„Role of Lymphotoxin-β Receptor Activation in Contact Hypersensitivity”

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

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Cindy Swett

To my mother because who I am today
Is because of her.

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Abbreviations

ACD	Allergic contact dermatitis
AIDS	Acquired immune deficiency syndrome
Ag	Antigen
APC	Antigen presenting cell
BAFF	B cell activation factor from the TNF family
BMCMC	Bone marrow-derived cultured mast cells
BMMCs	Bone marrow derived mouse mast cells
BSA	Bovine serum albumin
CHR	Contact hypersensitivity response
CHS	Contact hypersensitivity
DC	Dendritic cells
DcR3	Decoy receptor 3
DD	Death domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	2,4-dinitrofluorobenzene
dNTP	deoxyribonucleotide triphosphate
DTH	Delayed type hypersensitivity
EAE	Experimental allergic encephalomyelitis
ELC	Epstein-Barr virus-induced molecule-1 ligand chenokine
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FITC	Fluorescein-5-isothiocyanate
GM-CSF	Granulocyte macrophage-stimulating factor
HVEM	Herpes virus entry mediator
IC	Immune complex
ICAM-1	Intercellular adhesion molecule -1
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon gamma
IP-10	Inducible protein-10
KC	Keratinocytes

LC	Langerhans cells
LFA-1	Lymphocyte function-associated antigen-1
LIGHT	Lymphotoxin–like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells
LLNA	Local lymph node assay
LN	Lymph node
LT	Lymphotoxin
LTB_4	Leukotriene B_4
LTβR	Lymphotoxin beta receptor
MC	Mast cells
Mc	Macrophages
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein
MOG	Myelin oligodendrocyte glycoprotein
OVA	Ovalbumin
Oxa	Oxazolone
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll-*a* protein
PI	Propidium iodide
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear neutrophils
RANTES	Regulated upon activation normal T-cell expressed and presumably secreted
RNA	Ribonucleic acid
SDF-1α	Stromal derived factor-1alpha
SDS	Sodium dodecyl sulfate
sens	sensitised
SLC	Secondary lymphoid organ chemokine
TBS/Tw	Tris buffered salineTween-20
TCR	T cell receptor

TEMED	N,N,N’,N’-Tetramethyl-ethylenediamine
TGF	Transforming growth factor
TNCB	2,4,6-trinitrochlorobenzene
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
T reg	Regulatory T cells
Wt	Wild type

Abstract

Mast cells reside in close physical proximity to T cells in inflamed allergic tissues. Activation of T cells leads to the expression of $LT\alpha_1\beta_2$ or LIGHT, and since mast cells express lymphotoxin-β receptor (LTβR) on the membrane, this study examined the role of activated T cells interacting with mast cells via the LTβR-$LT\alpha_1\beta_2$/LIGHT system during the elicitation phase of contact hypersensitivity (CHS). To study whether the LTβR or its ligands are involved in CHS, mice deficient in either the LTβR or its ligand LIGHT were used. Both deficient mouse strains were not capable of developing a response 24 hours after challenge. The LTβR-Ig fusion protein was also used to block both ligands of the LTβR ($LT\alpha_1\beta_2$ and LIGHT) in CHS experiments but did not interfere with the regular CHS response. Adoptive transfer of sensitised lymph node cells into wild type, LTβR- and mast cell-deficient mice was also performed inducing the late phase (48 hours) response only. In conclusion, additional experiments are necessary to elucidate the role of the LTβR at the early (24 hours) phase in CHS. On the other hand, these results suggest that LTβR activation mediates a downregulatory effect on the late (48 hours) effector phase of CHS. However, additional experiments are necessary to demonstrate if the downregulatory effect is mediated by the activation of the LTβR on mast cells.

1 Introduction

1.1 Hypersensitivity reactions

The term hypersensitivity is applied when an adaptive immune response occurs in an exaggerated or inappropriate form. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately sometimes causing inflammatory reactions and tissue damage. Hypersensitivity reactions occur after re-exposure to foreign substances in previously sensitised individuals. Four types of hypersensitivity reactions (Type I, II, III and IV) have been described but in practice these types do not necessarily occur independently of each other. The first three types are antibody-mediated and are characterised by the different types of antigens recognised and the different classes of antibody involved. Type IV hypersensitivity reactions are mainly mediated by T cells (Roitt I. *et al*., 1998).

1.1.1 Type I hypersensitivity

Type I Hypersensitivity, also called immediate hypersensitivity or anaphylaxis, is mediated by allergen binding to IgE on mast cells (Serafin *et al*., 1987). This results in activation of Fcε receptors and degranulation and release of mast cell mediators, thus causing the allergic reactions (Brostoff J. *et al*., 1998). IgE-sensitised mast cells produce an acute inflammatory reaction with symptoms such as asthma or rhinitis.

1.1.2 Type II hypersensitivity

This type of hypersensitivity reaction is caused by antibodies other than IgE, which are directed against antigen on an individual's own cell-surface or extra-cellular matrix antigens. This leads to complement activation and recruitment of inflammatory cells and thus to tissue destruction (Roitt I. *et al*., 1998).

1.1.3 Type III hypersensitivity

In type III hypersensitivity, antibodies form circulating immune complexes with antigen, which are subsequently deposited in tissues, where they cause damage (Roitt I. *et al*., 1998).

1.1.4 Type IV hypersensitivity

Type IV hypersensitivity is also called delayed-type hypersensitivity (DTH) because takes more than 12 hours to develop clinically. This type of hypersensitivity is cell-mediated, which was demonstrated by induction of CHS with transfer of sensitised cells and not by serum (Barnetson R. *et al*., 1998). However, it was also shown that DTH is mediated by humoral immune reactions which will be explained later in this chapter. Three variants of DTH are recognised two of which, namely contact hypersensitivity (CHS) and tuberculin-type hypersensitivity, occur within 24 to 72 hours of antigen challenge. Granulomatous hypersensitivity reactions develop over a period of 21 to 28 days (Barnetson R. *et al*., 1998).

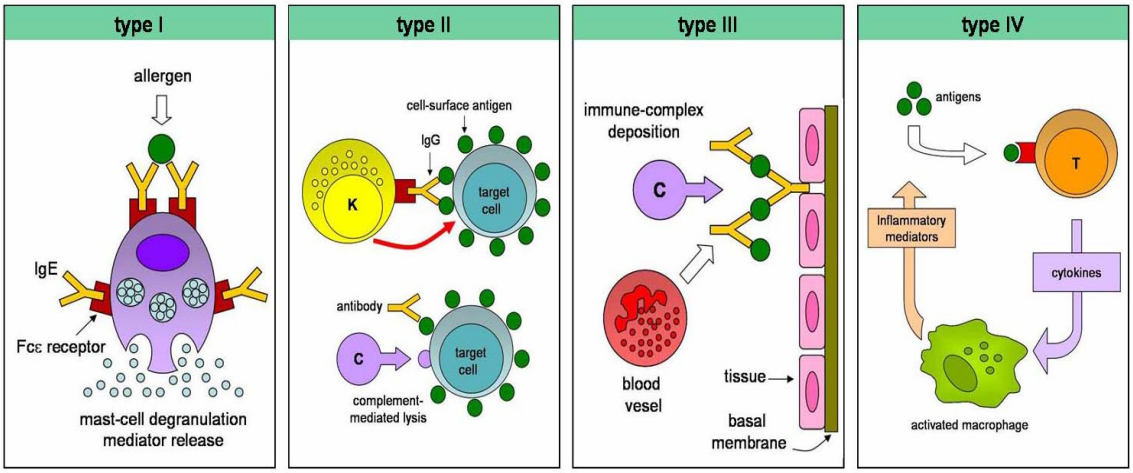


Figure 1.1 There are four types of hypersensitivity reaction that can cause tissue damage. Type I: mast cells bind to IgE via their Fc receptors and IgE becomes cross-linked after antigen recognition resulting in degranulation of mast cells. Type II: antibody is directed against antigen on the surface of individuals own cells of foreign antigen. Type III: immune complexes are deposited in tissue and induce infiltration of inflammatory cells. Type IV: sensitised lymphocytes release cytokines following second contact with the allergen, inducing infiltration of inflammatory cells (Brostoff J. *et al*., 1998).

1.2 Contact hypersensitivity response

Contact hypersensitivity (CHS) is a form of delayed-type hypersensitivity and is characterised by an eczematous reaction at the point of contact with allergens such as haptens (Janeway C. *et al*., 2001). After contact with an antigen, subsequent challenge with the same antigen induces a series of immunological events. CHS is easily

reproduced in mice and serves as an excellent model of T-cell mediated inflammation pertinent to autoimmunity and allergy (Barnetson R. *et al*., 1998).

Numerous studies demonstrated the importance of the skin in the generation of contact hypersensitivity and other forms of delayed-type hypersensitivity. Induction of allergic contact dermatitis requires the interaction of allergen with the skin (Macher *et al.*, 1969a; Macher *et al.,* 1969b). The skin is a unique site for sensitisation, because haptens injected into organs other than the skin induced specific unresponsiveness. Thus, the immunological outcome, which is either sensitisation or tolerance is influenced by the route of administration of the antigen. In addition, the response is also influenced by the antigen used and the concentration of the antigen.

