector control as standard.*

In a subsequent step, a clean plasmid preparation of the clone of interest was generated. To verify this plasmid as the completed target vector (="pEasyIRF1"), the sequences of all cloned fragments were confirmed and several control restrictions were performed *(Fig. 3.1.3)*.

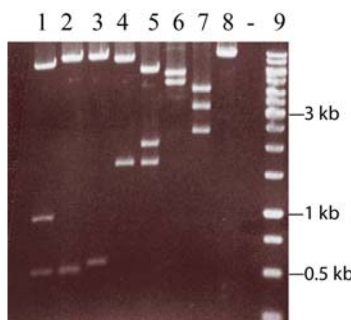

Figure 3.1.3: ***control digestions of pEasyIRF1***

1 HindIII/XhoI digestion to cut out SA5' and check for right orientation of the inner arm, resulting in 3 fragments of 8031 bp, 1024 bp and 555 bp
2 HindIII/NotI digestion to cut SA5' and check for functionality of the NotI restriction site, resulting in fragments of 9063 bp and 547 bp
3 BamHI/ClaI digestion to cut out SA3', resulting in fragments of 9023 bp and 587 bp
4 SalI digestion to cut out the inner arm, resulting in fragments of 7942 bp and 1668 bp
5 BamHI/SalI digestion to cut neo and IA, resulting in fragments of 5917 bp, 2025 bp and 1668 bp
6 EcoRI digestion for general vector control, resulting in fragments of 5185 bp and 4425 bp
7 EcoRV digestion for general vector control, resulting in fragments of 4034 bp, 3141 bp, 2331 bp and 104 bp which was hardly seen due to low amount of DNA in such a small band
8 NotI digestion for linearisation of the vector (9610 bp)
9 1kb ladder (10 8 6 5 4 3.5 3 2.5 2 1.5 1 0.75 0.5 0.25)

Insertions of all *Irf1* gene fragments were reconfirmed by cutting them out the way they had been inserted, using the restriction sites that had been added to the inserts during PCR-generation in the first place. SA5' was able to be cut out with HindIII and XhoI or NotI (resulting in fragments sized 8031 bp, 1024 bp and 555 bp or 9063 bp and 547 bp,

respectively), thereby also verifying the correct direction of IA, which comprises a XhoI restriction site asymetrically. SA3' was able to be cut out with BamHI and ClaI (resulting fragments were 9023 bp abd 587 bp) and finally IA insertion was proven by digestion with SalI (resulting fragments 1668 bp and 7942 bp). As an additional control of the target vector the neomycin cassette was removed by digestion with SalI and BamHI (lane 5), thereby again checking for the presence of IA. In addition, general vector single enzyme digestions with EcoRI or EcoRV were performed to rule out any additional insertions. The *in silico* predicted fragments of 5185 bp and 4425 bp for EcoRI digestion and 4034 bp, 3141 bp and 2331 bp forr EcoRV could be verified after digestion with these enzymes. Finally, the linearisation of pEasyIRF1 was tested by single restriction with NotI. A linearised plasmid increases the frequency of homologous recombination within the target cell after introduction of the target vector into embryonic stem cells (ES cells) by electroporation (ORR-WEAVER ET AL. 1981).

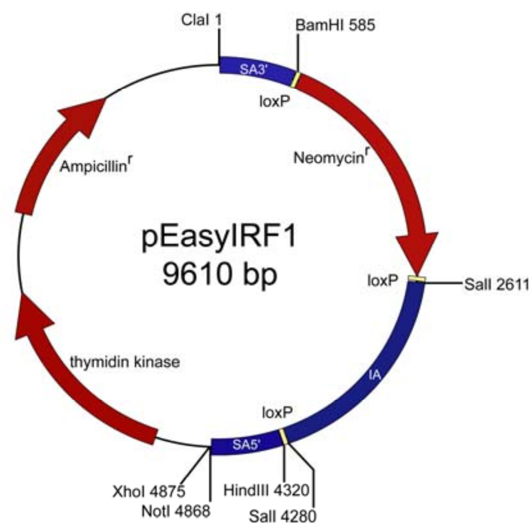

Figure 3.1.4: Plasmid map of the finalised targeting vector pEasyIRF1 for the generation of the conditional Irf1 knockout (9610 bp). *The short arm 3'(SA3') was cloned via ClaI and BamHI, adjacent to the first loxP site. Between the second and third loxP site, the inner arm (IA) was inserted via SalI. Bordering the third loxP site, the short arm 5' (SA5') was joined in by using HindIII and XhoI.*

After proven correctness by restriction analyses and sequencing, 50 µg of the vector pEasyIRF1 *(Figure 3.1.4)* were linearised with NotI and send to Düsseldorf for electroporation into E14.1 stem cells, derived from the SV129 mouse strain.

SV129 ES cells were electroporated with the linearised pEasyIRF1 and cultured as described (see 2.2.1.15). On day 10, 400 ES cell clones were picked from the Petri dishes and again cultured in 96 well plates (see 2.2.1.16). Genomic DNA was extracted after another 7 days of culturing and digested with XmaJI. The size of the included neomycin gene cassette of roughly 2000 bp enabled the easy identification of positively targeted ES cell clones by Southern blotting. If any, only one allele would have been altered by homologous recombination. Hence, 2 fragments, one for each allele would be detected in the successfully targeted ES cell clones. An 8 kb fragment of the targeted area was generated by restriction of the wildtype *Irf1* allele with AvrII (isoschizomere XmaJI); this fragment increased in size in the floxed allele due to the insertion of the neomycin gene cassette by approximately 2 kb (*Fig 3.1.5*).

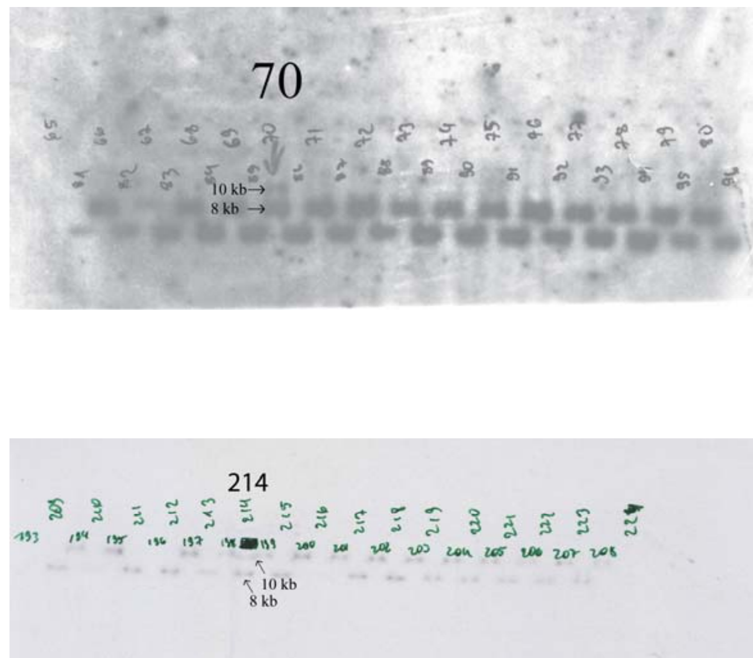

Figure 3.1.5: ***Southern blot autoradiography of large scale ES clone screening.*** *E14.1 ES cells were electroporated with pEasyIRF. 7 days after picking, the genomic DNA was digested with XmaJI and Southern blotting was performed. The clones #70 and # 214 show the 8 kb wildtype band and the 10 kb band of the targeted allele. For hybridisation, the 5'probe was used. Double combs were used in order to load as many samples as possible onto the gel, thereby producing a zigzag pattern of bands.*

The clones 70 and 214 showed a wildtype band of 8 kb as well as a fragment of 10 kb for the targeted allele, which includes the neomycin cassette.

Hence, both clones were positively targeted *(Fig. 3.1.5)* and verified separately *(Fig.3.1.6)*. For this purpose, the clones were thawed and cultured again as described to extract larger amounts of genomic DNA for reliable detection. Both clones could be validated as positively targeted ES cell clones. Untargeted ES cell DNA (E14.1) was used as control. Both clones #70 and #214 were most likely mixed clones, consisting of targeted and untargeted cells, because the wildtype band appeared to be stronger.

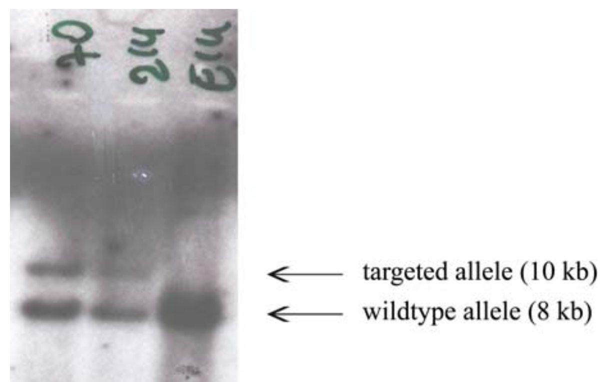

Figure 3.1.6: ***Southern blot autoradiography**. **Confirmation of the ES cell clones #70 and #214.** The clones were recultured for expansion and genomic DNA was digested with XmaJI. Untargeted E14.1 ES cell DNA was used as a wildtype control. The genomic DNA was digested with XmaJI and Southern blotting was performed. For detection, the blots were hybridised with the 5' probe.*

Although neomycin is very useful for identification of targeted ES cell clones, it can interfere with the expression of neighbouring genes, since it is placed into an intron and gene introns also include regulatory elements. Moreover, the target vetcor carries an independent promotor for expression of the neomycin gene cassette. This could lead to misdirected DNA sequence expression within the targeted cell. To generate a conditional knockout as close to wildtype as possible and to exclude any side effects of neomycin, the elimination of this resistance gene cassette was desired. Hence, the two targeted ES cell clones were transiently transfected by electroporation with the plasmid pICre that carries the gene for the Cre recombinase. During clonal expansion the low copy plasmid was "diluted out" of the cell culture. To do this, the clones #70 and #214

were thawed, cultured as for the electroporation with target vector, and finally electroporated with pICre. This second electroporation also offered the posibility of subcloning at the same time, resulting in a pure clone containing only targeted cells. Subsequently, ES cell clones were picked and analysed by Southern blotting.

For verification of neomycin excision and simultaneous detection of the floxed allele with distinction from the wildtype allele, BamHI restriction was applied. Since the target vector pEasyFlox carried 3 loxP sites, 3 different events could occur *(Figure 3.1.7)* and needed to be distinguished.

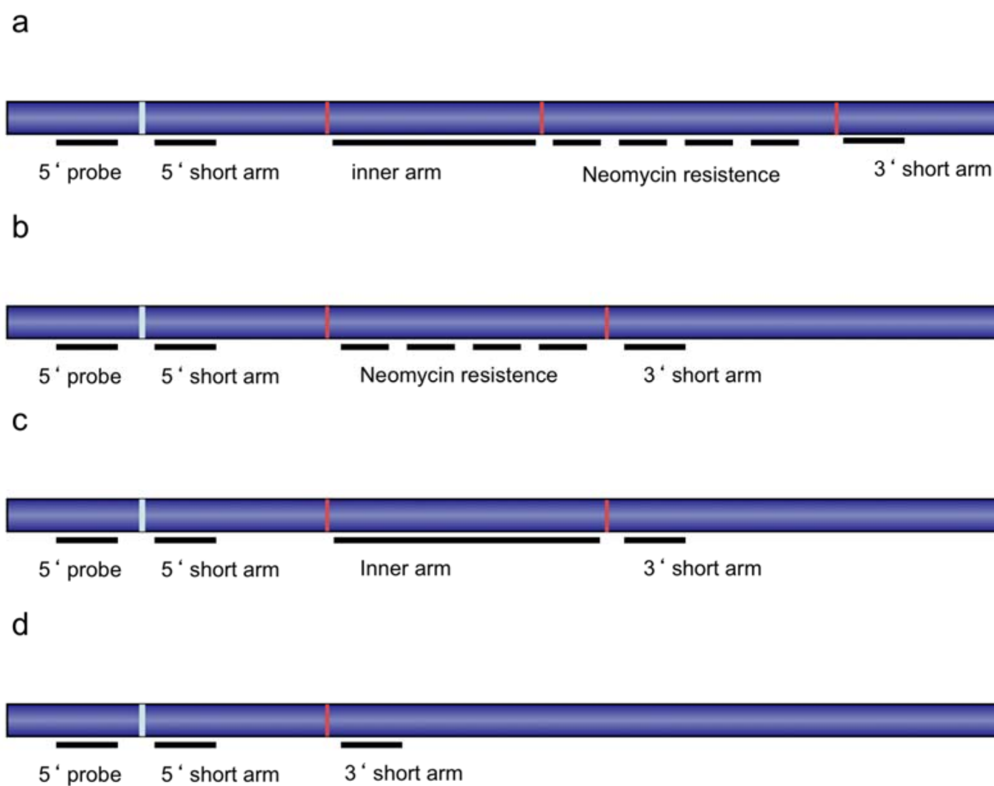

Figure 3.1.7: Scheme of events that could possibly occur after electroporation of targeted ES cell clones with pICre*. The Cre recombinase will operate all loxP sites (red) in equal measure. a) Targeted DNA with the indicated flanking short arms, inner arm and neomycin resistance cassette, and the location of the 5' probe. b) genomic situation after excision of the inner arm, c) the desired event: excision of neomycin, d) deletion of both the inner arm and the neomycin resistance gene. All events can be detected with the 5'probe, which was chosen at the border (light blue bar) of the manipuleted sequence*

In order to determine each possible event and to screen for the desired event of only neomycin gene cassette deletion, a reliable detection strategy had to be developed, which is depicted in the next graph *(Fig. 3.1.8)*. By using naturally occuring genomic restriction sites and inserted restriction sites in this specific location of the *Irf1* gene, it was possible to discriminate each event and to distinguish it.

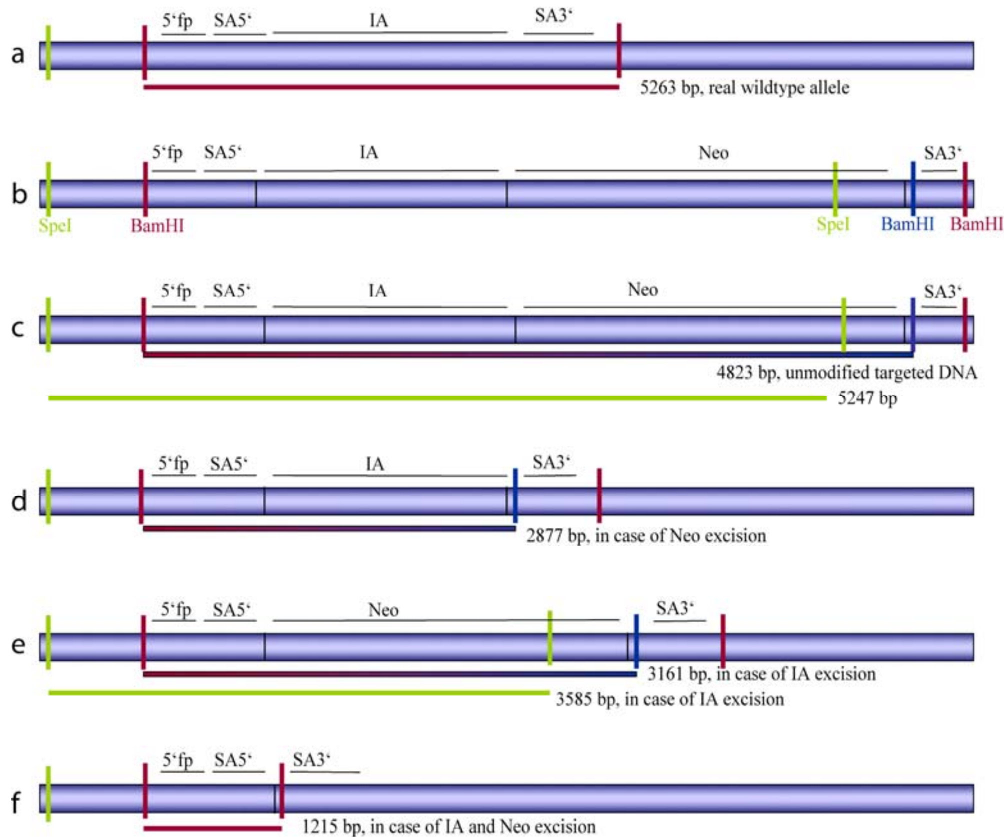

Figure 3.1.8: Restriction strategy to reveal each event and distinguish from the desired neomycin exision. *In the panel (a) the wildtype situation is depicted, in which two genomic BamHI restriction site are used to generate a fragment of 5263 bp. (b) indicates the location of the used restriction sites and loxP sites (black bars). The lower panels show the genomic situations of the targeted Irf1 locus depending on the activated loxP sites, flanking the inner arm and the neomycin gene cassette. SpeI restriction is only possible in case of presence of the neomycin gene cassette, unmodified targeted DNA can be detected by BamHI restriction (resulting fragment 4823 bp (c) and by SpeI digestion resulting in a fragment of 5247 bp. In case of neo excision BamHI restriction results in a 2877 bp fragment size (d). If IA is deleted the fragment size is 3161 bp in a BamHI restriction and 3585 bp after SpeI digestion (e) if IA and neo are deleted 1215 bp should be visible (f).*

The targeted region is located close to two BamHI restriction sites. With the cloning of the SA3' an additional BamHI restriction site was inserted. Therefore, by BamHI digestion it was possible to distinguish between the real, untouched wildtype allele and the manipulated targeted allele.