1.2.1 Irritants

Irritants and allergens have similarities but they induce different immunological reactions after application. Contact with irritants can also damage the skin by toxic mechanisms not mediated by hypersensitivity and produce eczema (Barnetson R. *et al*., 1998). Non allergic antigen responses are typically defined by a lack of measurable serum IgE or negative skin test reactivity because DTH is not a feature of the response to these antigens. Numerous investigations were preformed to elucidate the immune responses to irritants, which could contribute to the understanding of development of allergy and for identifying new targets for therapy (Woodfolk *et al*., 2002).

1.2.1 Haptens

Hapten is the term introduced for low molecular weight chemicals, which often have a molecular weight less than 1 kDa (Barnetson R. *et al*., 1998). Haptens are too small to be antigenic by themselves. They penetrate the epidermis and conjugate by covalent binding to proteins, thereby inducing the production of hapten-specific antibodies in experimental animals. Later it was demonstrated that not only B cells but also T cells might mount specific responses (Pohlit *et al*., 1979).

Many studies demonstrated the molecular basis for the recognition of haptens by T cells. Ortmann and co-workers demonstrated that hapten recognition by T cells required hapten-peptide conjugates covalently associated to MHC molecules (Martin *et al*., 1992; Ortmann *et al*., 1992). Moreover, other suggestions exist, such as hapten may also be covalently attached to MHC molecules or to proteins that are subsequently processed (Dieli *et al*., 1998). This mechanisms are further studied since haptenic chemicals and drugs represent major sources of allergens for human (Thierse *et al*., 2005). Also, certain contact allergens have unsaturated carbon bonds and are easily oxidised. Some haptens, such as dinitrochlorobenzene (DNCB), sensitise nearly all individuals and can be used to assess cell mediated immunity. Epicutaneous applied DNCB binds to epidermal proteins through the amino groups of the lysine residues. The sensitising potential of a hapten cannot be reliably predicted from its chemical structure, but there is a correlation with the number of haptens attached to the carrier and the ability of the molecule to penetrate the skin (Barnetson R. *et al*., 1998).

In this work oxazolone was used as a sensitising agent. The mechanism by which oxazolone is recognised by antigen presenting cells (APC) is not well understood. However, it has been shown that oxazolone is derived from 8-oxoguanine (8-oxo-G), which is one of the most common DNA lesions present in normal tissues due to exposure to reactive oxygen species (Tretyakova *et al*., 1999). 8-oxo-G is highly susceptible to secondary oxidation by endogenous oxidising agents like peroxynitrite, leading to the formation of 2'-deoxy-β-D-erythro-pentofuranosyl derivatives of oxaluric acid, oxazolone, and cyanuric acid (Luo *et al*., 2001). Mutation frequency and specificity of these three secondary oxidative products was analysed and it was shown that they were much more mutagenic than the parent 8-oxo-G (Henderson *et al*., 2002). Considering that oxazolone is a potent mutagen, it may modify exogenous or endogenous proteins of the dermis which are then further processed to yield MHC-binding oxazolone-peptide conjugates (Martin 2004). Oxazolone might also directly modify peptides already associated with MHC molecules on the surface of APC or may also function indirectly by altering the intracellular processing of self proteins (Weltzien *et al*., 1996)

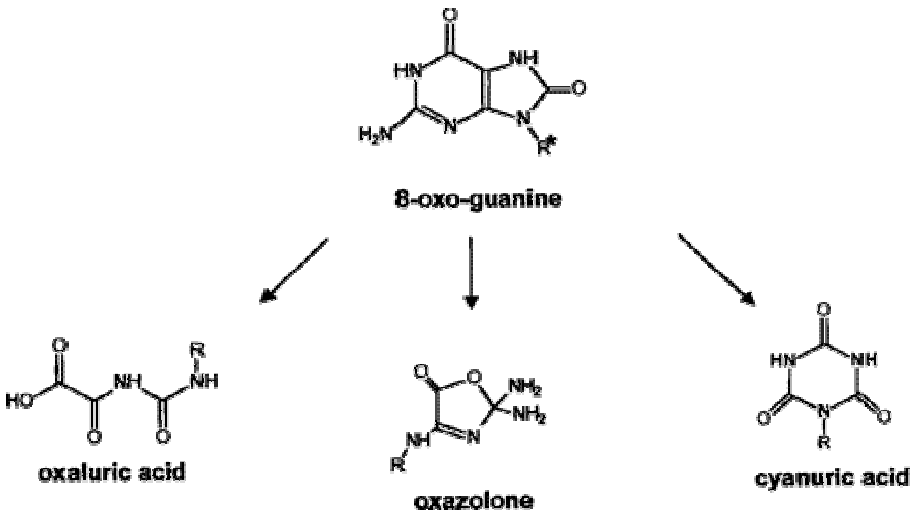


Figure 1.2 8-Oxo-G-Containing DNA treated with peroxynitrite results in secondary oxidation products: Oxaluric acid, oxazolone and cyanuric acid. (*R = rest of DNA) (Tretyakova *et al*., 2000).

1.2.3 T cell immune response to haptens

T lymphocytes comprise a central part of the immune defence system. Their antigen T-cell receptors (TCR) are specialised to interact with antigens on cellular surfaces in conjunction with class I ($CD8^+$ cytotoxic T cells) or class II ($CD4^+$ helper T cells) gene products of the major histocompatibility complex (MHC) (Davis *et al*., 1993). This event is known as MHC-restricted antigen recognition and results from rigorous selection processes during T cell maturation in the thymus. Surface of MHC-peptide which contacts the TCR leads to the activation of the respective T cell population. Typically, T cells are focused on the detection of protein determinants.

1.3 Sensitisation stage

Contact hypersensitivity responses are mediated by T cells following epicutaneous application of allergens (haptens) to the skin (Barnetson R. *et al*., 1998). Following primary application of the hapten to the skin, the hapten combines with a cutaneous protein and is recognised by epidermal APCs. Langerhans' cells (LCs) are the principal

APCs involved in CHS. They internalise the hapten-peptide conjugate and this induces their activation and maturation. They leave the epidermis and migrate to the paracortical areas of regional lymph nodes (Barnetson R. *et al*., 1998). LCs present the processed hapten-protein conjugates in association with MHC class II molecules to naïve CD4 lymphocytes, which induces activation and production of memory $CD4^+$ T cells. Activated T cells begin to recirculate to peripheral tissues ready to recognise the second application of the hapten to the skin (elicitation) (Janeway C. *et al*., 2001).

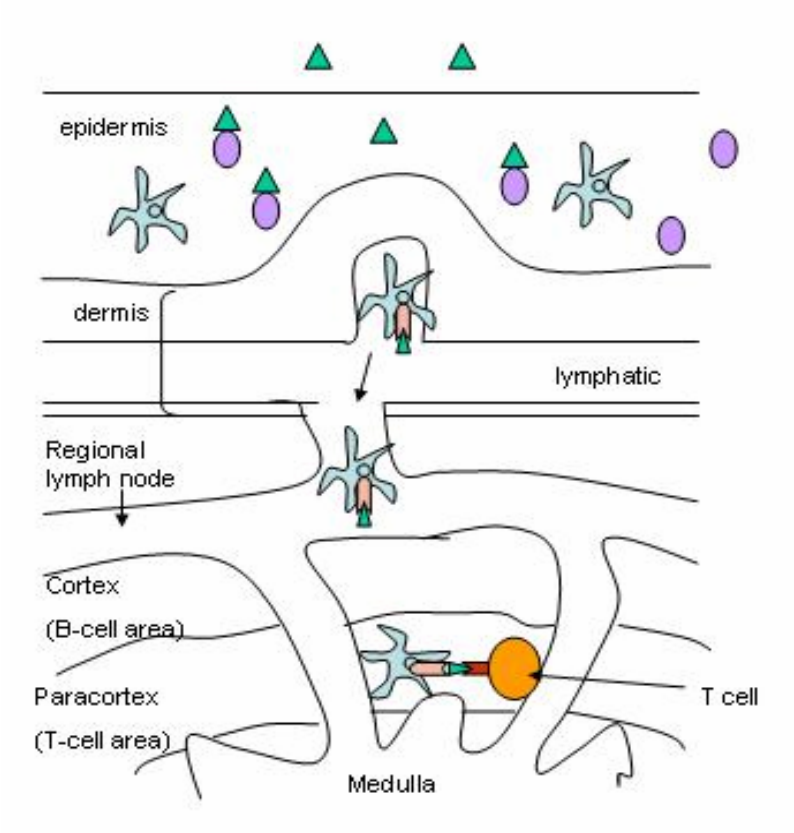


Figure 1.3 Sensitisation stage of contact hypersensitivity response. Hapten binds to a protein carrier and is recognised by Langerhans' cells, which internalise the antigen and migrate via afferent lymphatics to the paracortical area of the regional lymph nodes. Here they present processed hapten-protein conjugates in association with MHC class II molecules to $CD4^+$ lymphocytes, producing a population of memory $CD4^+$ T cells (Barnetson R. *et al*., 1998).

1.3.1 Role of Langerhans' cells and keratinocytes in contact hypersensitivity

In the sensitisation phase many cells are involved in the activation of antigen presenting cells. Contact hypersensitivity is primarily an epidermal reaction and the dendritic cell (Langerhans' cell) which is located in the suprabasal epidermis, is the principal antigen-

presenting cell involved (Barnetson R. *et al*., 1998). They are derived from the bone marrow and express MHC class II molecules, surface Fc and complement receptors. In vitro, Langerhans' cells act as antigen presenting cells and are more potent in this regard than monocytes. However, the mechanisms by which Langerhans' cells process the antigen is unknown. Antigens may be internalised and then presented via MHC molecules and stay on the surface for antigen presentation. Inactivation of Langerhans' cells with ultraviolet B radiation can prevent or alleviate the effects of contact hypersensitivity (Simon *et al*., 1991; Simon *et al*., 1994). Moreover, Langerhans' cells are also involved in the elicitation phase of contact hypersensitivity because depletion of Langerhans' cells previous to elicitation enhances the response (Grabbe *et al*., 1995).

Langerhans' cells activation is associated with induction of cytokine secretion (IL-1β, IL-6, IL-12, chemokines), enhanced expression of cell surface molecules (MHC class I and II, adhesion molecules, costimulatory molecules), and antigen uptake, processing and presenting capacity (Grabbe *et al*., 1998). In CHS, activation and migration of Langerhans' cells to the draining lymph nodes appears to depend on the capacity of haptens to induce IL-1β production in Langerhans' cells. IL-1β is a selective and almost immediate effect of epicutaneous hapten application and is not observed with irritants or tolerogens (Enk *et al*., 1992a; Enk *et al*., 1993; Enk *et al*., 1995). In addition, other studies demonstrated that the application of allergen, but not vehicle or irritant control, caused an up-regulation MHC class II molecules on Langerhans' cells in the epidermis within 24 hours after hapten application, as well as a change in Langerhans cell morphology (Aiba *et al*., 1990). Moreover, activated keratinocytes release tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which contributes to Langerhans' cells activation and migration. Activation of Langerhans' cells can be due to direct effects of hapten on Langerhans' cells themselves or can also be initiated by keratinocytes that secrete inflammatory cytokines as a result of hapten application (Barnetson R. *et al*., 1998).

Keratinocytes provide the structural integrity of the epidermis and have a central role in epidermal immunology (Barnetson R. *et al*., 1998). They produce a range of cytokines

important for contact hypersensitivity response including IL-1, IL-3, IL-6, IL-8, GM-CSF, M-CSF, TNF, TGFα, and TGFβ. IL-3 can activate Langerhans' cells, co-stimulate proliferative responses and recruit mast cells, and also induce the secretion of immunosuppresive cytokines (i.e. IL-10 and TGFβ) which induce clonal anergy (immunological unresponsiveness) in T_H1 cells. Activation of keratinocytes is induced by application of allergens or irritants on the skin and this induces the release of TNF and GM-CSF which activates Langerhans' cells. Keratinocytes may also express MHC class II molecules and ICAM-1 in their cell membrane (Janeway C. *et al*., 2001).

1.3.2 Cytokine profile in the sensitisation phase of contact hypersensitivity

Enk and Katz found that within the first hour of allergen application to the skin, expression of mRNA was enhanced for IL-1β (within 15 minutes), followed by TNF (within 30 minutes), and IFN-γ (within 1 hour) (Enk *et al*., 1992a). The upregulation of these cytokines was followed by enhanced expression of IP-10 (within 2 hours of hapten application), macrophage inflammatory protein-2 (MIP-2) and IL-1α (within 4 hours), as well as MHC class II (within 6 h), whereas β-actin signals used as a control remained unchanged. They also found that TNF and IFN-γ were up-regulated rather nonspecifically after application of the vehicle control to the skin. In contrast, signals for IL-1β, IL-1α, MIP-2, IP-10 and I-Aα were induced only after application of the allergen.

Epicutaneous application of TNCB or injection of IL-1β caused almost identical changes in the epidermal mRNA pattern, that is, enhanced expression (10-200 fold) of MHC class II I-Aα, MIP-2, TNF, IL-1α, IL-10, and even IL-1β (Enk *et al*., 1995). In contrast, injection of TNF and IL-1α induced only MIP-2 expression. The MIP-2 mRNA expression caused by IL-1β or TNCB was much more striking than that caused by IL-1α or TNF. However, a higher concentration of IL-1α (10 to 50 times) induced changes of epidermal cytokines qualitatively similar to those caused by IL-1β or TNCB. In addition, IL1β-deficient mice showed defective contact hypersensitivity responses to topically applied trinitrochlorobenzene (TNCB) (Shornick *et al*., 1996). This result demonstrated an important role for IL-1β in the initiation of the sensitisation phase.

1.4 Elicitation stage of contact hypersensitivity

Most studies of CHS have focused on the sensitisation phase, because it is at this point that naïve T cells are activated and differentiated into antigen specific effector T cells. However, clinically, the elicitation phase is much more important because the sensitisation phase is generally asymptomatic, whereas the elicitation phase results in the symptomatic allergic contact dermatitis (Grabbe *et al*., 1998).

After sensitisation, a secondary application of the same antigen to the skin (challenge) induces a series of immunological events which is known as the elicitation phase. In the elicitation phase many cell populations and mediators are involved to produce tissue damage. Briefly, antigen presentation by langerhans' cells to memory T cells occurs in the skin, with release of T cell cytokines (Janeway C. *et al*., 2001). Mast cells degranulate and release cytokines soon after contact with an allergen. In addition, TNF and IL-1 are secreted from many cell types, and from macrophages in particular, which are potent inducers of endothelial cell adhesion molecules (Barnetson R. *et al*., 1998). After challenge, locally released cytokines (TNF and IFN-γ) produce a gradient signal for movement of inflammatory cells towards the site of antigen challenge. Many cell types, cytokines and adhesion molecules are involved in the infiltration of inflammatory cells. In order to explain the molecular mechanisms involved, the elicitation stage is divided in two phases: an early (within 24 hours after challenge) and a late (48 hours after challenge) phase.

1.4.1 Early phase (within 24 hours) in contact hypersensitivity

1.4.1.1 Role of B lymphocytes and complement in contact hypersensitivity

Several groups demonstrated that the early and late elicitation phase was substantially impaired in B1-B cell deficient CBA/N-xid mice (Askenase *et al*., 1999; Paliwal *et al*., 2002). B1-B cells are the source of natural immunoglobulins (Igs) of the IgM isotype which are potent activators of the classical pathway of complement (Tsuji *et al*., 2002). Thus, hapten-IgM immune complexes may be formed when antigen enters the circulation and activate the complement system, which leads to the release of C5a.

Complement is considered to contribute to the pathogenesis of CHS. In a mouse model using either picryl chloride or oxazolone as a contact allergen C5a was shown to contribute significantly to the elicitation phase (Tsuji *et al*., 1996; Tsuji *et al*., 1997; Tsuji *et al.,* 2000). Tsuji and co-workers sensitised their mice at the shaved chest and abdomen. Challenge was performed by painting both pinnae which developed two distinct inflammatory reactions occurring at 2 and 24 hours after allergen challenge. The early 2 hours reaction was characterised by minor pinna swelling. Within the first 2 hours, ear extracts demonstrated strong chemotactic activity towards the J744A.1 macrophage cell line, which could be blocked by an anti-C5a antiserum and which was absent in C5aR deficient peritoneal macrophages. Thus, C5a is a crucial chemoattractant for neutrophils and lymphocytes generated during the first 2 hours after challenge (Nataf *et al*., 1999). In contrast, no C5a chemotactic activity could be found in pinna extracts 24 hours after challenge. The reaction at 24 hours was characterised by infiltration of $CD4^+$ T_H1 cells and release of IFN-γ. Then infiltration of mononuclear leukocytes and granulocytes was induced by chemokines such as MIP 1 α/β, IFN-γ inducible protein 10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) in the dermis. Moreover, this 24 hours inflammatory response was absent in C5- or C5aR-deficient mice and can be blocked by C5a-neutralising antibody. Thus, the early C5a mediated response is required to recruit T cells to elicit the 24 hours CHS (Kohl, 2001).

After challenge, C5a may also be generated locally by skin macrophages, Langerhans' cells, or keratinocytes. C5a may activate local mast cells, resulting in degranulation and release of MIP-2, TNF and serotonin as well as activation of endothelial cells and upregulation of adhesion molecules such as β_2-integrins and P-selectin (Foreman *et al*., 1994; Jagels *et al*., 2000). Circulating hapten-specific T cells will bind to the activated endothelial cells and then migrate across the endothelium into the challenged site of the skin. Then T_H1-cells interact with hapten-MHC molecules on local antigen presenting cells. The presence of T_H1 cells marks the onset of the 24 hours after challenge phase in CHS, with high amounts of IFN-γ in pinna extracts (Kohl, 2001).