As the neomycin gene imported a SpeI restriction site into this genomic region of the *Irf1* gene that otherwise did not appear in this genomic region of *Irf1,* digestion with SpeI offered a second control for the elimination of the neomycin gene cassette.

The bands expected in a correct ES cell clone were the following: After restriction with BamHI a band of 5263 bp in size should appear for the untargeted wildtype allele, 2877 bp should derive from the targeted allele after removal of the neomycin gene cassette, and after restriction with SpeI no band should be visible. The lengths of the fragments were calculated from the targeted genomic sequence, regardless of single nucleotides which might disappear during restriction with the indicated enzyme. But this is only a question of a few nucleotides that do not interfere with the analysis in general at all. This remark is only mentioned for complete and understandable traceability.

Figure 3.1.9: Southern blot autoradiography of large scale screening *for identification of neomycin deletion. Genomic DNA was digested with BamHI and Southern blotting was performed using the 5' probe. Clones 290 and 293 (arrows) showed bands approximately 2877 bp in size and larger than 5 kb.*

The screening procedure after the electroporation with pICre was the same as for the first electroporation with pEasyIRF1. Again, large scale screening of several hundreds of single clones was performed. As potential candidates the clone numbers 160, 351, 357 and the two clones depicted in *Fig. 3.1.9,* namely 290 and 293 were identified by Southern blotting. These five ES cell clones were thawed and recultured (2.2.1.19) to gain a larger amount of DNA to repeat this digestion and finally verify the targeting. DNA of surplus ES cell clones that were not picked for a closer look was pooled and used as control DNA on the blot as well as untargeted E14.1 ES cell DNA.
The DNA was digested with BamHI to confirm the deletion of the neomycin gene cassette and with XmaJI was used as an additional control, just as in the first screening for a positively targeted clone for confirmation. After deletion of the *neo* gene cassette, only a single fragment of 8 kb in size should be visible after digestion with XmaJI and two bands (5263 bp and 2877 bp) after BamH1 digestion *(Fig. 3.1.10).*

By BamHI digestion clone numbers 160, 290 and 293 out of these five clones could be verified for *neo* deletion, but only for the clones 290 and 293 restriction with XmaJI resulted in the expected single band of 8 kb. The band of the wildtype allele and the floxed targeted allele overlayed to one thick band. This band could not be detected for clone #160. The other clones (351 and 357, as well as the DNA pool) clearly showed a fragment lower than 5 kb (4823 bp) and one larger than 5 kb (5263 bp) in size, meaning *neomycin* was not deleted (4823 bp = untouched targeted allele). The other one displayed the wildtype allele, just as 160, 290 and 293 did.

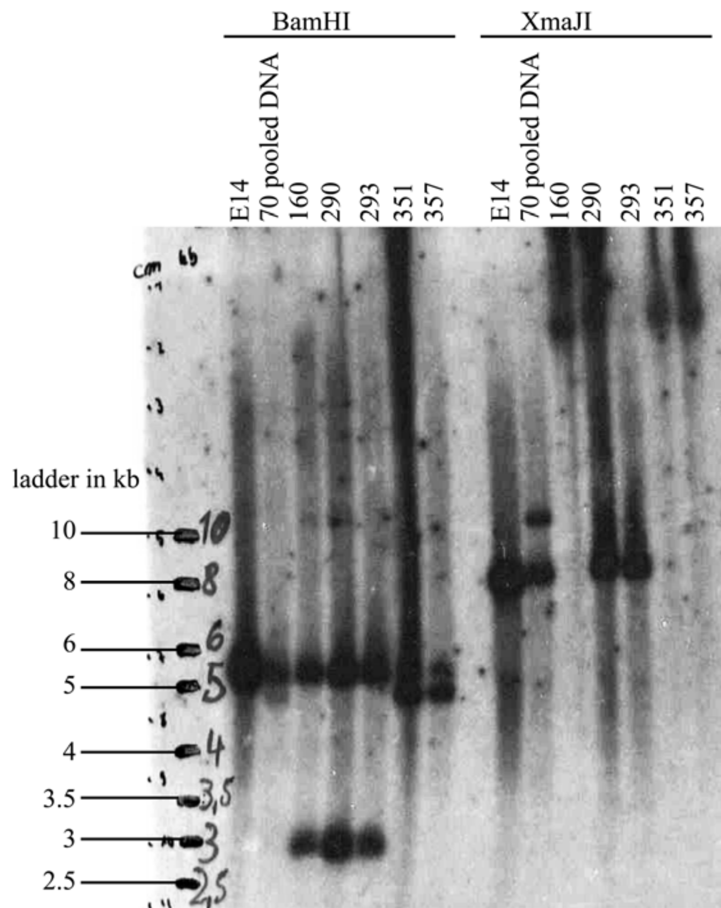

Figure 3.1.10: Southern blot autoradiography for confirmation of neomycin elimination in selected clones distinguished from the real Irf1 wildtype allele. *DNA was digested with BamHI and XmaJI restriction as control to perform Southern blotting with hybridisation of 5'probe. Clones 160, 290 and 293 show band sizes larger than 5 kb and smaller than 3 kb, indicating the desired fragment sizes of 2877 bp and 5263 bp. The XmaJI digestion revealed only one merged band for these clones at 8 kb. Clones 351 and 357 showed bands for 5263 bp (real wildtype) and 4823 bp targeted allele without Cre-recombination) after BamHI digestion.*

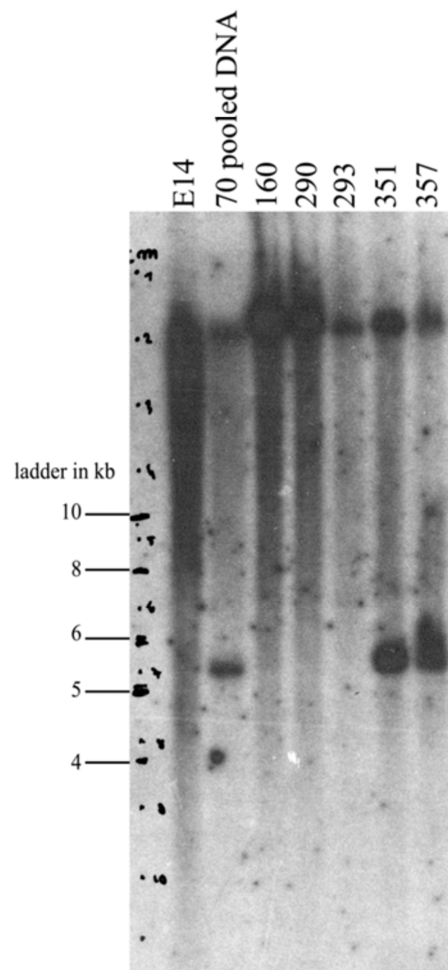

Figure 3.1.11: Southern blot autoradiography for confirmation of neomycin gene cassette elimination in selected clones. *The indicated clones were recultured and digested with SpeI to perform Southern blotting. The 5' probe was used for hybridisation.* The clones 160, 290 and 293 show no band, just like the control DNA E14.1.

As an additional control, restriction of genomic DNA with SpeI (*Fig 3.1.11*) should reveal the presence of *neomycin* gene cassette, since no other SpeI restriction site is available in this specific genomic region of *Irf1* than the one that comes with the *neomycin* resistance gene. As expected, in the clones 160, 290 and 293 no neomycin gene cassette was detectable, just as in the E14.1 ES cell DNA. In contrast, the other clones 351 and 357 also contained neomycin according to this analysis, just like the DNA pool that was used as a control. Not surprisingly, the band for the DNA pool is a bit fainter than the ones of the purified clones, since the DNA pool is most likely a mixture of all possible events induced during Cre activity.

Based on these results, clones #290 and #293 were used for blastocyst injection. Blastocysts were prepared from C57BL/6 animals. If transferred ES cells contributed to the blastocysts, the offspring should present a chimeric fur pattern. The colour of the fur of these brindled mice can appear in different shadings, from small dots of agouti within the black to pure bright cream. Clone #290 generated no offspring whereas clone #293 did do so *(scheme in Fig 3.1.12)*.

These chimeric animals were mated to C57BL/6 partners to check for germline transmission of the targeted allele. The offspring revealed the germline transmission by their agouti-brownish fur colour.

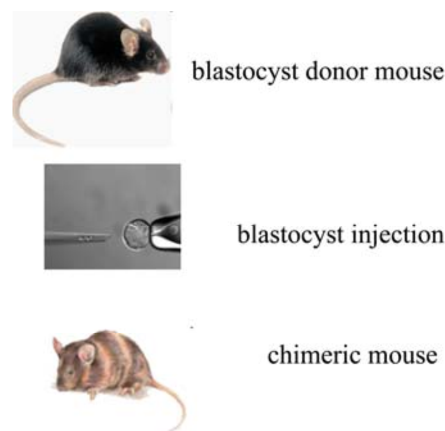

Figure 3.1.12: Scheme of blastocyst injection with targeted SV129 ES cells. *The blastocysts were taken from C57BL/6 mice. By injection of E14.1 ES cells derived from the SV129 mouse strain a chimeric mouse is generated. (modified from: http://www.unituebingen.de/Klinische_Genetik).*

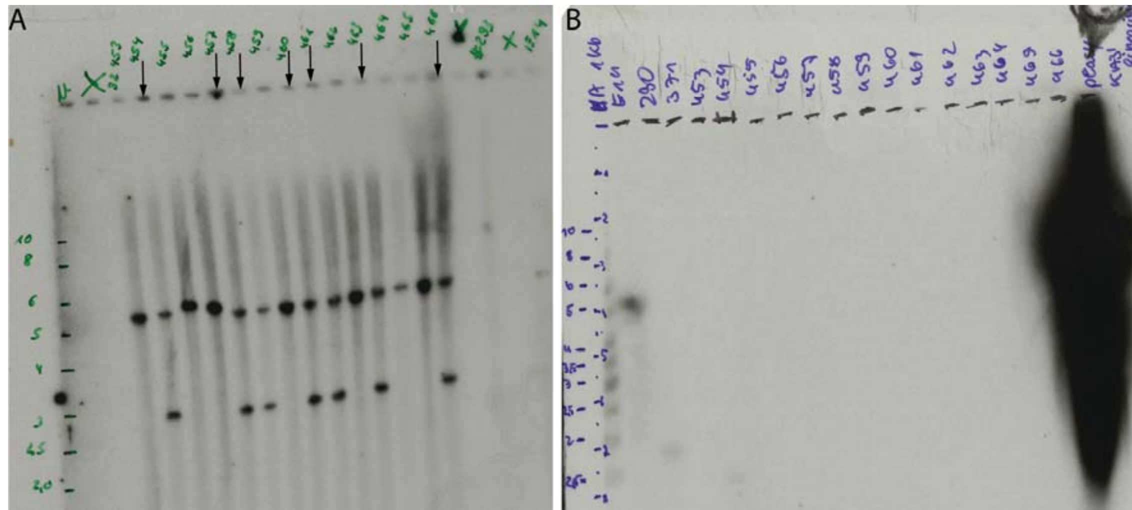

Figure 3.1.13: Southern blot autoradiography to check germline transmission in chimera offspring*.* *(A) DNA was digested with BamHI and Southern blotting was performed using the 5'probe. Arrows indicate germline transmitting animals showing the wildtype (5263 bp) and the floxed (2877 bp) allele. (B) DNA was digested with BamHI and Southern blotting was performed using the neo-probe. Only the positive control (pEasyKA3') bound the probe, none of the animals.*

In the germline transmitting animals, the presence of the floxed *Irf1* gene allele was proven by genetic analysis via Southern blotting (*Fig 3.1.13*), using the same strategy as for the ES cell screening after the deletion of the neomycin gene cassette. After BamHI digestion, the floxed allele produced a fragment size of 2877 bp whereas the wildtype allele resulted in a 5263 bp fragment. Additionaly, the presence of any *neomycin* gene cassette was excluded by hybridisation with the neo-probe, which was generated by digestion of the target vector pEasyFlox with BamHI and EcoRI, thereby cutting out a fragment of the *neomycin* resistance gene cassette. The purified DNA fragment was radioactively labelled like the 5'probe. The intermediate targeting vector pEasyKA3' served as positive control for *neo*.

The targeting for the conditional *Irf1* knockout was confirmed by this. The mice 32-454, -457, -458, -460, -461, -463 and -466 are heterozygous animals for the floxed allele of *Irf*. Due to the genetic background of the SV129-derived ES cell clone and the C57BL/6 blastocyst, the chimeric animals have a mixed background that needs to be backcrossed to a pure genetic background. Only then the mice should be mated with a given tissue-specific Cre-expressing mouse to generate a conditional knockout. A recommended

control is the generation of a complete knockout by using a Cre-deleter mouse. The offspring should express the same phenotype like the conventional *Irf1* knockout.

In order to verify the inhibition of any *Irf1* transcript, Northern blotting should be performed.

3.2 OVA MEDIATED ASTHMA INDUCTION IN *IRF1*$^{-/-}$ MICE

In parallel to the above described generation of the floxed *Irf1* mouse, the conventional knockout mouse for *Irf1* (MATSUYAMA *ET AL.* 1993) was to be investigated in the settings of asthma as a model of a Th2-mediated disease. IRF1 deficient mice show an enhanced production of IL-4 due to their genetical predisposition to develop a Th2-driven immune response. This phenomenon comes along with a stronger susceptibility for intracellular pathogens and higher IgG1 as well as IgE levels in serum (LOHOFF *ET AL.* 1997). These parameters are also seen during allergy. Hence, the hypothesis was to induce an acute phase asthma in these animals to study the presumably potentiated immune response in Irf1 knockout mice. Asthma is widely seen as a Th2-driven condition. The OVA-model offered the possibility to deduce a specific implication of an enhanced Th2 response that could be responsible for a potentially stronger allergic phenotype in these mice.
Several readout systems served as puzzle pieces to complete a comprehensive picture of the allergic reaction in the *Irf1* knockout mouse.

BAL fluid was taken from the mice and investigated for a variety of parameters. Cytokines were detected by ELISA and the cell content was morphologically identified by cytospin analysis. Surplus cells were directly restimulated for 4 hours with PMA and ionomycin in the presence of brefeldin A and stained immediately for CD4 and IL-4 to determine the percentage of IL-4-secreting T helper cells. These cells could be the main mediators of a worse asthma phenotype in IRF1 knockout mice, in case of verification of the hypothesis.
Figure 3.2.1a shows cell infiltrates in BAL fluid from wildtype and *Irf1* knockout mice, listed seperately for individual populations as determined by cytospin analysis. An OVA-dependent influx of leukocytes and eosinophils was significant in wildtype, yet surprisingly not in *Irf1* knockout mice. Migration of lymphocytes to the lung was hardly induced and did not reach statistical significance. Macrophages did not migrate treatment-dependently into the lung and neutrophils were not found at all. Thus, regarding cell migration there was no noteworthy difference found between the two mouse strains. The restimulated BAL cells were FACS analysed and showed an OVA-dependent increase for IL-4^{+}CD4^{+} cells, which turned out to be slightly, but

significantly increased in IRF1 deficient animals. Cytokine levels in BAL fluid (*Fig 3.2.1b*) were measured by ELISA and showed that IFN-γ production was reduced in OVA-treated groups, but yet to very low amounts. This effect was highly significant in wildtype mice, yet not in the knockout mice. This decline in IFN-γ levels upon OVA-stimulation was expected, because an allergic response is Th2-driven and IFN-γ is a cytokine mainly produced during a Th1 response. However, the absolute amounts of this cytokine were detected at the detection limit of the ELISA method. The same holds true for the IL-10 and the IL-6 measurement. No relevant amounts of these cytokines were produced. The secretion of IL-5 was not altered by OVA, nor were differences found in between the groups. In summary, an OVA-induced reaction is supported by the findings of an OVA-dependent influx of leucocytes and eosinophils in wildtype mice, the decrease of IFN-γ levels and the detection of IL-4^+CD4^+ cells. However, against our hypothesis no striking difference in the reaction to OVA was noted in *Irf1* knockout versus wildtype mice.

An allergic pathology is measured by determination of many factors. For a complete picture, several parameters in addition to BAL analyses were collected (*Fig. 3.2.2*). To assure that any effect that might be seen was not due to the size of an individual mouse, the weight of all mice in this experiment was measured at the end before they were killed.

The next parameter to be tested were OVA specific antibodies in the sera. In mice, IgG1 is Th2-dependently produced and can account for the severity of a Th2-driven disease. In the experiment depicted in *Fig. 3.2.2*, IgG1 was OVA-dependently produced with high significances in wildtype and even more in the *Irf1* knockout mice. In contrast, IgE levels did not shift in response to OVA, because comparable amounts were found in all groups. IgG2a indicates the part of counterregulation and is normally secreted in a Th1 response. Here, IgG2c was measured, because the C57BL/6 mouse strain carries a defective gene for IgG2a. Instead, they produce IgG2c (JOUVIN-MARCHE ET AL. 1989 AND MARTIN *ET AL*. 1997). No IgG2c was detectable in any of the serum samples, as expected from an allergic phenotype.