1.4.1.2 Role of adhesion molecules in contact hypersensitivity

Mast cell activation induces the release of mediators such as TNF, MIP-2, and serotonin which induces an increase in vascular permeability and activates endothelial cells (Feng *et al*., 1995; Hang *et al*., 1999; McHale *et al*., 1999a). Activated endothelial cells induce the expression of adhesion molecules such as VCAM-1, ICAM-1, P-selectin, and E-selectin on the luminal surface (McHale *et al*., 1999a; McHale *et al.*, 1999b) The expression of adhesion molecules mediates the recruitment of inflammatory cells into the challenged site.

In response to an inflammatory stimulus, leukocytes undergo a sequential cascade of events to leave the circulation, which involves initial tethering and rolling of leukocytes on endothelial cells. These events are entirely dependent on E- and P-selectins on the endothelium and are required for subsequent integrin-dependent firm adhesion and transendothelial migration of leukocytes (Granger *et al*., 1994; Kubes *et al*., 2000; Kubes *et al*., 2001). Staite and co-workers demonstrated that mice lacking E-selectin and P-selectin display attenuated leukocyte recruitment at 24 hours after challenge (Staite *et al*., 1996). Inhibition of α_4-integrin can also reduce leukocyte recruitment at the 24-hour phase of CHS (Chisholm *et al*., 1993; Issekutz *et al*., 2002; McHale *et al.*, 1999b). Since it is already known that selectins mediate rolling and the integrins mediate adhesion, and leukocyte recruitment is dependent on both rolling and adhesion, inhibition of either would reduce leukocyte recruitment. Interestingly, in the case of lymphocytes, the α_4-integrin can also support tethering and rolling (in addition to adhesion), allowing for lymphocytes to entirely bypass any need for selectins (Grabovsky *et al*., 2000; Hickey *et al*., 1999; Johnston *et al*., 1999).

Hwang and co-workers investigated leukocyte trafficking during the first 2 hours and 24 hours after challenge (Hwang *et al*., 2004). They used noninvasive intravital microscopy to visualise leukocyte rolling and adhesion at both time points in the dermal microvasculature. A very profound increase in leukocyte-endothelial cells interactions was noted within the first 2 hours. This was followed by a second wave of leukocyte recruitment between 4 and 24 hours after challenge. Within the first 2 hours the infiltrate

consisted mainly of neutrophils and a small subset of lymphocytes, this cells were recruited via E- and P- selectin. In contrast, leukocyte recruitment at 24 hours after challenge was primarily mediated by α_4-integrin (Johnston *et al*., 1999). Additional experiments with anti-E- and anti-P-selectin revealed that complete abrogation of the 2 hour phase of leukocyte-endothelial cell interactions eliminated leukocyte recruitment at 24 hours. $CD4^+$ lymphocyte recruitment depending on E- and P-selectin was demonstrated within the first 2 hours and these cells were essential for the subsequent 24 hour response. Despite the predominance of neutrophils in the first 2 hours after challenge, specific elimination of these cells did not affect the response after 24 hours. Therefore, the vascular endothelium was primed by $CD4^+$ lymphocytes (within the first 2 hours) to recruit various leukocyte populations at 24 hours after challenge in CHS (Hwang *et al*., 2004).

Recruitment of $CD4^+$ lymphocytes in the first 2 hours obviously modified the microenvironment to allow for the 24-hour recruitment of α_4-integrin-dependent leukocytes (Johnston *et al*., 1999). Hwang and co-workers suggested that the 2-hour leukocyte-endothelial cell interaction activated the endothelium to express VCAM-1, the α4-integrin ligand. In addition, it has been shown that VCAM-1 expression is elevated by 4 hours after challenge (McHale *et al.*, 1999b). Therefore, 2 hours after challenge leukocyte recruitment is selectin-mediated and 24 hours after challenge it may be α_4-integrin mediated.

1.4.1.3 Role of mast cells in contact hypersensitivity

Askenase and co-workers demonstrated that mast cells are involved in CHS (Askenase *et al*., 1983). They showed that two different strains of mast cell-deficient mice (W/W^v and SI/SId) developed a defective CHS response. Adoptive transfer experiments demonstrated that the defect in mast cell-deficient mice to develop CHS was due to defective elicitation, rather than a defect in the induction of effector T cells. They demonstrated this because sensitised cells from wild type mice could not transfer CHS responsiveness to mast cell-deficient mice, but CHS response could be transferred to wild type mice with sensitised cells from mast cell-deficient mice (SI/SId). This experiment

was performed in the two different strains of mast cell-deficient mice. Since both strains of mast cell-deficient mice with completely different genetic defects developed an impaired CHS, it is likely that mast cell deficiency is the cause of their inability to manifest regular CHS.

Different pathways exist for activation of mast cells in CHS (Secor *et al*., 2000). High affinity IgE receptor (FcεRI) on mast cells is a well characterised pathway of mast cell activation. IgG_1 and IgG_{2b} are also involved in activation of mast cells. In this mouse model of experimental allergic encephalomielitis (EAE), which is induced with myelin oligodendrocyte glycoprotein (MOG) to produce both IgG_1 and IgG_{2b}, these antibodies can activate mast cells since they express the receptors FcγRIIB/III. In addition, mast cells can also be directly activated independently of immunoglobulins (Ebertz *et al*., 1987; Theoharides *et al*., 1993). Mast cell can be activated by neuropeptides, such as substance P, certain complement components (i.e. C5a) and estradiol. Many studies indicate that direct interaction with activated T cells may be sufficient for mast cell activation, but this will be explained later.

Askenase and co-workers noted that CHS was preferentially induced in those cutaneous sites that were especially rich in serotonin (5-hydroxytryptamine (5-HT)-containing mast cells (pinnae and footpads) (Askenase *et al*., 1980). During the evolution of CHS response, local activated mast cells release serotonin (5-HT) between 6 and 18 hours after challenge. Examination of mast cells revealed surface activation, indicated by extension of surface filopodia, and degranulation by fusion and exocytosis. The endothelium of postcapillary venules at sites of CHS revealed the development of gaps between adjacent cells. The development of gaps permitted extravasation of tracers that was abolished by depletion (by reserpine) or antagonism of 5-HT. These endothelial gaps are important for the infiltration of inflammatory cells. Thus, mast cells degranulated and released 5-HT in CHS, and this 5-HT acted on local vessels. Transfer of non-Ig bearing sensitised lymphocytes also demonstrated similar mast cell degranulation and the formation of endothelial gaps. This indicated that mast cell degranulation and 5-HT release in murine CHS were probably T cell dependent (Askenase *et al*., 1980). In addition, pretreatment of

cutaneous sites with serotonin causes specific desensitisation of vascular serotonin receptors and inhibits subsequent elicitation of CHS response.

Biedermann and co-workers demonstrated that mast cells can determine the pattern of cells infiltrating into the sites of inflammation through the chemokines they produce (Biedermann *et al*., 2000). Mast cells are capable of producing high amounts of TNF and chemokines including macrophage inflammatory protein 2 (MIP-2), which is an orthologue of human IL-8. They showed that TNF and MIP-2 were essential for appropriate PMN recruitment during CHR and that both were dependent on the presence of mast cells. In addition, TNF was not required for MIP-2 induction during CHR. The biological function of TNF seems to be the induction of expression of adhesion molecules required for PMN attachment to endothelial cells (McHale *et al.*, 1999a). The chemokine MIP-2 establishes a chemotactic gradient required for diapedesis and directed migration of PMNs into the site of inflammation (Feng *et al*., 1995; Hang *et al*., 1999).

In CHS histamine a biogenic amine is released from mast cells in the skin, causing urticaria and itching. This chemical messenger displays numerous functions mediated through at least four pharmacologically distinct receptors i.e. H_1 to H_4 (Hill *et al*., 1997). H_1 receptor is expressed in peripheral nerves, keratinocytes and endothelial cells. The characteristic feactures of H_1 receptor activation in the skin are itching and increased vascular permeability. Because many of these functions contribute to allergic responses, H_1 receptor antagonists have been successfully used as drugs for treating allergies (Seike *et al.,* 2005).

1.4.1.4 Role of T cells in contact hypersensitivity

During sensitisation, Langerhans' cells present the antigen via MHC complexes to specific T cells resulting in primary activation. During the elicitation stage, primed T cells circulate through sites of subsequent hapten exposure and, following interaction with hapten-presenting cells, they are induced to produce inflammatory cytokines such as TNF and IFN-γ (Weigmann *et al*., 1997). As a result, further inflammatory cells are recruited to the site of challenge to develop CHS. Which T cell subpopulation mediates

CHS, however, remains unclear, as reports are conflicting. Many groups have reported that $CD4^+$ T cells (T_H1 and/or T_H2) are responsible for mediating CHS responses (Gautam *et al*., 1991; Hauser 1990; Miller *et al*., 1985). Other groups have provided evidence that both $CD4^+$ and $CD8^+$ T cells are responsible for inducing CHS (Gocinski *et al*., 1990), and that an additional subset of $CD4^+$ T cells has a down-regulatory role (Gocinski *et al*., 1990). Xu and co-workers demonstrated the involvement of IFN-γ producing $CD8^+$ effector cells (T_C1) in CHS, with a role of interleukin-4 (IL-4)/IL-10 secreting $CD4^+$ T cells (T_H2) as down-regulators (Xu *et al*., 1996). Thus, there is a role of $CD8^+$ T cells and $CD4^+$ T cells (T_H1 and T_H2) in CHS, and each $CD4^+$ T cells subset might influence either the early (within 24 hours) or late (48 hours) elicitation phase.

1.4.1.5 Role of $CD8^+$ T cell in contact hypersensitivity

$CD8^+$ T cells are cytotoxic and the killing can be receptor-mediated or granule-dependent mechanisms. In addition, they can also produce cytokines. The cytokines produced by many $CD8^+$ cytotoxic T cells are similar to the spectrum released from T_H1 cells (Xu *et al*., 1996). Therefore these cells are called T_C1. $CD8^+$ cells that produce T_H2-type cytokines (T_C2) are associated with regulatory and suppressor functions. The differentiation of $CD8^+$ T cells may be affected by the cytokines produced by $CD4^+$ T cells (Biedermann *et al*., 1999; Fiorentino *et al*., 1989). Therefore, IFNγ and IL-12 may induce T_C1 and IL-4 T_C2 generation.

Transfer experiments demonstrated the role of $CD8^+$ T cells in CHS (Ross *et al*., 2001). Transfer of hapten-sensitised $CD8^+$ T cells into naïve non-sensitised mice confers CHS after challenge. Moreover, depletion of $CD8^+$ T cells *in vivo* before challenge with DNFB diminished CHS and depletion of $CD4^+$ T cells enhanced the CHS response (Gocinski *et al*., 1990). The experiments performed in the mouse system correlate with the results obtained from AIDS patients. AIDS patients have a dramatic reduction of $CD4^+$ T cells and they still develop clinical symptoms upon contact with haptens (Viraben *et al*., 1994).

1.4.1.6 Role of $CD4^+$ T cells in contact hypersensitivity

$CD4^+$ T cells have two different profiles of cytokine production, which dissects the two basic types of response mediated by $CD4^+$ T-helper cells, in T_H1 and T_H2 T cells (Roitt I. *et al*., 1998). T_H1 cells secrete IFNγ, LT, and IL-2 and they are involved in cell-mediated inflammatory reactions (Mosmann *et al*., 1986). T_H1 cytokines activate cytotoxic, inflammatory and delayed type hypersensitivity reactions. In contrast, T_H2 cells cytokines include IL-4, IL-5, IL-9, IL-10 and IL-13 and they are specialised for B cell activation and support antibody production, especially IgE, which is involved in allergic responses (Janeway C. *et al*., 2001). Cytokines from T_H1 cells inhibit the actions of T_H2 cell and *vice versa* (Fiorentino *et al*., 1989). Thus, an immune response settles into a T_H1 or a T_H2-type of response.

1.4.1.6.1 $CD4^+$ T_H1 T cells are responsible for the early response (24 hours)

Cher and Mosmann showed that T_H1 clones have a major role in CHS (Cher *et al*., 1987). They performed adoptive transfer of T_H1 clones producing IL-2 and IFN-γ which was able to induce CHS. The CHS response was characterised by vascular leakage and infiltration of inflammatory cells. Some cytokines produced by T_H1 clones are IFN-γ, IL-2, and LT (Ferreri *et al*., 1992). IFN-γ has been shown to mediate some of the CHS characteristics like induration, erythema, and mononuclear phagocyte infiltration in human (Nathan *et al*., 1986). Treatment with anti-IFN-γ showed that this cytokine is an important mediator in the effector phase of CHS induced by T_H1 clones. However, anti-IFN-γ did not always suppress the increase in vascular permeability accompanying the T_H1-induced CHS response and had no effect in the histological profile (Fong *et al*., 1989). In the transfer model using two T_H1 clones derived from C57BL/6 mice, treatment with anti-IFN-γ before challenge did not inhibit the response measured at 24 hours after challenge. However, anti-IFN-γ treatment at 24 hours after challenge inhibited the response measured at 48 hours.

IFN-γ is a potent macrophage activating factor, which enhances their antimicrobial and tumoricidal activity, and stimulates their expression of MHC molecules and FcγR

(Schultz *et al*., 1983; Warren *et al*., 1985). IFN-γ has no chemotactic activity. However, activated macrophages release LTB_4 which is chemotactic for granulocytes and macrophages (Goetzl *et al*., 1981; Smith *et al*., 1980). LTB_4 stimulates the production of IL-2 and IFN-γ by lymphocytes and IFN-γ enhances the release of LTB_4 from macrophages. This reciprocal stimulatory effect of IFN-γ and LTB_4 is believed to be a mechanism by which the CHS response is propagated and amplified after challenge.

IFN-γ, produced by T_H1 clones, is involved in the CHS response (Fong *et al*., 1989). However, anti-IFN-γ was never able to completely suppress the response, therefore other factors must be involved in the CHS response. The adoptive transfer of two T_H1 clones derived from C57BL/6 mice induced a 24 hours response after challenge and anti-IFNγ treatment did not inhibit the response (Fong *et al*., 1989). Other factors involved in this response are probably IL-2, LT (from T_H1-specific cytokines) and also IL-3 and GM-CSF (T_H1/T_H2 common products), it has been suggested that IL-2 may have some effects in vascular permeability (Ettinghausen *et al*., 1988). Fong and Mosmann suggested that LT can stimulate macrophages to release reactive oxygen intermediates, thus it could contribute to the effector phase of CHS (Fong *et al*., 1989). In addition, IL-3 and GM-CSF, which are products of T_H1 and T_H2 T cells, have stimulating effects on macrophages (Metcalf *et al*., 1986; Weiser *et al*., 1987). GM-CSF is chemotactic for monocytes and granulocytes and these cells could contribute to the CHS response independently of IFN-γ. Another mechanism involved is secretion of IL-12 from activated macrophages, acting as a costimulator of T_H1 cell activation (Barnetson R. *et al*., 1998).

During the first 2 hours of CHR selective recruitment of $CD4^+$ lymphocytes takes place into the extravascular space at the challenged site (Hwang *et al*., 2004). As discussed earlier, it has been shown that C5a is critical during the first 2 hours after challenge to induce subsequent oedema formation and elevated levels of IFN-γ which is characteristic of the CHR after 24 hours (Tsuji *et al.*, 2000). Since transfer of isolated T lymphocytes from immunised mice can completely reconstitute the 24 hour CHR response (Ptak *et al*.,

1991), C5a apparently recruits T_H1 cells within the first 2 hours after challenge and these cells induce the 24 hours CHR response.