Based on these results, a Th1 immune response was not detectable in this experiment. Some parameters that indicate a Th2 response such as IgG1 or the IL-4 production by CD4^+ cells again confirm that the immunisation with OVA was in principle successful, but the expected stronger reaction expected in *Irf1* knockout mice was absent.

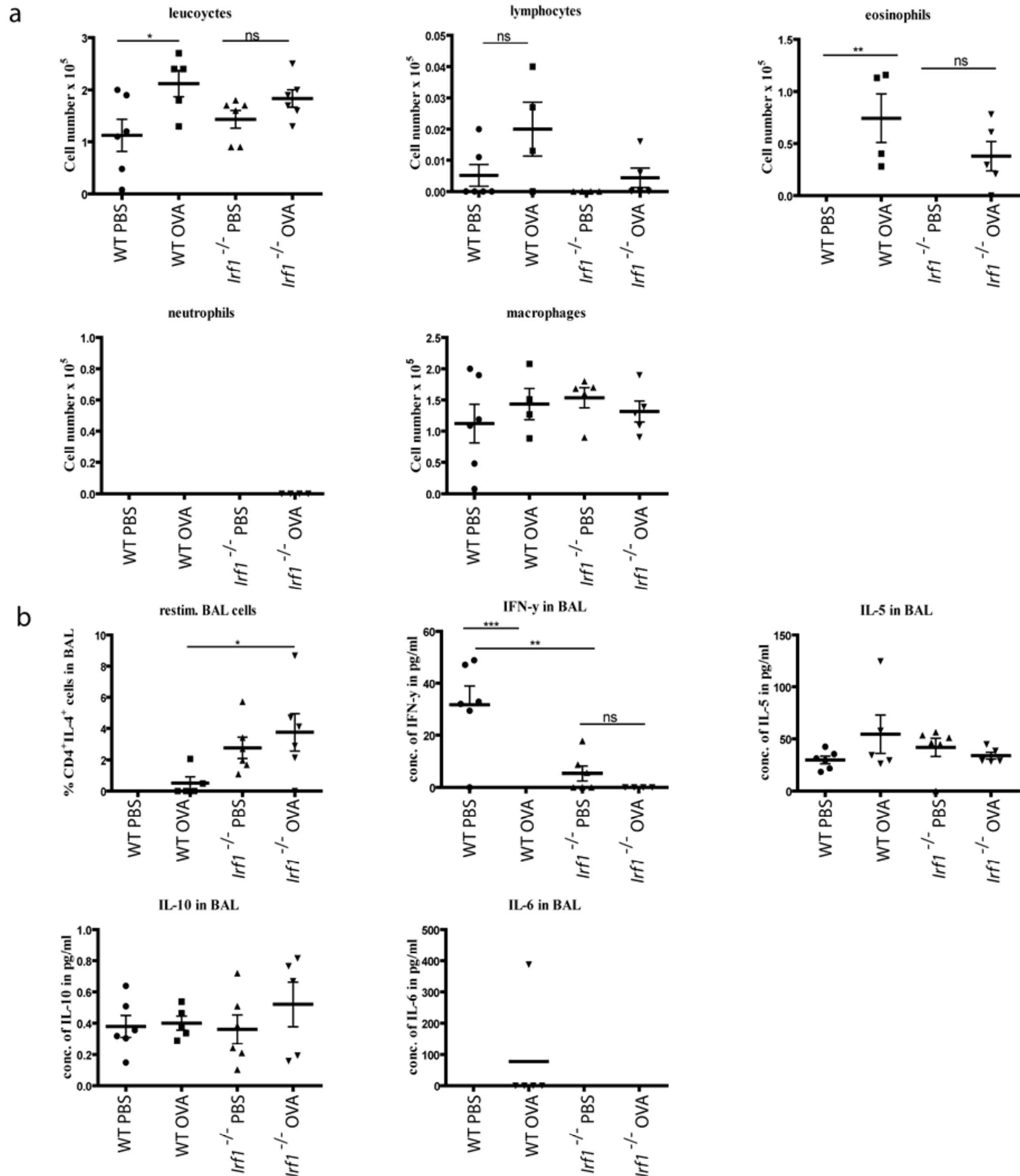

Figure 3.2.1: ***Acute asthma model.*** *Animals were treated with OVA or PBS s.c. on days 0, 7 and 14 and were challenged on days 26, 27 and 28. Analyses refer to day 29, when the mice where killed and samples were taken. Bronchoalveolar lavage fluid (BAL) analyses in wildtype (WT) and Irf1$^{-/-}$ animals for cell infiltrates (a) and cytokine levels as measured by ELISA (b) as well as FACS analysis of restimulated BAL CD4+ cells. Statistical analysis was done by one way ANOVA and Tukey's multiple comparison test. Standard deviation (SD) and significances (*p=<0.005; ** p=<0.001; *** p<0.0001; ns= not significant), if applicable are shown. Outliers were determined using Grubb's test and excluded from the analysis.*

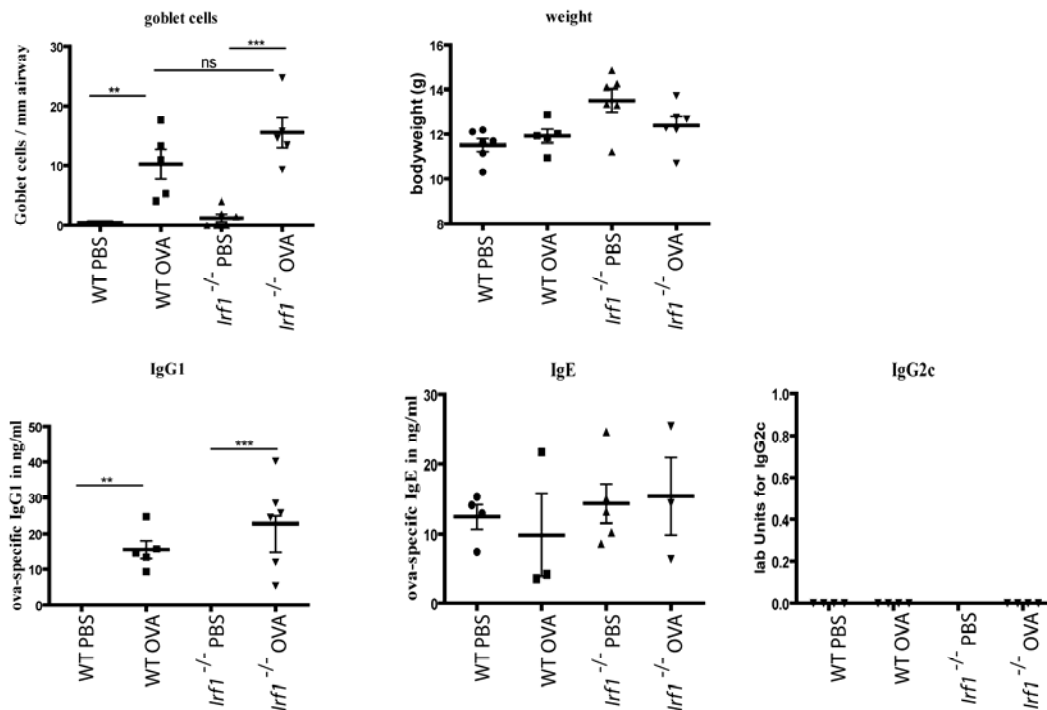

Figure 3.2.2: ***Acute asthma model****. Histological quantification of goblet cell counts, comparison of animal weight at the end of the experimental phase and ELISA results of OVA-specific serum immunoglobulin levels. Statistical analysis was done by one way ANOVA and Tukey's multiple comparison test. SD and significances (* p<0.005; ** p<0.001; *** p <0.0001; ns= not significant), if applicable are shown. Outliers were determined using Grubb's test and excluded from the analysis. .*

The predominant sign of allergic asthma is the lung inflammation accompanied with excessive mucus production. Therefore, histological analysis of the whole lung (proximal and distal region were statistically equally assessed) was the final and in fact the most important readout.

Lung slices were stained for mucus production by PAS staining and goblet cells were microscopally quantified in relation to the airway surface using the CellF software. The amount of goblet cells is a direct hint of the disease severity in the lung. The graph (*Fig. 3.2.2*) displays an increase in the goblet cell number per mm of airway in an OVA-dependent manner in both mouse strains, with slightly more significance in the *Irf1* knockout mouse. However, there was again no obvious difference seen between the two mouse strains.

The optical impression of the lung pathology in wildtype and *Irf1* knockout animals confirms the counted goblet cell analysis. As visualised by PAS staining, PBS control

mice had healthy lungs *(Figure 3.2.3 a and c)* whereas the asthmatic mice showed mucus-producing goblet cells within the lung epithelium *(Figure 3.2.3 b and d).*

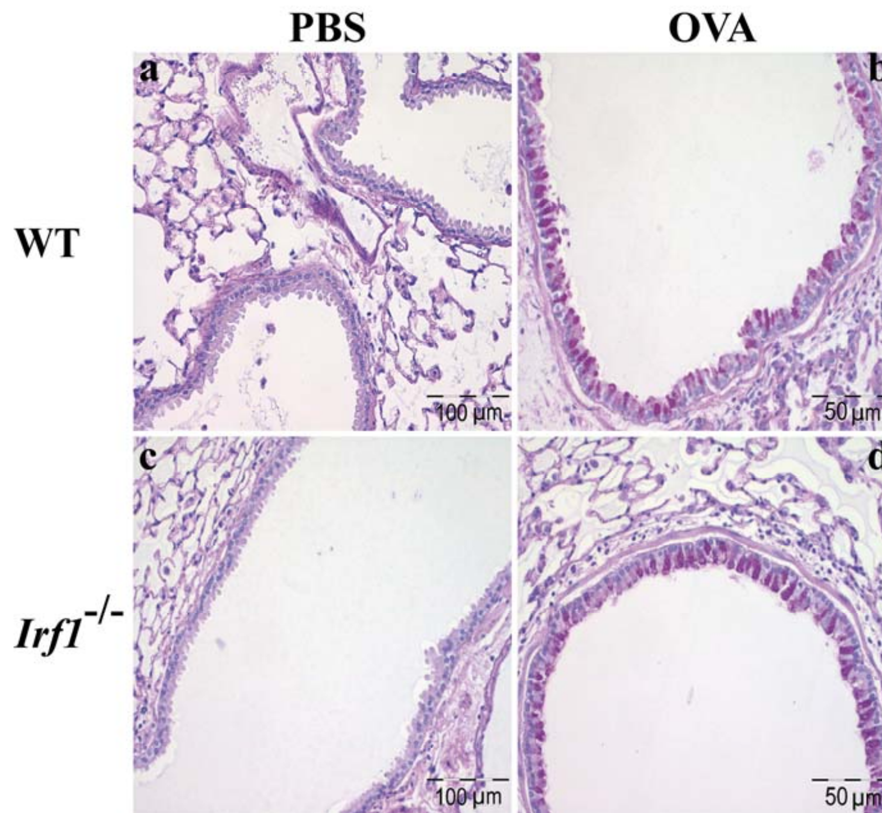

Figure 3.2.3: Histological slices of the lung. *Periodic acid Schiff (PAS)-stained for mucus producing goblet cells in PBS-(a and c, pictures taken at 40 x magnification) or OVA-treated (b and d, pictures taken at 20 x magnification) wildtype (WT) and Irf1$^{-/-}$ mice. Mucus-producing goblet cells are silhouetted in pink against the purple background of lung epithelium and tissue.*

Altogether, all analyses demonstrate clearly the same asthma phenotype in both mouse strains, wildtype C57BL/6 and IRF1 deficient mice.

No significant alterations compared to wildtype mice were notable in the *Irf1$^{-/-}$* mouse, despite the known overall "Th2 character" of this mouse strain in other models, such as infection with intracellular pathogens.

3.3 IL-17 PRODUCTION BY *IRF4*$^{-/-}$ γδ T CELLS

In a final and independent part of this thesis, my task was to analyse the capacity of T cells other than conventional αβ T cell receptor (TCR) expressing $CD4^+$ and $CD8^+$, to secrete IL-17. Here, the molecule that we were interested in was IRF4 rather than IRF1, because (as specified in the introduction) *Irf4*$^{-/-}$ γδ T cells are able to produce IL-17 to the same extent as wildtype γδ T cells *ex vivo* and also after *in vitro* culture in response to IL-23. This fact was very surprising, because *Irf4*$^{-/-}$ $CD4^+$ T cells have a severe defect in the production of IL-17. The third part of this thesis work was dedicated to closer characterise this phenomenon.

First, IL-17 production in IRF4 deficient γδ T cells was confirmed in different organs from wildtype and knockout mice. Peripheral and mesenterial lymphnodes, thymi, spleens and livers were excised, single cell suspensions were prepared and *ex vivo* restimulated for 4 hrs with PMA/ionomycin in the presence of the golgi blocker monensin. IRF4 deficient γδ T cells were capable to produce IL-17 in comparable amounts as wildtype cells in all tested organs. In contrast, $CD4^+$ IRF4 deficient T cells produced hardly any detectable amounts of IL-17 in this direct *ex vivo* analysis *(Fig 3.3.1)*.

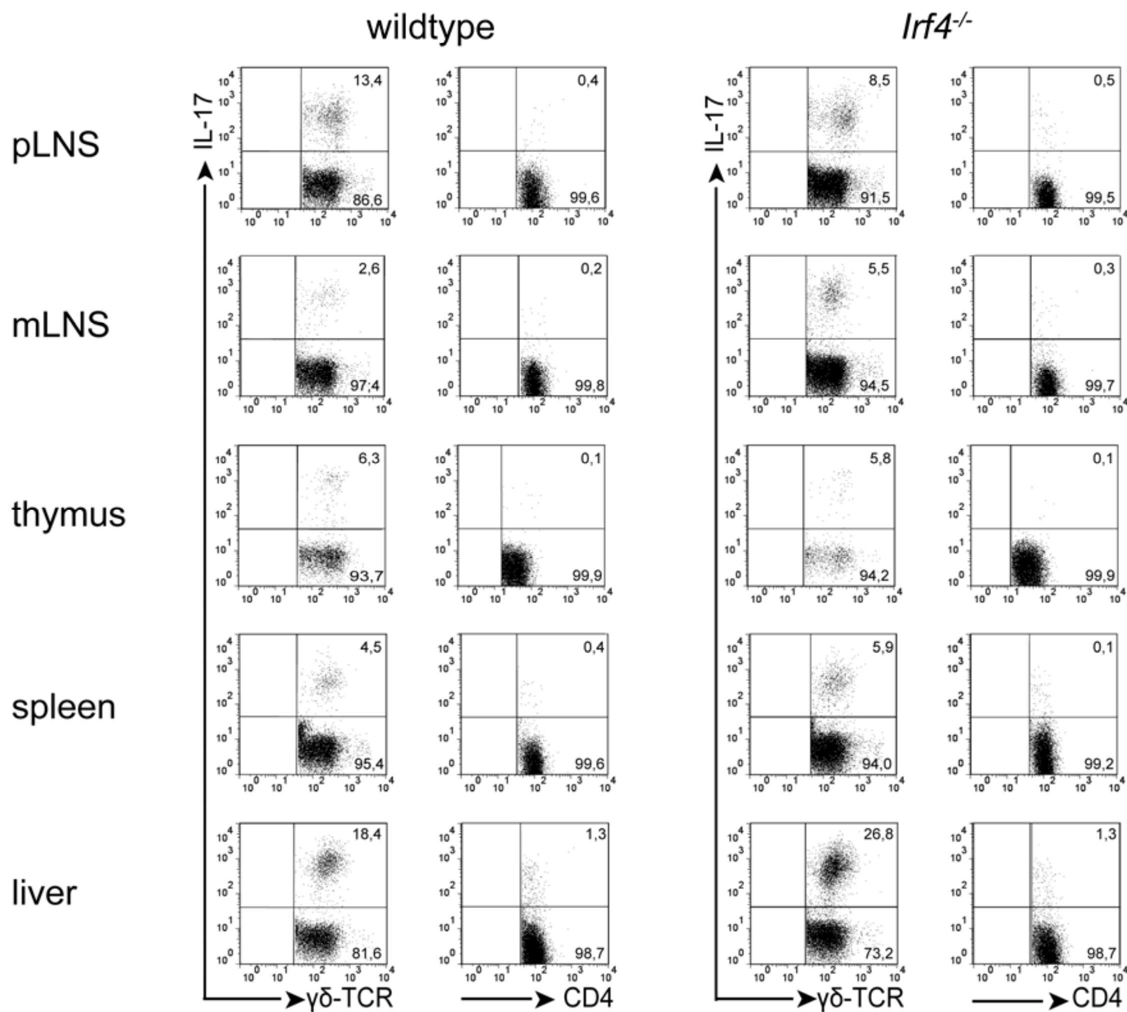

Figure 3.3.1: FACS staining for IL-17 and IFN-γ production in ex vivo PMA/ionomycin-stimulated cells from different organ homogenates of wildtype and Irf4 knockout mice. *Cells were stained for expression of the γδ TCR, CD4 or intracellular IL-17. Plots show cells gated on either CD4$^+$ T cells or γδ T cells. γδ T cells from IRF4 deficient animals produced IL-17. Irf4$^{-/-}$= deficient in interferon regulatory factor 4.*

Obviously, the defective IL-17 production in CD4$^+$T cells of the IRF4 deficient mouse is not applicable for γδ T cells. This phenomenon is also not a question of organ-specific distribution of this cell type.