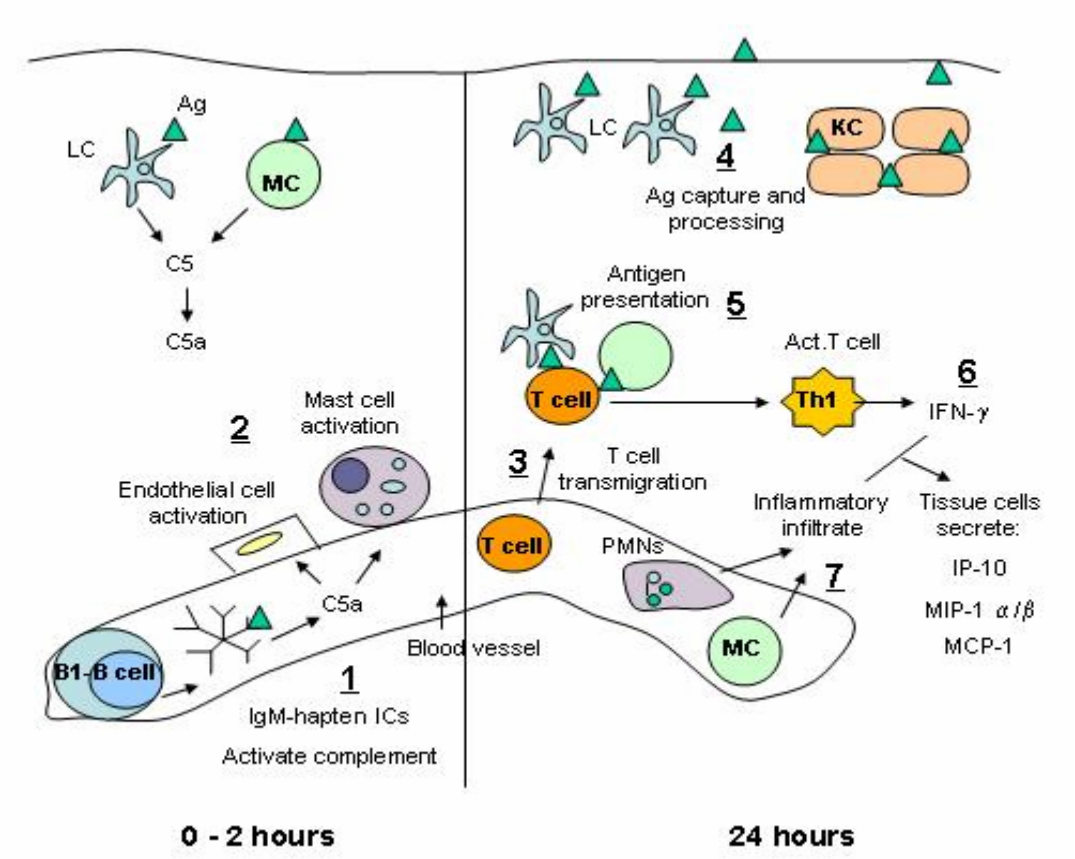


Figure 1.4 Model describing the early phase in contact hypersensitivity. The first 2 hours after challenge is characterised clinically by minor local edema. 1) Immune complexes are formed and activate complement. 2) C5a interacts with C5aR on mast cells, which results in the release of serotonin, histamine, and TNF; C5a also interacts with C5aRs on endothelial cells which become activated and upregulate adhesion molecules. In addition, LC and skin macrophages may release C5 to be cleaved into C5a by macrophages and mast cell proteases. At 4 hours after challenge 3) primed T cells migrate to the skin. 4) At the skin APCs capture the Ag and process it. 5) Then they present the Ag to primed hapten specific T cells. 6) Hapten specific T cells become activated to release T_H1 cytokines (IFN-γ) which interact with local tissue cells. These cells release several chemokines which lead to recruitment of non-specific leukocytes like monocytes and PMNs at 24 hours after challenge (7). Abbreviations: mast cell, macrophages; APC, antigen presenting cells; Ag, antigen; MC, mast cells, PMN, polymorphonuclear neutrophils; LC, Langerhans' cells; KC, keratinocytes (Kohl 2001).

1.4.2 Late phase (48 hours) in contact hypersensitivity

1.4.2.1 $CD4^+$ T_H2 T cells are responsible for the late response

Allergen specific T_H2 cells secrete the B-cell growth factors IL-4 and IL-5 (Janeway C. *et al.*, 2001). T_H2 cells are specialised for B cell activation and induce the production of IgE which subsequently activates mast cells. IL-4 can also activate local cells to produce chemo-attractants for polymorphonuclear neutrophils (PMNs) and lymphocytes. The role of IL-5 is to activate and induce the maturation of the infiltrating PMNs. As a result, inflammatory cells are recruited to the site of challenge to develop CHS.

Many studies demonstrated that IL-4 is important for the development of CHS. Salerno and co-workers demonstrated that IL-4 is an essential cytokine during the elicitation phase of CHS (Salerno *et al.*, 1995). Adoptive transfer of immune lymph node cells together with IL-4 anti-sense oligonucleotide into recipients, or treatment of recipients with anti-IL-4 monoclonal antibodies, resulted in the inhibition of CHS. Thus, IL-4 seems necessary for the development during the elicitation phase of a CHS response. In patients with tuberculosis and positive tuberculin reactions, serum IgE which is directed against the mycobacterial antigen was found (Barnetson R. *et al.*, 1998). In addition, PMNs stimulated with antigen produced IL-4. $CD4^+$ T_H2 T cells secrete IL-4 which acts as IgE switch factor (Roitt I. *et al.*, 1998). Thus, DTH in humans is not necessarily an exclusively T_H1 response. Mosmann has modified the T_H1/T_H2 theory claiming that IL-4 is critical for generating a systemic DTH response (Mosmann *et al.*, 1986). This is consistent with the fact that IL-4-deficient mice cannot mount a late (48 hours) CHS response (Weigmann *et al.*, 1997).

To fully understand the molecular mechanism of T_H2/T_C2 T cells and whether cytokines released by these cells are involved in CHS, IL-4-deficient mice were used. IL-4 is required for the differentiation of T_H2 and Tc2 cells, therefore, it is conceivable that these mice lack both cell types (Kopf *et al.*, 1993; Kuhn *et al.*, 1991). Weigmann and co-workers showed that the pinna swelling in wild type mice compared to IL-4-deficient-mice was similar at 24 hours after challenge (Weigmann *et al.*, 1997). However, 48 hours after challenge the pinna swelling of IL-4-deficient mice was significantly reduced

compared to wild type. Interestingly, the difference in pinna swelling was maintained during the subsequent course of the effector phase of the CHR. Based on these results, the elicitation stage can be divided in two phases, the first phase which induces CHS independently of IL-4 (T_H1 mediated) and the second phase which is dependent on IL-4 (T_H2 mediated) (Weigmann *et al*., 1997). These results were consistent with the results by Berg and co-workers who demonstrated that using oxazolone as a contact allergen in IL-4-deficient mice on a C57BL/6 background induced a reduced pinna swelling response at 48 and 72 hours after challenge (Berg *et al*., 1995).

The data by Weigmann and co-workers suggested that T_H1/Tc1 cells mediated the early 24 hours and that T_H2/Tc2 cells mediated the late 48 to 72 hours effector phase of a CHR (Weigmann *et al*., 1997). Moreover, T_H2 cells seem to be responsible for intensifying the reaction. The enhancing effect of IL-4 might be due to its function as a proinflammatory mediator. IL-4 up-regulates the expression of the vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells resulting in increased adhesion of lymphocytes, a necessary step for extravasation and migration into the site of challenge (Masinovsky *et al*., 1990). Moreover, IL-4 has chemotactic and activating activity for macrophages (Hiester *et al*., 1992), induces mast cell proliferation and enhances development of cytotoxic T cells (Janeway C. *et al*., 2001).

1.5 The close proximity of mast cells and T cells might facilitate the elicitation of immune responses

Immunoglobulin-E-dependent mast cell activation represents a major effector mechanism in many immediate hypersensitivity reactions (type I), whereas various T cell subsets are the major effector cells in DTH (Type IV) (Roitt I. *et al*., 1998). However, type I and type IV hypersensitivity reactions do not work independently of each other. Today it is clear that both mast cell- and effector T cell-dependent mechanisms contribute to the manifestations of some host defence or immunological disorders (Nakae *et al*., 2005). For example, experiments using mast cell-deficient mice demonstrated that mast cells can enhance the development and/or magnitude of certain T cell-associated responses. These responses include models of CHS, DTH, and asthma, and models of autoimmune diseases such as EAE, antibody-induced arthritis, and inflammatory bowel disease

(Askenase *et al*., 1983; Biedermann *et al*., 2000; Malone *et al*., 1986; Marsh *et al*., 1985; Mekori *et al*., 1990; Secor *et al*., 2000).

Mast cells and T cells may be activated in parallel by independent mechanisms and influence each other's function. For example, activated T cells in contact with mast cell can induce mast cells to secrete histamine, TNF, and metalloproteinase 9, and to exhibit enhanced IL-4 mRNA transcription (Baram *et al*., 2001; Bhattacharyya *et al*., 1998; Inamura *et al*., 1998). Also, purified populations of mast cells can present antigens to T cells by either MHC class I- or class II-restricted mechanisms *in vitro*, inducing activation of T cells (Fox *et al*., 1994; Frandji *et al*., 1993).

Furthermore, it is known that certain mast cell products (TNF, histamine and several chemokines) can influence T cell function (Nakae *et al*., 2005). Mast cells represent a potential source of TNF, which can exert effects on T cell recruitment, activation, and function (Gordon *et al*., 1990; McLachlan *et al*., 2003). Also, TNF juxtacrine effects can contribute to the mechanism by which T cell contact induces mast cells to secrete metalloproteinase 9 (Baram *et al*., 2001). Histamine is the major product of mast cells and it can promote T_H1 and T_H2 cell activation. Activated mast cells also secrete many chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), MIP-2 and CCL5 (RANTES), which can enhance T cell recruitment to sites of inflammation (Nakajima *et al*., 2002; Sayama *et al*., 2002). In addition, leukotrine B4 produced by mast cells also may have a role in regulating T cell migration (Ott *et al*., 2003).

Nakae and co-workers used cDNA microarray analysis and showed that human umbilical cord blood derived mast cells are able to express certain costimulatory molecules including OX40 ligand and 4-1BB ligand (Nakae *et al*., 2005). These ligands can promote T cell activation through cell-cell contact. In addition, stimulation of such mast cells through FcεRI aggregation enhances the expression of mRNA for these molecules (Sayama *et al*., 2002). *In vitro* studies showed that mast cells markedly enhanced proliferation and cytokine production in activated T cells that had been stimulated through the CD3/TCR complex. Moreover, mast cells stimulated via the FcεRI enhanced

the proliferation of several activated T cell subsets including $CD8^+$, $CD4^+$, T_H1, T_H2, T_C1, T_C2, γδ TCR^+, and $CD4^+$CD62L- T cells (Nakae *et al*., 2005). In contrast, even IgE/antigen-stimulated mast cells had little or no effect on resting splenic T cells. These observations suggest that mast cell-dependent enhancement of T cell activation may occur most readily *in vivo*, when T cells are already undergoing some activation, e.g. at sites of allergen exposure in sensitised mice (Nakae *et al*., 2005). Interestingly, it has been suggested that activated mast cells can migrate to local lymph nodes (Wang *et al*., 1998), indicating their potential to influence naive T cell activation and differentiation.