The fact that γδ T cells can produce IL-17 upon stimulation with IL-23 is well accepted in the recent literature (GRAY *ET AL.* 2011, PETERMANN *ET AL.* 2010, NESS-SCHWICKERATH AND MORITA 2010). Since IRF4 deficient γδ T cells showed IL-17 secretion in many different organs *per se*, the question raised, whether they would also make IL-17 upon IL-23 and

IL-1β stimulation. These two cytokines have been shown to induce IL-17 production in γδ T cells upon *in vitro* culture for several days. So, γδ T cells were purified from spleens and peripheral lymphnodes by using the MACS® technology and were activated with αCD3/αCD28 activation beads and stimulated with IL-23 and IL-1β or each of these cytokines alone for 48 or 96 hrs. For intracellular cytokine staining, monensin was added after 44 hrs for 4 hrs. For testing of soluble IL-17, IL-22 and IFN-γ levels in culture, supernatants were quantified by ELISA at both time points. The results clearly show that IRF4 deficient γδ T cells were able to produce IL-17 upon individual stimulation with IL-23 or IL-1β as nicely as the wildtype cells did. This effect was even potentiated by stimulation with both cytokines together. The ELISA data supported these findings, though the *Irf4* knockout mice produced overall even higher amounts of cytokines.

The analyses revealed also IL-22 expression in a similar profile as for IL-17 production. Again the IRF4 deficient γδ T cells produced higher amounts of cytokine compared to wildtype.

However, IFN-γ secretion seemed to be affected rather by IL-1β than by IL-23. The simultaneous stimulation with both cytokines, IL-1β and IL-23 resulted in a further increased IFN-γ secretion in the IRF4 knockout cells.

The extended culture period of 96 hrs did not reveal any other results. The induction profile of cytokines upon stimulation with IL-23 and/or IL-1β was comparable.

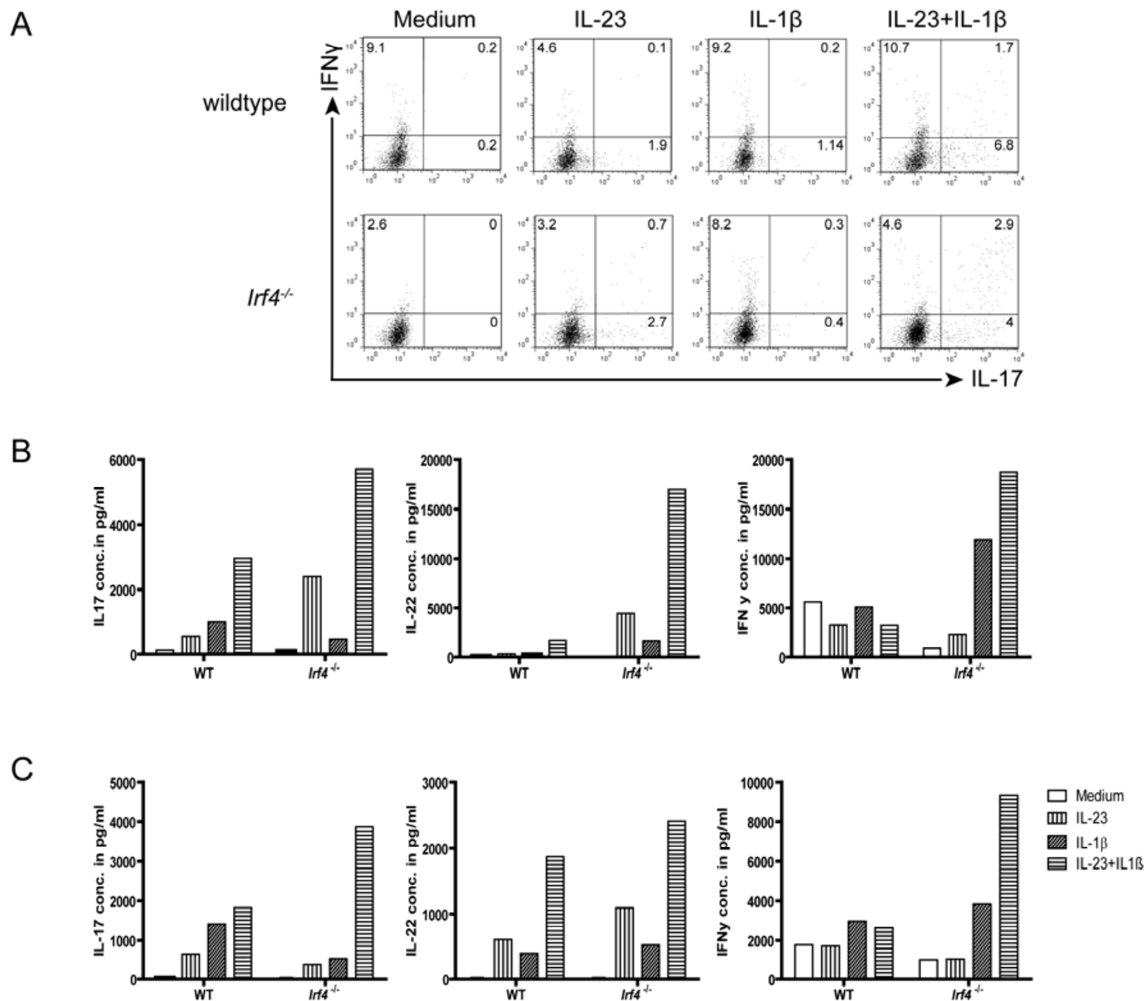

Figure 3.3.2. Cytokine production by γδ T cells upon stimulation with IL-23 and/or IL-1β. *MACS®-purified γδ T cells from C57BL/6 or IRF4 deficient mice were stimulated with αCD3/αCD28 beads (2 beads/3 cells) and the indicated cytokines (IL-23 25 ng/ml; IL-1β 10 ng/ml) and were cultured for 48 h or 96 hrs. (A) FACS analysis for intracellular IFN-γ and IL-17 was performed on day 2 after adding monensin to the culture during the final 4 hrs of culturing. Graphs show gated cells positively stained for γδ TCR. Numbers indicate percentages of positive cells located in the respective quadrant (B) culture supernatants were taken after 48 hrs and analysed by ELISA for IL-17, IL-22 and IFN-γ production. (C) culture supernatans were taken after 96 hrs and analysed by ELISA for IL-17, IL-22 and IFN-γ production. Three experiments were performed with similar outcome. WT = wildtype, Irf4*

In addition to a normal environment, the presence and *in vivo* expansion of γδ T cells were to be assessed in the settings of an inflammation, thereby identifying possible misfunctional properties of IRF4 deficient γδ T cells compared to wildtype. To do this, *Irf4*$^{-/-}$ mice and heterozygous littermates were infected with *Streptococcus pneumoniae* intranasally. Three days post infection, the lungs were harvested and cell homogenates were prepared. After 4 hours of restimulation with PMA/ionomycin in the presence of monensin, the cells were FACS stained for γδTCR, CD4 and intracellular IFN-γ as well as IL-17 (*Fig. 3.3.3*).

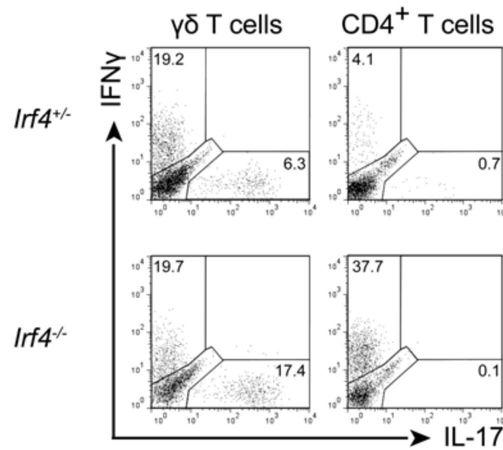

Figure 3.3.3: FACS analysis of lung homogenates after infection with Strep. pneumoniae: *IRF4 deficient and heterozygous mice were infected with 20000 cfu Strep. pneumoniae. Lungs were harvested on day 3 post infection, homogenised and the cells were stained for γδ TCR, IFNγ and IL-17. The FACS plots depict gated γδ T cells. Numbers indicate percentages of positive cells located in the respective quadrant. Two experiments were performed with similar outcome.*

The investigation of the infected lungs demonstrated the presence of γδ T cells in both genetic backgrounds and these cells produced similar amounts of IFN-γ. The IRF4 deficient mice showed an even higher production of IL-17 compared to control mice. Also CD4^{+} T cell were present, but IL-17 was not produced at all by them, while only the IRF4 deficient CD4^{+} cells were highly positive for IFN-γ. Interestingly, there were not any double positive cells detectable at all.

Since *Irf4*$^{-/-}$ CD4^{+} T cells cannot differentiate into IL-17 producing Th17 cells, the question raised, how the signaling pathway was altered in γδ T cells. To investigate, if the IL-17 production by γδ Tcells in the *Irf4*$^{-/-}$ mouse correlates with the expression of a transcription factor that is known to be in general involved in the expression of IL-17 and/or IL-23 signaling, realtime PCR analysis for *rora, rorc* and *Tbx21* was perfromed. For this purpose, γδTCR^{+} cells were enriched by staining of the γδ TCR, followed by labelling with anti-fluorchrome beads and a purification step via MACS® colums. The resulting suspension was then sorted with an Aria III FACS sorting machine for high purity. From this fraction, RNA and cDNA was prepared and used for TaqMan PCR.

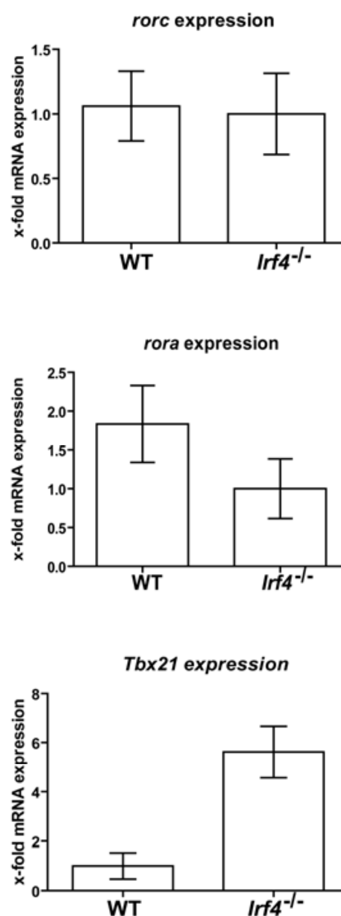

Figure 3.3.4: TaqMan® analysis of transcription factors in γδ T cells. *RNA was prepared from ex vivo sorted γδ T cells and tested for expression of rora, rorc and Tbx21. The result show respresentative data out of 3 independent experiments.*

By quantitative realtime PCR all 3 transcription factors could be detetcted in *ex vivo* analysed γδ T cells. *Rora* and *rorc* were expressed to similar extent in wildtype and IRF4 deficient γδ Tcells. In contrast, T-bet-expression was slightly up-regulated in the *Irf4* knockout cells. These findings are not surprising, since RORγt and RORα are involved in the development of Th17 cells while inhibition of T-bet by IL-23 is a pre-condition for IL-22 (MUS *ET AL.* 2010). Hence, transcription factors known to be of relevance in IL-23 singaling and/or important for Th17 differentiation are also expressed in IRF4 deficient γδ Tcells.

Another attractive candidate for mediating IL-17 production in γδ T cells is NFκB whose signaling molecules RelA and RelB have recently been demonstrated to determine IL-17 expression by γδ T cells *ex vivo* (POWOLNY-BUDNICKA *ET AL.* 2011). Hence, the strong suspicion was obvious that the NFκB pathway was also activated in IL-17 expression by IRF4 deficient γδ T cells during stimulation with IL-23 and/or IL-1β. In order to assess this question, the NFκB inhibitor PS1145 was used in the further experiments to test the respective pathway. This inhibitor blocks both, the RelA (classical) and the canonical activation of NFκB via BAFF which involves RelB (YEMELYANOV *ET AL.* 2006 AND TAI *ET AL.* 2006).

The proper function of the inhibitor was tested in a luciferase assay, in which the NFκB–reporter cell line 293-mTLR9-luc was used. The luciferase production upon CpG stimulation was almost totally blocked by administration of PS1145 *(Fig 3.3.5 A).* However, the cytokine expression by γδ T cells, either in wildtype or in IRF4 deficient cells was not affected. *(Fig. 3.3.5 B*). Therefore, in cytokine stimulated γδ T cells, NFκB is neglectable for IL-17 production.

STAT molecules have been reported to be important mediators in IL-23 signaling (PARHAM *ET AL.* 2002). However, the induction of IL-17 by IL-23 in STAT4 deficient γδ T cells was also comparable between wildtype *(Fig. 3.3.5 C)* and STAT4 deficient γδ T cells (as shown in *Fig.3.3.2*), thereby also precluding STAT4 as critical actor in this pathway.

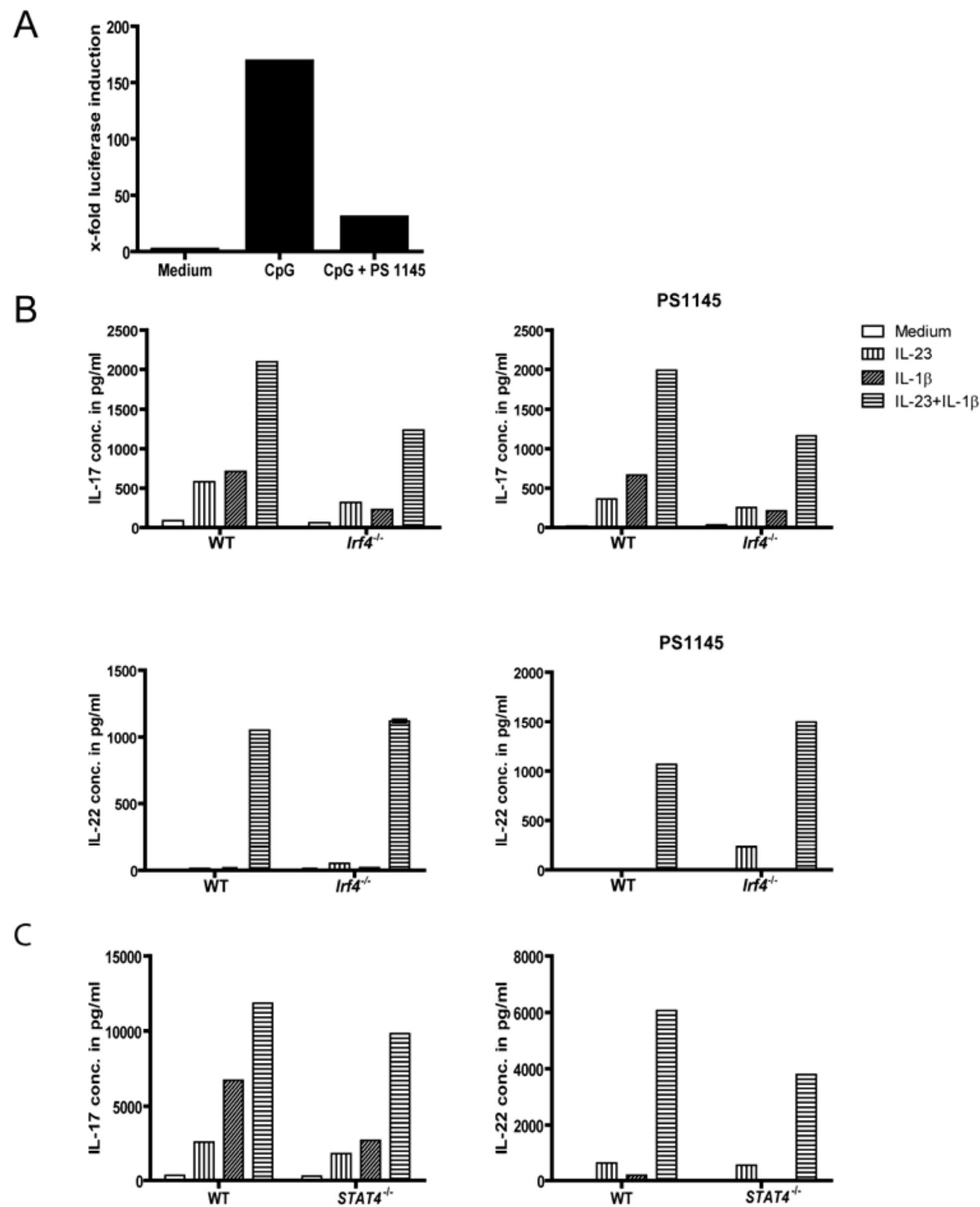

Figure 3.3.5: ***Analysis of the potential signaling pathway***. *(A) Luciferase assay in 293-mTLR-luc cells, stably transfected with luciferase under the control of the NFκB-promoter and inducible by CpG administration. The NFκB inhibitor PS1145 was used at 5 nM. Luciferase measurement after 15 hrs. (B) γδ T cells from wildtype (WT) and Irf4 knockout mice (Irf4$^{-/-}$) were stimulated with or without PS1145 in the presence of IL-23 (25 ng/ml) and/or IL-1β (10 ng/ml) or in medium alone. IL-17 and IL-22 production was determined by ELISA from supernatants harvested after 15 hrs. (C) γδ T cells from wildtype (WT) and STAT4 knockout mice (STAT4$^{-/-}$) were stimulated with IL-23 (25 ng/ml) and/or IL-1β (10 ng/ml) or in medium alone. IL-17 production was determined by intracellular staining after 48 hrs. Two experiments with similar outcome.*

Together, this final part of my thesis establishes that IRF4 is required for IL-17 production only in some, but not in all types of T cells. Apparently, a so far unknown pathway for production of this cytokine exists in non-conventional T cells expressing the γδ TCR. When IL-17 production in these cells is induced in response to the cytokines IL-1β and IL-23 *in vitro*, this pathway acts independently not only of IRF4, but also of NFκB and STAT4.

4. DISCUSSION

4.1 GENERATION OF A CONDITIONAL KNOCKOUT MOUSE LINE FOR *IRF1*

The transcription factor *Irf1* is expressed in a variety of cell types and was first described for its properties as a tumor suppressor gene. Later on its essential function in Th1 cell differentation came more into focus. These two capabilities differ completely and are not related to each other. It is also known that the IRF1 knockout mouse has a strong defect in thymic $CD8^+$ T cell development. Only about 3 % of circulating T cells are $CD8^+$ T cells and it remains uncertain whether the cells have no other intrinsic defect. To question the effects of IRF1 in a given experimental setting *in vivo*, a conditional knockout system would be of great benefit, because due to the tissue specificity of this system one can exclude any other than the desired developmental defect in the organism.
In this thesis work, a targeted chimeric mouse for *Irf1* was successfully generated by using the Cre-lox system. The flox sites-containing target vector was cloned for gene-specific targeting and introduced into embryonic stem cells. After blastocyst injection, chimeric mice were generated.

The Cre-lox sytem has been widely used to generate knockout mice, and Cre-expressing mice under cell-specific promoters are similarly accessible. The herein used target vector pEasyFlox offered indeed a simple system, but also bears possibilities for improvement. The deletion of the neomycin resistance cassette is eligible to avoid unpredictable regulatory effects due to destruction or modification of potential regulatory sites in the intron of the target gene by the additional neomycin sequence. Several reports suggest that the presence of an intron contributes to regulation of general gene expression, even if derived from a different gene (BUCHMANN AND BERG 1988, CHUNG AND PERRY 1989, REID *ET AL.* 1990). As an example, a targeted reporter knock-in mouse for *Irf1* exists that includes the insertion of a lacZ gene and still carries the neomycin resistance cassette. This mouse has been established by the European Conditional Mouse Mutagenesis Programm (http://www.eucomm.org/). An overview of all available mouse strains and ES cell lines regarding *Irf1* targeting are examinable on

the webpages of the International knockout mouse Consortium (http://www.findmice.org//index.jsp). All of these constructs still contain the antibiotic resistance gene as well as additional non-*Irf1*-related sequences, which were designed for better homologous recombination (personal communication).
To avoid these disadvantages in the current conditional knockout *Irf1* preparation, the selection marker neomycin was to be deleted by a second transfection with a Cre-expression vector. Unfortunately, all three loxP sites were addressed this way and the excision of *neo* had to be distinguished from the other events. In any future project, this problem can be circumvented by turning towards the flippase-flippase recognition target (FLP-FRT) system. It has similarities with the Cre-lox system and is derived from baker's yeast *Sacharomyces cerevisiae* (REVIEWED BY SCHWEITZER 2003). The combination of loxP sites flanking the target gene and FRT sites flanking the antibiotic resistance cassette in a target vector of this system will decrease the number of stem cells clones to be screened drastically and no extra screening strategy for this step would be necessary, which makes the whole procedure easier and more efficient.

Irf1 is expressed in a variety of different cell types, but its function in a specific cell type in the context of a given disorder is only poorly understood. Therefore, it would be of great interest to study the effects of IRF1 in different disease settings using a conditional knockout.

In the following, examples for putative future projects will be given.

In allergic asthma, the contribution of IRF1 in airway epithelial cells could shed a light on a possible function during inflammation, manifestation of the disease or airway remodelling. A mouse expressing Cre in Clara cells is available (BERTIN 2005). The expression of IRF1 in epithelial cells in general has been demonstrated by several studies. In HK2 cells, derived from proximal tubule epithelium of human kidney, *Irf1* was induced upon glucose stimulation, underlining also the implication of IRF1 in the development of diabetes (ALI *ET AL.* 2010, SUK 2001). A complete suppression of insulitis and diabetes was observed in NOD mice lacking IRF1 (NAKAZAWA *ET AL.* 2001). The implication of $CD8^+$ T cells in this disease has been well established. Since the complete *Irf1* knockout mouse shows a defect in $CD8^+$ T cell development, one could argue that the resistance of IRF1 deficient NOD mice is secondary to a defect in

surviving $CD8^+$ T cells in the periphery. To answer this question and study the exact function of IRF1 in diabetes at the same time, a conditional knockout for IRF1 in $CD8^+$ T cells would be helpful.

In 2000, Clavell and colleagues demonstrated *Irf1* expression in lamina propria mononuclear cells (CLAVELL *ET AL.* 2000), and it may also play a role for the integrity of the epithelium. For the investigation of ciliated epithelia such as in the lung and in the gut, a transgenic Cre-system designed for ciliated cells is available (ZHANG *ET AL.* 2007). The Jackson Laboratory database lists a wide range of Cre-expressing mice, which includes an even finer possibility to investigate the gut epithelium apart from the lung: Cre-mediated deletion can be achieved in only small and large intestine (jaxmice.jax.org/models/cre_flp_loxp). Beside organ-specific knockout, cell type-specific elimination of *Irf1* might be desired, to determine an effect mediated by one type of cell. Since IRF1 is a key transcription factor in T helper cell differentiation, it could also be interesting to disrupt the gene in all $CD4^+$ T cells. Could asthma then be prevented? This could also help to reveal IRF1-related functions in other T helper cell subsets than Th1.

Taken together, the massive impact of IRF1 on many different kinds of diseases can hardly be foreseen, but the usefulness of a conditional knockout is incontestable. So, there will be many applications for the mouse which was generated in the current thesis!

4.2 OVA-MEDIATED ASTHMA INDUCTION IN *IRF1*$^{-/-}$ MICE

Undifferentiated $CD4^+$ T cells from *Irf1*$^{-/-}$ mice fail to properly respond to an adequate immune stimulus and do not differentiate into Th1 cells. This effect is mainly mediated by the incapacity of antigen presenting cells (APCs) to produce IL-12, due to the lack of IRF1 that activates the transcription of the IL-12p40 subunit of this cytokine (TAKI *ET AL.* 1997, LOHOFF *ET AL.* 1997). Its absence leads to induction of Th2 cells instead. Consistent with these findings is the observation that *Irf1* knockout mice are susceptible for intracellular pathogens such as *Leishmania major* and *Listeria monocytogenes*, but resistent to extracellular parasites such as *Nippostrongulus brasiliensis* (TAKI *ET AL.* 1997; LOHOFF *ET AL* 1997).

The paradigm of asthma being a Th2-mediated disease is well established (LARCHÉ *ET AL.* 2003). Hence, the assumption that IRF1-deficient mice could develop a significant more severe asthma phenotype seemed to be likely. To test this hypothesis, asthma was induced in conventional *Irf1* knockout mice in comparison with wildtype mice using the acute OVA model. Surprisingly, the simple idea of a worse pathology was a bad shot. In almost every parameter measured, the IRF1 deficient mice reacted to the same extent as the wildtype mice. Even if one might determine a faint tendency of *Irf1*$^{-/-}$ mice towards a stronger reaction, these values were not significant. Although the restimulated BAL cell content of the *Irf1* knockout mice showed significantly more CD4^{+}IL-4^{+} cells, this most likely only reflects the overall tendency of these animals to react in a Th2 response *per se,* since this effect was not very strong. Besides, the expression of the cytokines in the BAL was measured at the detection limit of the system, so any statements, especially regarding IFN-γ, IL-10 and IL-6 production and relevance should be made and considered only with caution. Overall, the pathology arising during the induction of asthma was not stronger in the *Irf1*$^{-/-}$ compared to wildtype mice. Hence, this mouse does not react any stronger in response to OVA as the wildtype. Altogether, the analyses on the asthma phenotype in *Irf1* knockout mice were very disappointing. It should be stressed, however, that all analyses were made in the genetic C57BL/6 background which is far less susceptible to allergy than the BALB/c background. Therefore, I have backcrossed *Irf1* knockout mice to the BALB/c background, to provide the opportunity for the lab to repeat the experiments in a better suited setting in the future.

Many different cell types are involved in the pathogenesis of asthma (AFSHAR *ET AL.* 2008), suggesting many cellular cooperations, where contributions of single cell types may be negligible and taken over by other cells. Beside Th cells, CD8^{+} T cells were shown to play a crucial role in the early development of asthma (KOYA *ET AL.* 2007). Here, a finely tuned interplay of CD4^{+} and CD8^{+} T cells was demonstrated in OT-I mice, in which CD8^{+} T cells express the transgenic TCR for OVA$_{257-264}$ peptide (SINFEKL). Without presence of CD4^{+} as well as CD8^{+} T cells already at the time of the first sensitisation, these mice failed to develop asthma syptoms and measurable paramaters such as airway hyperresponsiveness, airway eosinophilia and Th2 cytokine elevation. The *Irf1* knockout mouse is unable to release a normal level of CD8^{+} T cells into the periphery

mainly due to thymic misregulation of MHC I molecules. Transferred donor wildtype CD8 cells develop normally in the *Irf1* knockout environment (PENNINGER *ET AL.* 1997). Hence, the lack of a bias towards asthma in the *Irf1*$^{-/-}$ mice may arise due to the fact that there are either not enough CD8^{+} T cells in the periphery for sufficient intercellular crosstalk or that these CD8^{+} T cells are defective for certain functions. Koya and colleagues showed that an initial priming step for both CD4^{+} and CD8^{+} T cells is necessary to mount an allergic immune response (2008).

Adoptive transfer experiments where wildtype CD8^{+} T cells are introduced into *Irf1* knockout mice could shed a light on the interaction of CD4^{+} and CD8^{+} T cells in this knockout setting. However, CD8 is not solely expressed on T cells. Dendritic cells (DCs) do express CD8 as well and encounter T cells in the spleen. An interesting study suggests evidence for CD8^{+}DCs to enable a specific γδ T cell population expressing Vγ1, but not Vγ4 T cell receptor chains thereby mediating airway hyperresponsiveness (COOK *ET AL.* 2008), suggesting another CD8-dependent function that might be insufficient in IRF1 knockout mice. The fact that γδ T cells are involved in the asthmatic disease is also supported by the observation in human patients having increased Th2 cytokines all produced by gammadelta T cells in line with a decreased level of Th1 cytokines (KRUG *ET AL.* 2001; SPINOZZI *ET AL.* 1995). In addition, IL-4 and IL-5-producing γδ T cells are resident in nasal passages of asthmatic patients (PAWANKAR *ET AL.* 1996). These data all support a contribution of these cells in asthma. These finding can also be found in experimental mouse studies, where contradicting functions for γδ T cells have been demonstrated. There are reports of enhanced airway inflammation mediated by γδ T cells (ZUANY-AMORIM *ET AL.* 1998) and that mice deficient in γδ T cells are protected from developing airway hyperresponsiveness (AHR) (LAHN *ET AL.* 1999), while Hamelmann *et al.* demonstrate that γδ T cells contribute to AHR, but have no significant role in regulating airway inflammation (1996). This can be explained by the above mentioned distinct subsets of γδ T cells.

As for DCs, they can be found throughout the airways and lung interstitium, lung vasculature and bronchial lymphnodes (REVIEWED BY GEURTSVANKESSEL AND LAMBRECHT 2008) and have sentinel function since they express an amazing variety of receptors for inflammatory mediators as well as pattern recognition receptors, such as Toll-like recpetors or C-type lectin receptors (BARRETT *ET AL.* 2009). Some of the receptors are released upon tissue damage. CD8^{+} DCs receive antigen and activating signals from phagocytes which commute between the periphery such as lung or skin and the

lymphnodes (BELZ *ET AL.* 2004; ALLAN *ET AL.* 2006). Hereupon, the DCs present the antigen and can prime T cells to differentiate into cytotoxic T cells or T helper cells (DEN HAAN *ET AL.* 2000; BELZ *ET AL.* 2005; BELZ *ET AL.* 2004A). These features give the DCs a dominant position in connecting the innate and the adaptive immune sytem (VERMAELEN *ET AL.* 2001) and make the activation of specific cell types such as γδ T cells even more plausible.

Very recently, a new cell type has been introduced and characterised: nuocytes expand *in vivo* in response to IL-7, IL-25 and IL33 and represent an early source of IL-13 (NEILL *ET AL.* 2010). Nuocytes were examined in the context of helminth infection, but keeping the hygiene hypothesis in mind and that allergic diseases may be a misled “substitute reaction” of the immune system, these cells may also contribute for a “Th2” response in asthma. This hypothesis is supported by an independent study that reports of a $CD4^+$ T cell subset producing IL-5 and IL-13 independently of IL-4 upon IL-33-polarisation and that these cells exacerbate OVA-specific airway inflammation (KUROWSKA-STOLARSKA *ET AL.* 2008).

Altogether, there are plenty of possibilities, why it is not solely the Th1/Th2 paradigm that determines the severity of asthma. This may explain, why IRF1 deficient mice are not affected by stronger asthma phenotype despite of having a bias towards Th2 responses. It would be interesting to address the question, if IRF1 also orchestrates the intercellular crosstalk of the other above mentioned cells during asthma, e.g. by regulating cytokine expression other than for Th1 cell differentiation. It is also concievable that IRF1 deficient DCs have reduced amounts of surface CD8 and are impaired to properly prime T cells and to comply with their surveillance duties.

Despite these considerations, there is no doubt that the recuitment of T cells towards the lung is crucial in the development of asthma. Here, chemokines and lipid mediators critically lead the way. Thus, it is also possible that IRF1 alters the expression of homing receptors, lipid mediators or the receptors for them. IRF1 takes action in CC chemokine ligand 5/RANTES production (MATSUZAKI *ET AL.* 2010), so it would be worthwhile to investigate homing and cell trafficking capabilities in the *Irf1* knockout mouse. Besides, increased levels of leukotriene B_4 (LTB_4) have been found in the blood (SHINDO *ET AL.* 1995, SAMPSON *ET AL.* 1995), BAL (WENZEL *ET AL.* 1995, ZAITSU *ET AL.* 1998) and exhaled condensates (CSOMA *ET AL.* 2002, MONTUSCHI AND MARNES 2002) of asthmatic patients. Pathogenic functions of LTB_4 include survival signals for leukocytes, neutrophils and eosinophils (HÉBERT *ET AL.* 1996), as well as a role in T cell trafficking (TAGER *ET AL.* 2003).

Apart from distinct T cell subsets or/and other cell types that may be involved in the development of allergic pulmonary inflammation, one should not forget the role played by the environment. IRF1 is an important mediator of IFN-γ signaling and Schmitz *et al*. reported of a connection of IFN-γ and signaling via the Toll like receptor (TLR) 9 that recognizes bacterial and viral nucleotides (2007). IFN-β-production in myeloid DCs requires IRF1, which is recruited and activated by MyD88, which, in turn is involved in many TLR signalling pathways. Although IFN-γ can induce high amounts of IRF1, it does not care for its activation and transport into the nucleus, which results in an insufficient IFN-β-response (NEGISHI *ET AL.* 2006). However, if TLR9 is activated at the same time, IRF1 is also activated and migrates into the nucleus. This synergy of TLR9 and IFN-γ demonstrates a tight interaction of the innate and the adaptive immune system and proves a function of IRF1 also in innate immunity, although its importance in T helper cell differentiation is unquestioned. Hence, one could argue that the main function of IRF1 in allergic asthma lies not within the T cells, but instead in the innate immune system, and a modified experimental setup for acute asthma by also giving TLR9 ligands to a conditional *Irf1* knockout mouse could be applied, keeping in mind that among others, viral infections may trigger the development of asthma. TLR9 binds unmethylated CpG containing DNA fragments derived from bacteria and viruses, which results in a strong immunomodulatory reaction, but implies anti-allergic mechanisms (BAUER *ET AL.* 2001, HORNER AND RAZ 2003). However, certain SNPs in the promotor of TLR9 were associated with a moderately increased risk for the development of asthma and insufficient TLR9 expression could account for a higher risk to develop asthma (KORMANN 2007) and chronic lung inflammation (ITO *ET AL.* 2009). It could be worthwhile to test the contribution of IRF1 in this context, because TLRs could be used as possible therapeutic target molecules in asthma treatment. SNPs in the IRF1 gene have also been associated with a higher risk to develop asthma (SCHEDEL *ET AL.* 2008, WANG *ET AL.* 2006). If a given set of SNPs in both genes (for TLR9 and IRF1) occurred at the same time, a possible effect of a drug was circumvented. Indeed, targeting TLRs as preventive strategy is already used in hyposensitisation. First studies suggested that indoor endotoxin levels (derived from house dust, most likely house dust mite or certain bacteria) in early childhood may lower allergen sensitisation later on (GEREDA *ET AL.* 2000A, GEREDA *ET AL.* 2000B). Animal studies reported contradictory results (PURKERSON

&ISAKSON 1994, GERHOLD *ET AL.* 2002 AND 2003). However, asthma protection in offspring can be mediated by TLR signaling in the respiratory tract of the mother as induced by *Acinetobacter lwoffii* application during pregnancy (CONRAD ET AL. 2009). Still, the exact beneficial, microbial components have yet to be identified as well as their recognition pathways in order to develop a proper treatment on this basis; also, one should be aware of possible limitations when addressing these targets (AGRAWAL *ET AL.* 2003, EISENBARTH *ET AL.* 2004, REDECKE *ET AL.* 2004). It is conceivable that TLR9 expression in patients is insufficient, thus resulting in a much higher risk of asthma. In this situation, the therapeutic potential of the TLR targets CpG to raise a protective immune response dominated by IFN-γ may be limited (IKEDA *ET AL.* 2003), along with a limited potential to inhibit Th2-induced factors, produced by lung mast cells (CHRISHOLM *ET AL.* 2004). Moreover, severe side effects of therapeutic TLR ligands are possible. Malignant B cells do express TLR9 (DECKER *ET AL.*2000, JAHRSDÖRFER *ET AL.* 2002 AND 2005) and CpG ODN can induce their proliferation as most extensively studied in the context of chronic lymphocytic leukemia (CLL) (DICKER *ET AL.* 2006). Of interest, CpG ODN can up-regulate the IL-21 receptor and the combination of CpG ODN and IL-21 resulted syergistically in apoptosis (JAHRSDÖRFER *ET AL.* 2005A), but also in the production of functional granzyme B in CLL cells (JAHRSDÖRFER *ET AL.* 2006). These results demonstrate that unexpected mechanisms might cause significant damage that does not counterbalance the initially intended benefits.