Mast cells reside in close physical proximity to T cells in inflamed allergic tissues. These close appositions between mast cells and T cells have led investigators to propose a functional relationship between these two cell populations that might facilitate the elicitation of the immune response. Physical contact between mast cells and T cells has been demonstrated in humans and mice. Human mast cells (HMC-1) or primary bone marrow derived human mast cells were cocultured with activated and with resting T cells (Bhattacharyya *et al*., 1998). Mast cells cocultured with activated T cells released histamine, β-hexosaminidase and produced TNF, and this effect peaked at 30 hours. In contrast, mast cells cocultured with resting T cells were not activated. Thus, it is possible that in a T cell-mediated inflammatory process like DTH, human mast cells can be activated by effector T cells. Likewise, mouse mast cells were cocultured with activated and resting T cells (Inamura *et al*., 1998). Coculturing of bone marrow-derived cultured mast cells (BMCMC) with activated T cells and not with resting T cells resulted in activation of mast cells and histamine release. Coculture of activated T cells in addition to crosslinking of Fcε RI augmented degranulation of mast cells. To demonstrate that activation of mast cells was mediated by a cell-cell contact with T cells, the two populations were separated with a porous membrane. Separation of mast cell and T cells prevented degranulation of mast cells indicating that mast cell activation was dependent on cell to cell contact. Moreover, the kinetics of histamine release paralleled the kinetics of the formation of heterotypic aggregates, which peaked after 12 hours of coculture. In addition, introduction of anti-LFA-1 and anti-ICAM-1 monoclonal antibodies inhibited the adhesion-induced mast cell degranulation, suggesting a mast cell activation pathway

induced by LFA-1/ICAM-1-mediated heterotypic aggregation with activated T cells (Inamura *et al*., 1998).

1.6 Lymphotoxin-β receptor (LTβR)

1.6.1 Ligands of the LTβR

The nomenclature of the ligands reflects the biochemical structures involved. Typically, individual proteins within an oligomeric structure are designated with Greek letters and accordingly LTα and LTβ refer to the subunits of the complex. The actual name "lymphotoxin" (LT), meaning cytotoxic activity, comes from the biochemical characterisation as a cytotoxic protein which was the earliest mechanism postulated to be used by cytotoxic T lymphocytes (Ware *et al*., 1995)

LTα, formerly called TNFβ, was characterised biochemically as a cytotoxic protein secreted from a human B lymphoblastoid line (Ware *et al*., 1995). LTα is a classical secretory protein which lacks a transmembrane domain (Ware, 2005). LTα can exist as a homotrimer ($LT\alpha_3$) which is secreted upon cleavage of its signal peptide. $LT\alpha_3$ binds to two TNF receptors, TNFR-1 and TNFR-2 and is solely produced by activated lymphocytes. LTα can also be incorporated into a heterotrimeric complex with a second subunit LTβ (Ware, 2005).

The genes encoding LTβ, TNF and LTα reside in tightly linked locus within the MHC on chromosome (Chr) 17 in the mouse (Chr 6 in humans) (Ware *et al*., 1995). The gene encoding LTβ has 4 exons and is located approximately 2 kb from TNF. The mRNA transcript for LTβ in activated T cells has a length of approximately 0.9 kb and codes for a type II transmembrane protein, and so far no soluble form of the protein has been identified. LTβ has a single N-linked glycosylation site at the intracellular domain and also has a single cystein residue at the extracellular domain. The conserved amino acids located within the internal β strand regions present a basis for the heterotrimeric complex formation with LTα. LTβ does not seem to function by itself but only in association with LTα. The predominant form of the LTαβ heterotrimer expressed by activated

lymphocytes contains two LTβ subunits associated with one LTα subunit to form $LT\alpha_1\beta_2$. This ligand binds with high affinity to the lymphotoxin beta receptor (LTβR).

Another ligand that binds to the LTβR is LIGHT, also called Herpes-Barr virus entry mediator-ligand (HVEM-L) or mouse tumor necrosis factor superfamily (mTNFSF14), and its gene *tnfsf14* (Misawa *et al*., 2000). LIGHT consists of 239 amino acid residues, and the calculated molecular weight is 26,338 Da. The mRNA transcript for *tnfsf14* has a size of 2.1 kb and was detected in spleen and lung, and a 4.2 kb transcript was detected in the heart. Moreover, LIGHT mRNA was also found in heart, brain, liver and to a lesser extent in kidney and testis (Misawa *et al*., 2000). *Tnfsf14* is located on chromosome 17 at region D-E1 which showed that LIGHT is closely associated with LTα, LTβ and TNF. LIGHT is expressed as a homotrimer on activated T cells (Morel *et al*., 2000), natural killer cells (Cohavy *et al*., 2005), immature dendritic cells (Tamada *et al*., 2000a), monocytes and granulocytes. LIGHT can bind to three receptors: HVEM, DcR3 and LTβR (Scheu *et al*., 2002). HVEM is expressed on immature dendritic cells, T and B lymphocytes, NK cells, monocytes, and endothelial cells, and in addition is also a receptor for $LT\alpha_3$ (although binding is weak). Dc3R (TR6) belongs to the TNFR family and lacks a transmembrane region. This receptor competes with the HVEM and LTβR for LIGHT engagement, acting as a negative regulator. In addition to LIGHT, the Dc3R also binds to FasL. The LTβR is another receptor for LIGHT and is expressed on follicular dendritic cells (FDCs) and stromal cells (Browning *et al*., 1997; Murphy *et al*., 1998). The LTβR have two ligands LIGHT and $LT\alpha_1\beta_2$. The phenotype of LIGHT deficient mice showed a full complement of lymph node, although LIGHT can contribute to lymph node development (Scheu *et al*., 2002), as revealed by LIGHT-deficient LTβ-deficient double knockout mice (Ware, 2005). In addition, absence of LIGHT also leads to defects in dendritic cell maturation (Morel *et al*., 2001).

1.6.2 Lymphotoxin-beta receptor

The Lymphotoxin-β receptor (LTβR) is a member of the TNF receptor superfamily. The LTβR plays a role in the development of peripheral lymphoid organs and cellular

immunity (Rennert *et al*., 1998). The absence of $LT\alpha_1\beta_2$-LTβR signalling leads to the lack of all lymph nodes, defects in T and B cell segregation, and follicular dendritic cell network formation within the spleen. This phenotype is shared by LTα-deficient mice. However they occasionally retain mesenteric lymph nodes. In contrast, the mice deficient in LTβ retain mesenteric lymph nodes and in some cases cervical lymph nodes. Moreover, the splenic architecture appears less disturbed than in LTα- and LTβR-deficient mice. In adult mice, LTβR signalling is required for maintaining the splenic architecture, the integrity of the marginal zone, and compartmentalisation of T and B cells (Dejardin *et al*., 2002). Moreover, during antigen-dependent responses, germinal centres and development of follicular dendritic cells fail to form in LTβR-deficient mice.

The LTβR mRNA transcripts are constitutively expressed in all visceral organs and lymphoid tissues (Force *et al*., 1995). This is expected because the sequence of the putative promoter region lacks TATA and CAAT sequences. Like several housekeeping genes and *tnfr1*, *Ltbr* expression is directed by a so called housekeeping-type promoter (Muller *et al*., 2001). The amino acid sequence of the LTβR comprises 408 amino acids (Ware *et al*., 1995). The LTβR has a ligand-binding domain with four cysteine-rich pseudorepeats followed by a short proline-rich membrane proximal region. In the extracellular region two potential N-glycosylation sites are present. The C-terminal cytoplasmic domain binds to the putative signalling molecule TRAF3.

Activation of the TNF receptor family leads to different signalling pathways (Ware, 2005). The TNFR1 contains a death domain (DD) and start the apoptotic cascade via TNFR-associated DD (TRADD). LTβR, TNFR2 and HVEM utilize TNFR receptor associated factors (TRAFs) and a family of zinc RING finger proteins to connect to the intracellular signalling pathways (Ware, 2005). In the LTβR, the TRAF-interacting region is composed of short peptide motifs (TRAF 2 and 3). The TRAF domain contains a peptide binding crevice in each subunit that allows the accommodation of several sequences, receptors and regulators. The TRAF crevice accommodates the LTβR and activates the signalling pathway. The zinc RING finger moiety functions as part of an ubiquitin ligase complex leading to proteosome degradation, which is a common feature

for the activation or turnover of many components in these signalling pathways. TRAF 2 and 3 are important in enabling NF-κB activation. The LTβR induces different pattern of gene expression via two NF-κB pathways (Dejardin *et al*., 2002). The first pathway leads to the expression of inflammatory genes such as VCAM-1, MIP-1β and MIP-2. The second pathway leads to transcription of genes which are implicated in secondary lymphoid organogenesis and homeostasis such as secondary lymphoid tissue chemokine (SLC), Epstein-Barr virus-induced molecule 1 ligand (ELC), stromal derived factor-1alpha (SDF-1α), and B cell activation factor from the TNF family (BAFF). NF-κB activation leads to the expression of inducible genes which regulate inflammation underlying innate and adaptive immune responses, cell growth, and apoptosis (Ware, 2005).