In summary, the fact of a great variety of different cell types which are involved in the pathogenesis of allergic asthma explains well, why the *Irf1* kockout mouse does not mount a significantly stronger pathology in the OVA model of acute asthma. The single contribution of Th2 cells does apparently not play the overwhelming role in this disease and the newly dicovered cell types and subsets produce cytokines formerly ascribed to Th2 cells!

The identification of new T helper subsets and investigation of the underlying mechanisms of intercellular crosstalk and their involvement in the pathogenesis of asthma took a step forward in understanding the disease. However, more questions raised, but these new cell populations may also represent targets for medical treatment of allergic diseases such as asthma.

4.3 IL-17 PRODUCTION BY *IRF4*$^{-/-}$ γδ T CELLS

In contrast to conventional αβ T cells, which leave the thymus as naïve cells and develop effector functions after antigen contact in the periphery, γδ T cells raise effector capabilities within the fetal thymus as early as of day 14 to day 18 (TONEGAWA *ET AL.* 1989; RAULET 1989). At least parts of these cells are released from the thymus into the periphery as IL-17-producing cells (JENSEN *ET AL.* 2008; RIBOT *ET AL.* 2009; SHIBATA *ET AL.* 2008). One of their most striking features is their ability to rapidly produce IL-17 and IFN-γ (ROARK *ET AL.* 2007 AND 2008). For this reason, they are considered to belong to the innate immune system. Th17 cells need IL-6 and TGF-β for differentiation and subsequent IL-17 production. However, in peripheral γδ T cells, IL-23 and IL-1β were shown to enhance IL-17 production, and by that support Th17 effector function and co-operate with Th17 cells in mediating autoimmune pathology (SUTTON *ET AL.* 2009). The differentiation of Th17 cells is dependent of IRF4. In its absence, even EAE development is completely blocked (BRÜSTLE *ET AL.* 2007).

Surprisingly, my thesis shows that *Irf4* knockout γδ T cells are indeed perfectly capable to produce IL-17. Direct restimulation of organ homogenates of *Irf4* knockout mice resulted in IL-17 detection in γδ T cells to the same extent as in wildtype controls, proving the general capability of these cells to express IL-17 independently of the organ, they were found in. On order to test the *in vivo* expansion of these cells in a natural setting, mice were infected with *Streptococcus pneumoniae*. The results revealed the presence of IL-17^{+} γδ T cells in the lung of *Irf4* knockout mice, which produced even more IL-17 than heterozygous littermates (*Fig. 3.3.3*). This finding underlines that γδ T cells from *Irf4* knockout mice are fully immune competent. In humans, γδ T cells are able to respond and display cytolytic activity to a variety of bacterial superantigens (REVIEWED IN RUST AND KONING 1993). They have also functions in host-micobial homeostasis at mucosal surfaces and protect the host from invasion of commensal bacteria in the small intestine (ISMAIL *ET AL.* 2011). Mucosal epithelia produce antimicrobial proteins to defend these surfaces against microbial attacks. This is in line with a report for γδ T cells being the major source of IL-17 during infection with *Mycobacterium tuberculosis* (LOCKHART *ET AL.* 2006) and with Listeria or Nocardia (KING *ET AL.* 1999, RIOL-BLANCO *ET AL.* 2010, SKEEN AND ZIEGLE 1993). These data also suggest that γδ T

cells might orchestrate the production of this defense mechanism and could be an attractive therapeutic target for example in intestinal or pulmonary inflammatory diseases.

Of course, we attempted to get more insight into why γδ but not Th cells of *Irf4* knockout mice can produce IL-17. Meanwhile, RORγt is well established as lineage-specific transcription factor for Th17 differentiation, with support by RORα (IVANOV *ET AL.* 2006). Herein, realtime analysis of freshly isolated and sorted *Irf4* knockout and wildtype γδ T cells showed no difference in the expression of these transcription factors as well as of the Th1 master regulator Tbet (*Fig. 3.3.4*). Recently, it has been shown that mRNAs of these transcription factors are expressed in γδT cells in response to PMA/ionomycin, in support of the production of IL-17 and IFNγ by these cells (POWOLNY-BUDNICKA *ET AL.* 2011).

In addition the same authors also present evidence for a NFκB function during differentiation of γδ T cell precursors into IL-17 producing cells which involved the lymphotoxin receptor β (LTβR). They claim that the activation of the NFκB member RelB as a result of LTβR signal transduction is inevitable, because LTβR-deficient mice failed to develop IL-17 producing γδ T cells *in vivo*.

Our experiments primarily tested for IL-17 production *in vitro* rather than *in vivo*. In this experimental setting, our results clearly refute any NFκB involvement for the IL-17 production of this T cell subset in the *Irf4* knockout mice, as demonstrated in a suppression assay with the NFκB inhibitor PS 1145 *(Fig. 3.3.5).* The cytokine production was not impaired at all. During these experiments, we made use of previous finding that IRF4 deficient γδ T cells are not only capable to express IL-17 *per se*, but also produce IL-17 in response to IL-23. This effect was even potentiated by stimulation with IL-1β at the same time. Both cytokines help to promote and sustain the Th17 phenotype in normal mice, but also IL-17 production in γδ T cells, thereby supporting Th17 responses (SUTTON *ET AL.* 2009).

For further investigation of the underlying pathway of this IL-17 production, I repeated the experiments in STAT4 deficient mice, because STAT4 has previously been shown to be involved in the signaling of IL-23 (PARHAM *ET AL.* 2002). Interestingly, in this case, STAT4 was not addressed and dispensable for IL-17 production by γδ T cells in response to IL-23. This was equally true for cells from *Irf4* knockout and wildtype

mice. Besides STAT4, STAT3 is activated during IL-23 signaling (PARHAM *ET AL* 2002). In order to generate a complete picture, it will therefore be necessary to also address this molecule in the IL-17 production of the IRF4 deficient γδ T cells. Yet, Shibata *et al.* also declared STAT3 to be dispensable for IL-17 cytokine production in γδ T cells (SHIBATA *ET AL.* 2011). However, just as for the importance of NFκB, this statement stems from a different experimental design, and certainly was not tested in the case of the IRF4 deficient mouse. Hence, the involvement of STAT3 should still be formally excluded in this mouse.

All findings together could unfortunately not reveal the mechanism, by which IL-17 production is initiated in IRF4 deficient γδ T cells. So, what may be the underlying signaling pathway? On the one hand the chances that several evolutionally independently developed signaling pathways lead to the same gene product might be rare due to an apparent inefficacy. On the other hand, our data suggest a pathway different from NFκB but also independent of IRF4.

Obviously, this pathway involves signaling via IL-1 and IL-23, which are major factors in chronic inflammation (CUA *ET AL.* 2003; YEN *ET AL.* 2006). The function of IL-23 is not yet completely understood, but it may act as growth factor for Th17 cells (KORN *ET AL.* 2009) and prevents conventional αβ T cells to convert into Foxp3$^+$ regulatory T cells (PETERMANN *ET AL.* 2010). In turn, IL-1β is a proinflammatory cytokine that mediates fever, recruitment of neutrophilic granulocytes and triggers the secretion of IL-6. Its signalling also involves NFκB (LIEB *ET AL.* 1996). Together, stimulation of γδ T cells by IL-23 and IL-1β leads to a further increase of the IL-17 production by these cells, more than by each of them alone. It is very likely that the mechanism that has been activated in the thymus is simply reinforced in the periphery by stimulation with these two cytokines. In chondrocytes, IL-1β is able to induce the Notch-hairy enhancer of split (Hes)1 pathway and thereby provides a signal for the expression of certain activation marker genes in chondrocytes, such as matrix metalloproteinase 13 and agrecanase disintgrin and metallopreotease (ADAM) with thrombospondin type 1 motif, 5 (ADAMTS5) independently of Notch (OTTAVIANI *ET AL.* 2010). Interestingly, this pathway has recently been shown to be responsible for the development of IL-17-producing γδ T cells in the thymus and periphery (SHIBATA *ET AL.* 2011). This could mean that likewise in peripheral γδ

T cells, the induction of IL-17 by IL-23 and IL-1β could be mediated by the Hes1 pathway. This signaling may or may not be related to RelA and RelB (POWOLNY-BUDNICKA *ET AL.* 2011), but may circumvent all STAT molecules that have been connected to the IL-23 receptor signaling. However, the fact that in my hands a functional NFκB inhibitor does not have even the slightest effect on IL-17 production, suggests that if the Notch-Hes1-pathway is involved in the herein reported IL-17 production of IRF4 deficient γδ T cells, it acts independent of RelA and RelB. These assumptions, of course, have to be fully proven in the *Irf4* knockout mouse in the future.

A final remark refers to the implications of our findings for the role of γδ T cells in the pathogenesis of autoimmune diseases (ODYNIEC *ET AL.* 2004). These cells have been found in lesions of multiple sclerosis (MS) patients (RAJAN *ET AL.* 1998). Apparently, they clonally expand by the restricted usage of TCR variable chain segment (V)γ9 (WUCHERPFENNIG *ET AL.* 1992) and by this even represent a cell fraction in blood, which can be considered as predictor for disease activity (POGGY *ET AL* 2007, RINALDI *ET AL* 2006). This fact is especially interesting with regard to the *Irf4* knockout mouse, because this mouse is completely resistant to the murine model of MS, namely EAE and strongly requires Th17 cells. Therefore γδ T cells my not play a role in the initiation of the disease, but their functions lies rather in supporting Th17 cells.

All together, despite of many attempts to reveal the underlying mechanism of IL-17 production by IRF4 deficient γδT cells, it was not yet possible to find the final clue. However, several hints might be given here for further investigation.

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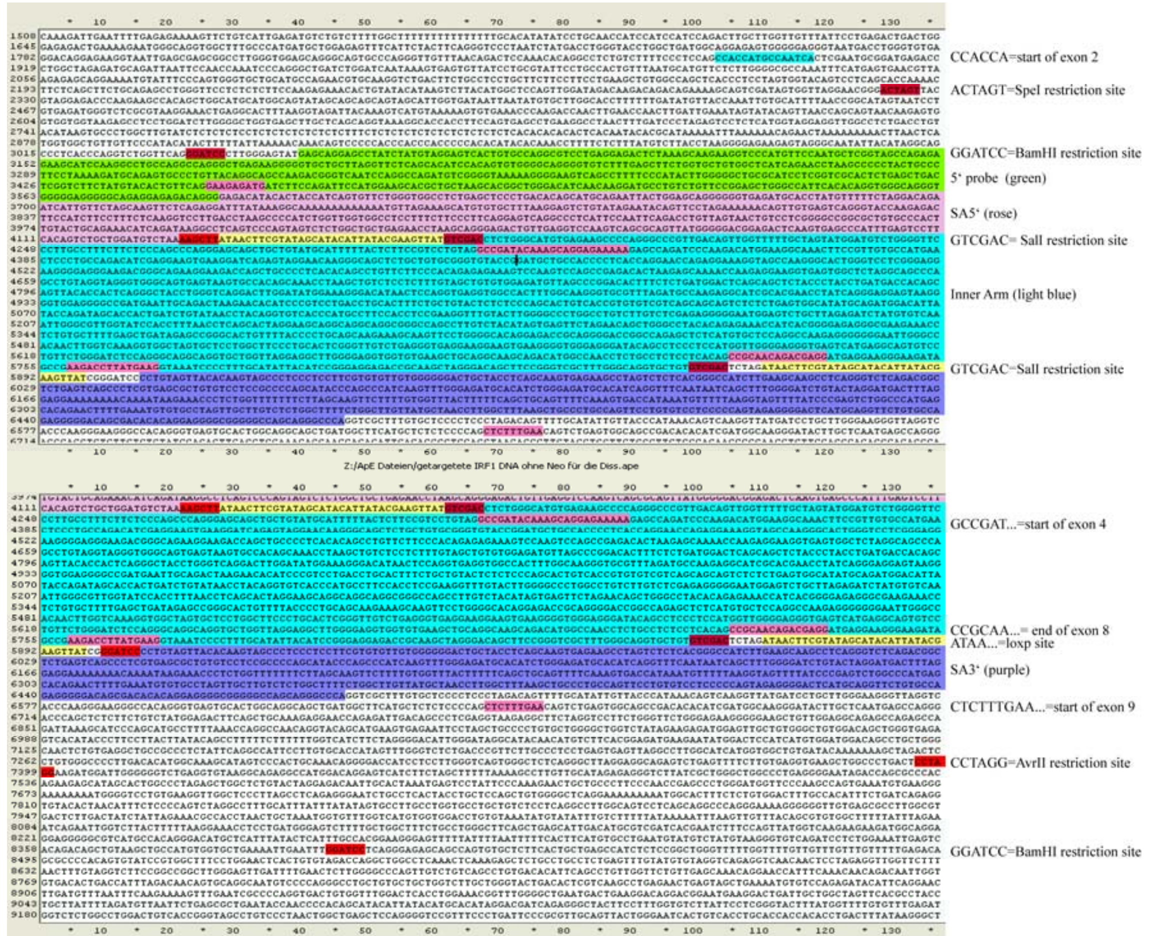


Figure A1: ***Targeted locus of the Irf1 gene.*** *Upper panel includes the sequence in front (further 5') of the targeted site; the lower panel includes a section behind (further 3') of the targeted site.*

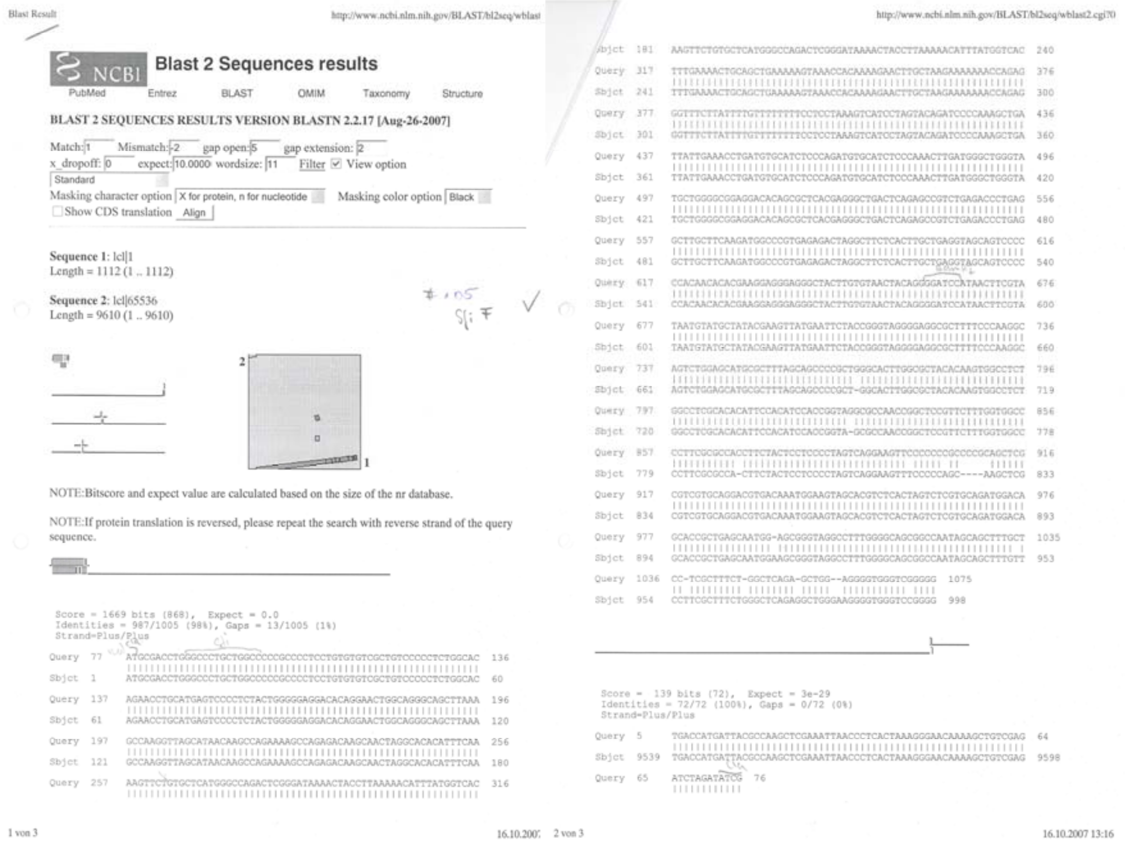


Figure A2: ***Sequence verification of SA3***. *The sequence between the two restriction sites ClaI and BamHI matches 100 %. The genomic Sfi restriction site is also correct.*

Blast Result http://www.ncbi.nlm.nih.gov/BLAST

Blast 2 Sequences results

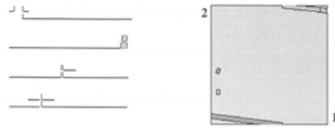
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.0000 wordsize: 11 Filter ☑ View option
Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|1
Length = 1109 (1 .. 1109)

Sequence 2: lcl|65536
Length = 9610 (1 .. 9610)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the sequence.

```
 Score = 1244 bits (647),  Expect = 0.0
 Identities = 677/677 (100%), Gaps = 0/677 (0%)
 Strand=Plus/Minus

Query  1     AGCGCATGCTCCAGACTGCCTTGGGAAAAGCGCCTCCCCTACCCGGTAGAATTCATAACT
Sbjct  677   AGCGCATGCTCCAGACTGCCTTGGGAAAAGCGCCTCCCCTACCCGGTAGAATTCATAACT

Query  61    TCGTATAGCATACATTATACGAAGTTATGGATCCCCTGTAGTTACACAAGTAGCCCTCCC
Sbjct  617   TCGTATAGCATACATTATACGAAGTTATGGATCCCCTGTAGTTACACAAGTAGCCCTCCC

Query  121   TCCTTCGTGTGTTGTGGGGGGACTGCTACCTCAGCAAGTGAGAAGCCTAGTCTCTCACGG
Sbjct  557   TCCTTCGTGTGTTGTGGGGGGACTGCTACCTCAGCAAGTGAGAAGCCTAGTCTCTCACGG

Query  181   GCCATCTTGAAGCAAGCCTCAGGGTCTCAGACGGCTCTGAGTCAGCCCTCGTGAGCGCTG
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1 von 3

http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi?0

```
Sbjct  497   GCCATCTTGAAGCAAGCCTCAGGGTCTCAGACGGCTCTGAGTCAGCCCTCGTGAGCGCTG  438

Query  241   TGTCCTCCGCCCCAGCATACCCAGCCCATCAAGTTTGGGAGATGCACATCTGGGAGATGC  300
Sbjct  437   TGTCCTCCGCCCCAGCATACCCAGCCCATCAAGTTTGGGAGATGCACATCTGGGAGATGC  378

Query  301   ACATCAGGTTTCAATAATCAGCTTTGGGGATCTGTACTAGGATGACTTTAGGAGGAAAAA  360
Sbjct  377   ACATCAGGTTTCAATAATCAGCTTTGGGGATCTGTACTAGGATGACTTTAGGAGGAAAAA  318

Query  361   AAACAAAATAAGAAACCCTCTGGTTTTTTTCTTAGCAAGTTCTTTTGTGGTTTACTTTTT  420
Sbjct  317   AAACAAAATAAGAAACCCTCTGGTTTTTTTCTTAGCAAGTTCTTTTGTGGTTTACTTTTT  258

Query  421   CAGCTGCAGTTTTCAAAGTGACCATAAATGTTTTTAAGGTAGTTTTATCCCGAGTCTGGC  480
Sbjct  257   CAGCTGCAGTTTTCAAAGTGACCATAAATGTTTTTAAGGTAGTTTTATCCCGAGTCTGGC  198

Query  481   CCATGAGCACAGAACTTTTGAAATGTGTGCCTAGTTGCTTGTCTCTGGCTTTTCTGGCTT  540
Sbjct  197   CCATGAGCACAGAACTTTTGAAATGTGTGCCTAGTTGCTTGTCTCTGGCTTTTCTGGCTT  138

Query  541   GTTATGCTAACCTTGGCTTTAAGCTGCCCTGCCAGTTCCTGTGTCCTCCCCCAGTAGAGG  600
Sbjct  137   GTTATGCTAACCTTGGCTTTAAGCTGCCCTGCCAGTTCCTGTGTCCTCCCCCAGTAGAGG  78

Query  601   GGACTCATGCAGGTTCTGTGCCAGAGGGGGACAGCGACACACAGGAGGGGCGGGGGCCAG  660
Sbjct  77    GGACTCATGCAGGTTCTGTGCCAGAGGGGGACAGCGACACACAGGAGGGGCGGGGGCCAG  18

Query  661   CAGGGCCCAGGTCGCAT  677
Sbjct  17    CAGGGCCCAGGTCGCAT  1

 Score =  802 bits (417),  Expect = 0.0
 Identities = 431/433 (99%), Gaps = 2/433 (0%)
 Strand=Plus/Minus

Query  678   CGATATCTAGATCTCGACAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCG  737
Sbjct  9610  CGATATCTAGATCTCGACAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCG  9551

Query  738   TAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC  797
Sbjct  9550  TAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC  9491

Query  798   ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACA  857
Sbjct  9490  ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACA  9431

Query  858   TTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT  917
Sbjct  9430  TTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT  9371

Query  918   TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCC  977
Sbjct  9370  TAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCC  9311

Query  978   TCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTC-  1036
Sbjct  9310  TCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCA  9251

Query  1037  AAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGC-  1095
```

2 von 3 16.10.2007 13:16

Figure 2Aa: ***Sequence verification of SA3', reverse strand reaction**. The sequence between the two restriction sites ClaI and BamHI matches 100 %. The genomic Sfi restriction site is also correct.*

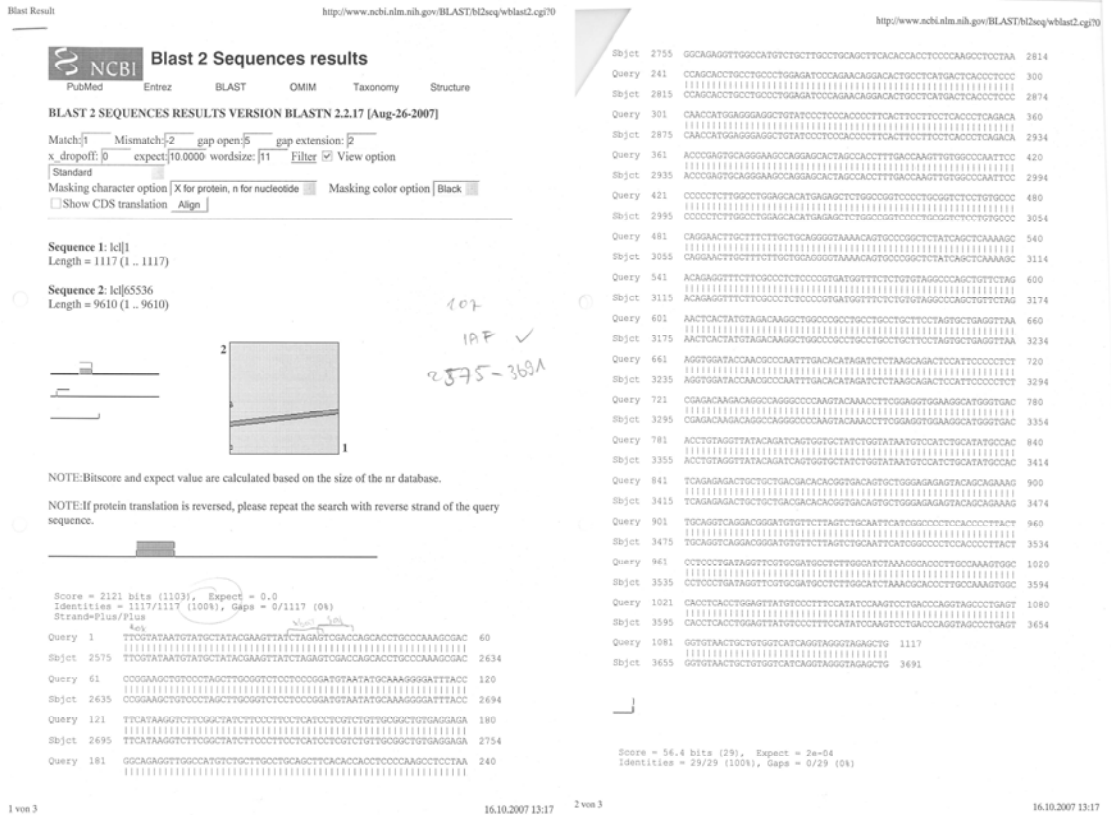


Figure A3: ***Sequence verification of IA.*** *The first part of the sequence matches 100 %. The restiction sites are also correct.*

```
Blast Result                                  http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi?0

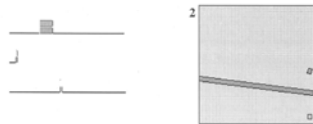
NCBI  Blast 2 Sequences results
PubMed   Entrez   BLAST   OMIM   Taxonomy   Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1   Mismatch: -2   gap open: 5   gap extension: 2
x_dropoff: 0   expect: 10.0000   wordsize: 11   Filter [x] View option
Standard
Masking character option  X for protein, n for nucleotide   Masking color option  Black
[ ] Show CDS translation   Align

Sequence 1: lcl|1
Length = 1123 (1 .. 1123)

Sequence 2: lcl|65536
Length = 9610 (1 .. 9610)
```



```
NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query
sequence.
```

```
 Score = 2113 bits (1099),  Expect = 0.0
 Identities = 1113/1113 (100%), Gaps = 0/1113 (0%)
 Strand=Plus/Minus

Query  1     GCAGTTACACCACTCAGGGCTACCTGGGTCAGGACTTGGATATGGAAAGGGACATAACTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3665  GCAGTTACACCACTCAGGGCTACCTGGGTCAGGACTTGGATATGGAAAGGGACATAACTC  3606

Query  61    CAGGTGAGGTGGCCACTTTGGCAAGGGTGCGTTTAGATGCCAAGAGGCATCGCACGAACC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3605  CAGGTGAGGTGGCCACTTTGGCAAGGGTGCGTTTAGATGCCAAGAGGCATCGCACGAACC  3546

Query  121   TATCAGGGAGGAGTAAGGGGTGGAGGGGCCGATGAATTGCAGACTAAGAACACATCCCGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3545  TATCAGGGAGGAGTAAGGGGTGGAGGGGCCGATGAATTGCAGACTAAGAACACATCCCGT  3486

Query  181   CCTGACCTGCACTTTCTGCTGTACTCTCTCCCAGCACTGTCACCGTGTGTCGTCAGCAGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

1 von 3                                       16.10.2007 13:18
```

```
http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi?0

3485  CCTGACCTGCACTTTCTGCTGTACTCTCTCCCAGCACTGTCACCGTGTGTCGTCAGCAGC  3426

241   AGTCTCTCTGAGTGGCATATGCAGATGGACATTATACCAGATAGCACCACTGATCTGTAT  300
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3425  AGTCTCTCTGAGTGGCATATGCAGATGGACATTATACCAGATAGCACCACTGATCTGTAT  3366

301   AACCTACAGGTGTCACCCATGCCTTCCACCTCCGAAGGTTTGTACTTGGGGCCCTGGCCT  360
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3365  AACCTACAGGTGTCACCCATGCCTTCCACCTCCGAAGGTTTGTACTTGGGGCCCTGGCCT  3306

361   GTCTTGTCTCGAGAGGGGGAATGGAGTCTGCTTAGAGATCTATGTGTCAAATTGGGCGTT  420
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3305  GTCTTGTCTCGAGAGGGGGAATGGAGTCTGCTTAGAGATCTATGTGTCAAATTGGGCGTT  3246

421   GGTATCCACCTTTAACCTCAGCACTAGGAAGCAGGCAGGCAGGCGGGCCAGCCTTGTCTA  480
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3245  GGTATCCACCTTTAACCTCAGCACTAGGAAGCAGGCAGGCAGGCGGGCCAGCCTTGTCTA  3186

481   CATAGTGAGTTCTAGAACAGCTGGGCCTACACAGAGAAACCATCACGGGGAGAGGGCGAA  540
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3185  CATAGTGAGTTCTAGAACAGCTGGGCCTACACAGAGAAACCATCACGGGGAGAGGGCGAA  3126

541   GAAACCTCTGTGCTTTTGAGCTGATAGAGCCGGGCACTGTTTTACCCCTGCAGCAAGAAA  600
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3125  GAAACCTCTGTGCTTTTGAGCTGATAGAGCCGGGCACTGTTTTACCCCTGCAGCAAGAAA  3066

601   GCAAGTTCCTGGGGCACAGGAGACCGCAGGGGACCGGCCAGAGCTCTCATGTGCTCCAGG  660
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3065  GCAAGTTCCTGGGGCACAGGAGACCGCAGGGGACCGGCCAGAGCTCTCATGTGCTCCAGG  3006

661   CCAAGAGGGGGGGAATTGGGCCACAACTTGGTCAAAGGTGGCTAGTGCTCCTGGCTTCCC  720
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3005  CCAAGAGGGGGGGAATTGGGCCACAACTTGGTCAAAGGTGGCTAGTGCTCCTGGCTTCCC  2946

721   TGCACTCGGGTTGTCTGAGGGTGAGGAAGGAAGTGAAGGGGTGGGAGGGATACAGCCTCC  780
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2945  TGCACTCGGGTTGTCTGAGGGTGAGGAAGGAAGTGAAGGGGTGGGAGGGATACAGCCTCC  2886

781   CTCCATGGTTGGGGAGGGTGAGTCATGAGGCAGTGTCCTGTTCTGGGATCTCCAGGGCAG  840
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2885  CTCCATGGTTGGGGAGGGTGAGTCATGAGGCAGTGTCCTGTTCTGGGATCTCCAGGGCAG  2826

841   GCAGGTGCTGGTTAGGAGGCTTGGGGAGGTGGTGTGAAGCTGCAGGCAAGCAGACATGGC  900
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2825  GCAGGTGCTGGTTAGGAGGCTTGGGGAGGTGGTGTGAAGCTGCAGGCAAGCAGACATGGC  2766

901   CAACCTCTGCCTCTCCTCACAGCCGCAACAGACGAGGATGAGGAAGGGAAGATAGCCGAA  960
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2765  CAACCTCTGCCTCTCCTCACAGCCGCAACAGACGAGGATGAGGAAGGGAAGATAGCCGAA  2706

961   GACCTTATGAAGGTAAATCCCCTTTGCATATTACATCCGGGAGGAGACCGCAAGCTAGGG  1020
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2705  GACCTTATGAAGGTAAATCCCCTTTGCATATTACATCCGGGAGGAGACCGCAAGCTAGGG  2646

1021  ACAGCTTCCGGGTCGCTTTGGGCAGGTGCTGGTCGACTCTAGATAACTTCGTATAGCATA  1080
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2645  ACAGCTTCCGGGTCGCTTTGGGCAGGTGCTGGTCGACTCTAGATAACTTCGTATAGCATA  2586

1081  CATTATACGAAGTTATAGCTTCTGATGGAATTA  1113
      |||||||||||||||||||||||||||||||||
2585  CATTATACGAAGTTATAGCTTCTGATGGAATTA  2553


e = 66.1 bits (34),  Expect = 3e-07
tities = 34/34 (100%), Gaps = 0/34 (0%)

                                              16.10.2007 13:18
```

Figure A3a: ***Sequence verification of IA****. The middle part matches 100 %.*

Blast Result http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi?0

Blast 2 Sequences results

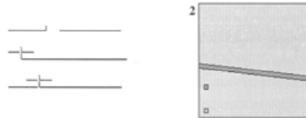
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.0000 wordsize: 11 Filter ☑ View option
Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|1
Length = 1122 (1 .. 1122)

Sequence 2: lcl|65536
Length = 9610 (1 .. 9610)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score = 2126 bits (1105),  Expect = 0.0
 Identities = 1114/1118 (99%), Gaps = 1/1118 (0%)
 Strand=Plus/Minus

Query  1     GGAGACTCAAGTGAGCCCATTTGAGTCCTTCACAGTCTGCTGGATGTCTAAGCCAAGCTT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4376  GGAGACTCAAGTGAGCCCATTTGAGTCCTTCACAGTCTGCTGGATGTCTAAGCCAAGCTT  4317

Query  61    ATAACTTCGTATAGCATACATTATACGAAGTTATGTCGACCTCTGGGCATGTGAGAAGCC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4316  ATAACTTCGTATAGCATACATTATACGAAGTTATGTCGACCTCTGGGCATGTGAGAAGCC  4257

Query  121   CCAGGGCCCGTTGACAGTTGGTTTTTGCTAGTATGGATGTCTGGGGTTCCCTTGCCTTTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4256  CCAGGGCCCGTTGACAGTTGGTTTTTGCTAGTATGGATGTCTGGGGTTCCCTTGCCTTTC  4197

Query  181   TTCTCCCAGCCCAGGGAGCAGCTGCTGTATGCATTTTTACTCTTCCGTCCTGTAGGCCGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```



```
Sbjct  4196  TTCTCCCAGCCCAGGGAGCAGCTGCTGTATGCATTTTTACTCTTCCGTCCTGTAGGCCGA  4137

Query  241   TACAAAGCAGGAGAAAAAGAGCCAGATCCCAAGACATGGAAGGCAAACTTCCGTTGTGCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4136  TACAAAGCAGGAGAAAAAGAGCCAGATCCCAAGACATGGAAGGCAAACTTCCGTTGTGCC  4077

Query  301   ATGAACTCCCTGCCAGACATCGAGGAAGTGAAGGATCAGAGTAGGAACAAGGGCAGCTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4076  ATGAACTCCCTGCCAGACATCGAGGAAGTGAAGGATCAGAGTAGGAACAAGGGCAGCTCT  4017

Query  361   GCTGTGCGGGTGTACCGGATGCTGCCACCCCTCACCAGGAACCAGAGGAAAGGTAGCCAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4016  GCTGTGCGGGTGTACCGGATGCTGCCACCCCTCACCAGGAACCAGAGGAAAGGTAGCCAA  3957

Query  421   GGGCACTGGGTCCTCGGGAGGAAGGGGAGGGAAGACGGGCAGAAGGAAGACCAGCTGCCC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3956  GGGCACTGGGTCCTCGGGAGGAAGGGGAGGGAAGACGGGCAGAAGGAAGACCAGCTGCCC  3897

Query  481   CTCACACAGCCTGTTCTTCCCACAGAGAGAAAGTCCAAGTCCAGCCGAGACACTAAGAGC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3896  CTCACACAGCCTGTTCTTCCCACAGAGAGAAAGTCCAAGTCCAGCCGAGACACTAAGAGC  3837

Query  541   AAAACCAAGAGGAAGGTGAGTGGCTCTAGGCAGCCCAGCCTGTAGGTAGGGTGGGCAGTG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3836  AAAACCAAGAGGAAGGTGAGTGGCTCTAGGCAGCCCAGCCTGTAGGTAGGGTGGGCAGTG  3777

Query  601   AGTAAGTGCCACAGCAAACCTAAGCTGTCTCCTCTTTGTAGCTGTGTGGAGATGTTAGCC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3776  AGTAAGTGCCACAGCAAACCTAAGCTGTCTCCTCTTTGTAGCTGTGTGGAGATGTTAGCC  3717

Query  661   CGGACACTTTCTCTGATGGACTCAGCAGCTCTACCCTACCTGATGACCACAGCAGTTACA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3716  CGGACACTTTCTCTGATGGACTCAGCAGCTCTACCCTACCTGATGACCACAGCAGTTACA  3657

Query  721   CCACTCAGGGCTACCTGGGTCAGGACTTGGATATGGAAAGGGACATAACTCCAGGTGAGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3656  CCACTCAGGGCTACCTGGGTCAGGACTTGGATATGGAAAGGGACATAACTCCAGGTGAGG  3597

Query  781   TGGCCACTTTGGCAAGGGTGCGTTTAGATGCCAAGAGGCATCGCACGAACCTATCAGGGA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3596  TGGCCACTTTGGCAAGGGTGCGTTTAGATGCCAAGAGGCATCGCACGAACCTATCAGGGA  3537

Query  841   GGAGTAAGGGGTGGAGGGGCCGATGAATTGCAGACTAAGAACACATCCCGTCCTGACCTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3536  GGAGTAAGGGGTGGAGGGGCCGATGAATTGCAGACTAAGAACACATCCCGTCCTGACCTG  3477

Query  901   CACTTTCTGCTGTACTCTCTCCCAGCACTGTCACCGTGTGTCGTCAGCAGCAGTCTCTCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3476  CACTTTCTGCTGTACTCTCTCCCAGCACTGTCACCGTGTGTCGTCAGCAGCAGTCTCTCT  3417

Query  961   GAGTGGCATATGCAGATGGACATTATACCAGATAGCACCACTGATCTGTATAACCTACAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3416  GAGTGGCATATGCAGATGGACATTATACCAGATAGCACCACTGATCTGTATAACCTACAG  3357

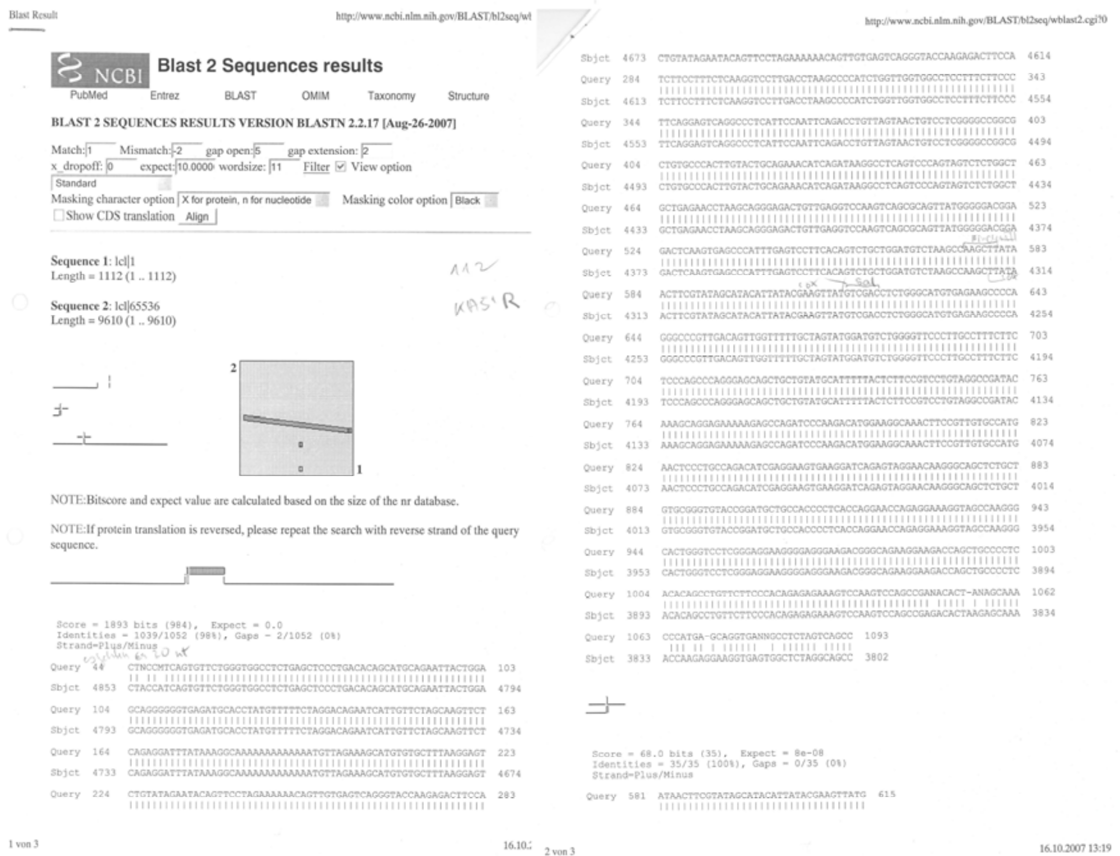
Query  1021  GTGTCACCCATGCCTTCCACCTCCGAAGGTTTGTACTTGGGGCCCTGGCCTGTCTTGTCT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3356  GTGTCACCCATGCCTTCCACCTCCGAAGGTTTGTACTTGGGGCCCTGGCCTGTCTTGTCT  3297

Query  1081  CGAGAGGGGGAATGGARNCTGC-TAGAGATCTATNTGT  1117
             ||||||||||||||||  |||| ||||||||||| |||
Sbjct  3296  CGAGAGGGGGAATGGAGTCTGCTTAGAGATCTATGTGT  3259


 Score = 68.0 bits (35),  Expect = 8e-08
 Identities = 35/35 (100%), Gaps = 0/35 (0%)
```

Figure A3b: ***Sequence verification of IA, reverse strand reaction.*** *The rearmost part of the sequence matches 100 %. Restriction sites and loxP sites are also correct.*

Figure A4: ***Sequence verification of SA5'****. The sequence matches 100 %.*



Blast Result http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wt

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.0000 wordsize: 11 Filter ☑ View option
Standard
Masking character option: X for protein, n for nucleotide Masking color option: Black
☐ Show CDS translation Align

Sequence 1: lcl|1
Length = 1112 (1 .. 1112)

Sequence 2: lcl|65536
Length = 9610 (1 .. 9610)

NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score = 1893 bits (984),  Expect = 0.0
 Identities = 1039/1052 (98%), Gaps = 2/1052 (0%)
 Strand=Plus/Minus

Query  44    CTNCCMTCAGTGTTCTGGGTGGCCTCTGAGCTCCCTGACACAGCATGCAGAATTACTGGA  103
             || || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4853  CTACCATCAGTGTTCTGGGTGGCCTCTGAGCTCCCTGACACAGCATGCAGAATTACTGGA  4794

Query  104   GCAGGGGGTGAGATGCACCTATGTTTTTCTAGGACAGAATCATTGTTCTAGCAAGTTCT  163
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4793  GCAGGGGGTGAGATGCACCTATGTTTTTCTAGGACAGAATCATTGTTCTAGCAAGTTCT  4734

Query  164   CAGAGGATTTATAAAGGCAAAAAAAAAAAAATGTTAGAAAGCATGTGTGCTTTAAGGAGT  223
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4733  CAGAGGATTTATAAAGGCAAAAAAAAAAAAATGTTAGAAAGCATGTGTGCTTTAAGGAGT  4674

Query  224   CTGTATAGAATACAGTTCCTAGAAAAAACAGTTGTGAGTCAGGGTACCAAGAGACTTCCA  283
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

1 von 3 16.10.2

http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi?0

```
Sbjct  4673  CTGTATAGAATACAGTTCCTAGAAAAAACAGTTGTGAGTCAGGGTACCAAGAGACTTCCA  4614

Query  284   TCTTCCTTTCTCAAGGTCCTTGACCTAAGCCCCATCTGGTTGGTGGCCTCCTTTCTTCCC  343
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4613  TCTTCCTTTCTCAAGGTCCTTGACCTAAGCCCCATCTGGTTGGTGGCCTCCTTTCTTCCC  4554

Query  344   TTCAGGAGTCAGGCCCTCATTCCAATTCAGACCTGTTAGTAACTGTCCTCGGGGCCGGCG  403
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4553  TTCAGGAGTCAGGCCCTCATTCCAATTCAGACCTGTTAGTAACTGTCCTCGGGGCCGGCG  4494

Query  404   CTGTGCCCACTTGTACTGCAGAAACATCAGATAAGGCCTCAGTCCCAGTAGTCTCTGGCT  463
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4493  CTGTGCCCACTTGTACTGCAGAAACATCAGATAAGGCCTCAGTCCCAGTAGTCTCTGGCT  4434

Query  464   GCTGAGAACCTAAGCAGGGAGACTGTTGAGGTCCAAGTCAGCGCAGTTATGGGGGACGGA  523
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4433  GCTGAGAACCTAAGCAGGGAGACTGTTGAGGTCCAAGTCAGCGCAGTTATGGGGGACGGA  4374

Query  524   GACTCAAGTGAGCCCATTTGAGTCCTTCACAGTCTGCTGGATGTCTAAGCCAAGCTTATA  583
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4373  GACTCAAGTGAGCCCATTTGAGTCCTTCACAGTCTGCTGGATGTCTAAGCCAAGCTTATA  4314

Query  584   ACTTCGTATAGCATACATTATACGAAGTTATGTCGACCTCTGGGCATGTGAGAAGCCCCA  643
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4313  ACTTCGTATAGCATACATTATACGAAGTTATGTCGACCTCTGGGCATGTGAGAAGCCCCA  4254

Query  644   GGGCCCGTTGACAGTTGGTTTTTGCTAGTATGGATGTCTGGGGTTCCCTTGCCTTTCTTC  703
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4253  GGGCCCGTTGACAGTTGGTTTTTGCTAGTATGGATGTCTGGGGTTCCCTTGCCTTTCTTC  4194

Query  704   TCCCAGCCCAGGGAGCAGCTGCTGTATGCATTTTTACTCTTCCGTCCTGTAGGCCGATAC  763
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4193  TCCCAGCCCAGGGAGCAGCTGCTGTATGCATTTTTACTCTTCCGTCCTGTAGGCCGATAC  4134

Query  764   AAAGCAGGAGAAAAAGAGCCAGATCCCAAGACATGGAAGGCAAACTTCCGTTGTGCCATG  823
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4133  AAAGCAGGAGAAAAAGAGCCAGATCCCAAGACATGGAAGGCAAACTTCCGTTGTGCCATG  4074

Query  824   AACTCCCTGCCAGACATCGAGGAAGTGAAGGATCAGAGTAGGAACAAGGGCAGCTCTGCT  883
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4073  AACTCCCTGCCAGACATCGAGGAAGTGAAGGATCAGAGTAGGAACAAGGGCAGCTCTGCT  4014

Query  884   GTGCGGGTGTACCGGATGCTGCCACCCCTCACCAGGAACCAGAGGAAAGGTAGCCAAGGG  943
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4013  GTGCGGGTGTACCGGATGCTGCCACCCCTCACCAGGAACCAGAGGAAAGGTAGCCAAGGG  3954

Query  944   CACTGGGTCCTCGGGAGGAAGGGGAGGGAAGACGGGCAGAAGGAAGACCAGCTGCCCCTC  1003
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3953  CACTGGGTCCTCGGGAGGAAGGGGAGGGAAGACGGGCAGAAGGAAGACCAGCTGCCCCTC  3894

Query  1004  ACACAGCCTGTTCTTCCCACAGAGAGAAAGTCCAAGTCCAGCCGANACACT-ANAGCAAA  1062
             ||||||||||||||||||||||||||||||||||||||||||||| ||||| | ||||||
Sbjct  3893  ACACAGCCTGTTCTTCCCACAGAGAGAAAGTCCAAGTCCAGCCGAGACACTAAGAGCAAA  3834

Query  1063  CCCATGA-GCAGGTGANNGCCTCTAGTCAGCC  1093
              ||| || | ||||||  | |||||| |||||
Sbjct  3833  ACCAAGAGGAAGGTGAGTGGCTCTAGGCAGCC  3802


 Score = 68.0 bits (35),  Expect = 8e-08
 Identities = 35/35 (100%), Gaps = 0/35 (0%)
 Strand=Plus/Minus

Query  581   ATAACTTCGTATAGCATACATTATACGAAGTTATG  615
             |||||||||||||||||||||||||||||||||||
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Figure A4a: ***Sequence verification of SA5', reverse reaction**. The sequence matches 98 %. The missing nucleotides at position 46 and 49 were verified in the forward reaction, and were additionally verified in an additional sequencing reaction.*

Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer in Berlin und *Marburg* waren die Damen und Herren:

Prof. Dr. R.	Achazi	Prof. Dr. H.	Kreß
Prof. Dr.	Bartolomäus	Prof. Dr.	Kurreck
Dr.	Boshardt	Prof. Dr. H.	Kürschner
Prof. Dr. P.	Brouwer	Dr. O.	Liesenfeld
Dr.	Bunte	*Prof. Dr. M.*	*Lohoff*
Dr. M.	Christel	Priv.-Doz. Dr. J.	Lopez-Pila
Prof. Dr. W.	Dohle	Prof. Dr. Dr. h.c. R.	Menzel
Dr.	Drost	Prof. Dr.	Mielke
Dr. H.	Ellerbrok	Dr.	Müller-Gronbach
Dr. K.	Fiebig	Prof. Dr. R.	Mutzel
Prof. a.D. Dr. W.	Frey	Prof. Dr. C.	Niemitz
Prof. Dr. R.	Gorenflo	Prof. Dr. G.	Pauli
Prof. Dr. P.	Götz	Prof. Dr. H.-D.	Pfannenstiel
Prof. Dr. K.	Graszynski	Prof. Dr. H.-J.	Pflüger
Dr. J.	Gross	C.	Rasmus
J.	Groß	Prof. Dr. D.	Riegel
Prof. Dr. E	Hahn	Prof. Dr. M.-D.	Sacristán
Herr Prof. Dr. E.	Hartmann	Prof. Dr. J.	Schmitt
Prof. Dr. K.	Hausmann	Prof. Dr.	Schnarrenberger
Prof. Dr. R.	Heilbronn	Prof. Dr.	Schricker
Prof. Dr.G.	Heller	Dr. v.	Schwartzenberg
Prof. Dr. R.	Hengge-Aronis	Dr.	Skiebe-Corrette
Dr. I.	Hensen	Prof. Dr. J.	Steidle
Prof. Dr. H.	Hilger	Prof. Dr.	Sudhaus
Prof. Dr. M.	Hilker	Dr.	Tebbe
Dr. M.	*Huber*	Dr. D.	Tischendorf
Dr.	Ignatius	Prof. Dr. D.	Todt
Dr.	Kern	Prof. Dr. E.	Wachmann
Prod. Dr. G.	Korge	Dr.	Woltmann
Prof. Dr.	Koßmehl	Prof. Dr. I.	Zerbst

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1. Huber M, Brüstle A, Reinhard K, Guralnik A, Walter G, **Mahiny A**, von Löw E, LohoffM. (2008). „IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype“. *Proc Natl Acad Sci U S A*. 105(52):20846-51

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Parts of this thesis are prepared for publication.