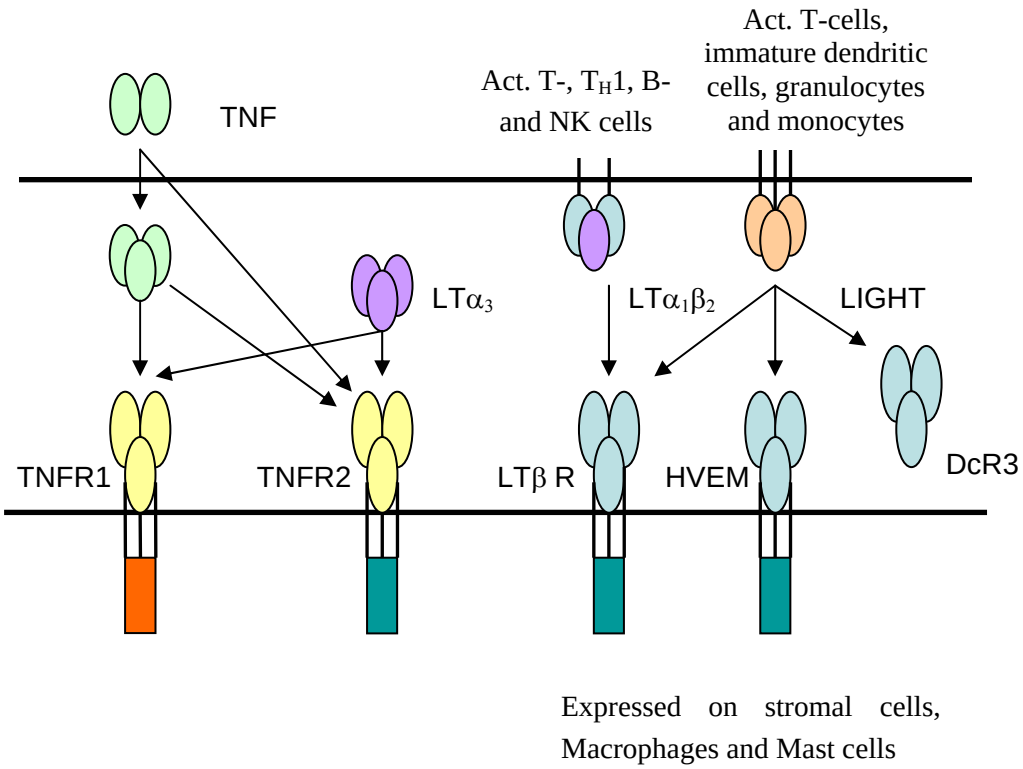


Figure 1.5 The Tumor Necrosis Factor (TNF) receptor family and its ligands. Ligands are depicted in the upper portion. Arrows indicate their respective high affinity receptors (lower portion). The orange box in the cytosolic region indicates presence of a death domain and green boxes indicate a TRAF binding motif (Hehlgans and Männel, 2002).

LTβR mRNA has been found to be constitutively expressed in mouse tissues like lung, liver, kidney, heart, spleen, and lymph nodes, skin, muscle, bone, stomach, small and

large intestine, thymus, ovaries and uterus (Browning *et al*., 2002; Force *et al*., 1995; Nakamura *et al*., 1995). In contrast, LTβR expression is lacking on peripheral blood T cells and on T cell lines. In the embryonic stage, LTβR was expressed on epithelial cells, especially when derived from the small and large intestine. LTβR was also found on stromal cells of the developing human thymus, on follicular dendritic cells (FDCs), and on reticular dendritic cells of the thymus and the spleen (particularly in the white pulp) (Murphy *et al*., 1998). Moreover, LTβR is expressed on cell lines of hemopoietic origin like RAW cells and peritoneal mouse macrophages (Browning *et al*., 1997). Since the LTβR is expressed on cells of hemopoietic origin its expression on mast cells was also investigated, and it was clearly shown that LTβR is also expressed on mast cells (Stopfer *et al*., 2004).

1.7 Aims

This project is based on previous studies that indicated that mast cells and T cells are involved in contact hypersensitivity (Askenase *et al*., 1983; Bour *et al*., 1995). Moreover, close apposition of T cells and mast cells in inflammation led to the investigation of physical interactions between these two cell populations. Interaction of activated T cells with mast cells induced mast cell activation as a result. Activation of T cells leads to the expression of several proteins on the membrane such as $LT\alpha_1\beta_2$ and since mast cells express LTβR on the membrane, the aim of this project was to investigate the role of activated T cells interacting with mast cells via the LTβR-$LT\alpha_1\beta_2$ system during the elicitation phase of a contact hypersensitivity response.

In this study mice deficient in LTβR and LIGHT were used in CHS experiments. Mice were sensitised and challenged with 0.3% oxazolone, which had been previously shown to be an optimal concentration to induce a maximal CHR and to reduce the possibility that excess hapten application could cause penetration beyond the epidermis, or induce other unanticipated responses (Rennert *et al*., 2001). To test whether LTβR is involved in CHS responses, treatment with the LTβR-Ig fusion protein just before challenge was used with the intention to block the LTβR-$LT\alpha_1\beta_2$ interaction at the elicitation stage. In addition, the biological activity of the LTβR-Ig fusion protein, which captures the LTβR ligands, $LT\alpha_1\beta_2$ and LIGHT on sensitised lymph node cells, was analysed by FACS. Moreover, expression of LTβ on lymph node cells was also analysed by FACS analysis with monoclonal antibodies.

With the aim to investigate the function of LTβR on mast cells in the elicitation phase of CHS, adoptive transfer experiments were performed. For this, sensitised lymph node cells were transferred to LTβR- and to mast cell-deficient mice. Sensitised lymphocytes express the ligands for LTβR and transfer to LTβR- and to mast cell-deficient mice should give a clear result concerning the interactions between mast cells and lymphocytes via the LTβR-$LT\alpha_1\beta_2$ /LIGHT system.

2 Materials

2. 1 Chemicals and reagents

Agarose	GIBCO/Invitrogen, Karlsruhe, Germany
Acrylamide 30 %	Roth, Karlsruhe, Germany
BioWithtaker Insect-Xpress, L-Glutamine	Cambrex, Bio Science Walkerville, Inc. USA
Bovine Serum Albumin	Boehringer, Mannheim, Germany
Chelating Sepharose Fast Flow	Amersham, Biosciences, Germany
Chloroform	Fluka, Buchs, Switzerland
Coomassie Brilliant Blue R250	Fluka, Buchs, Switzerland
Desoxynucleoside Trisphosphate Set, PCR Grade	Roche, Mannheim, Germany
Dimethyl sulfoxide	Sigma Aldrich, Steinheim, Germany
Ethidium Bromide	Sigma Aldrich, Steinheim, Germany
Fetal calf serum	PAN Biotech, Aidenbach, Germany
Fluorescein Isothiocyanate	Sigma Aldrich, Steinheim, Germany
Gentamycin	ICN, Meckenheim, Germany
Giemsa Stain, Modified	Sigma Aldrich, Steinheim, Germany
Imidazole	Sigma Aldrich, Steinheim, Germany
Ionomycin	Sigma Aldrich, Steinheim, Germany
β-Mercaptoethanol	Sigma Aldrich, Steinheim, Germany
Sodium dodecyl sulfate	Sigma Aldrich, Steinheim, Germany
Ni-NTA Agarose	Qiagen, Hilden, Germany
Oxazolone	Sigma Aldrich, Steinheim, Germany
PCR Buffer, 10x concentrated	Roche Diagnostics, Mannheim, Germany
Penicillin/Streptomycin	Sigma Aldrich, Steinheim, Germany
Phorbol myristate acetate	Sigma Aldrich, Steinheim, Germany
Propidium iodide	Sigma Aldrich, Munich, Germany
RPMI 1640- Powder medium	Sigma Aldrich, Steinheim, Germany
TEMED	Sigma Aldrich, Steinheim, Germany
Triton X-100	Serva, Heidelberg, Germany
Trypan Blue	Sigma Aldrich, Steinheim, Germany

Tween 20	Fluka, Buchs, Switzerland

2.2 Equipment and laboratory supplies

Cell culture flasks (70, 250, 750 ml)	Falcon, Heidelberg, Germany
Centrifuge: Beckmann J2-21 M/E Centrifuge	Beckmann, Munich, Germany
Beckmann L-70 Ultracentrifuge	Beckmann, Munich, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany
Cytospin 3 Centrifuge	Shandon, Pittsburgh, USA
Centrifuge Tubes (15, 50 ml)	Falcon, Heidelberg, Germany
Cell Strainers	Falcon, Heidelberg, Germany
CryoTube Vials	Nunc, Kamstrup, Denmark
Eppendorf cups (1.5 ml/ 2 ml)	Eppendorf, Hamburg, Germany
Eppendorf plastic pestle	Eppendorf, Hamburg, Germany
FACS tube, 5 ml Polystyrene round bottom	Becton Dickinson, Heidelberg, Germany
FACSCalibur Flow Cytometer	Becton Dickinson, California, USA
WinMDI Version 2.8	Joseph Trotter, FACS Scribbs Research Institute La Jolla, CA, USA
Gel Electrophoresis machine	BioRad, Munich, Germany
Hyperfilm ECL	Amersham/GE-Healthcare, Freiburg, Germany
Immobilon-P Transfer Membrane	Millipore Corporation, MA, USA
Incubator Heraeus 6000	Heraeus, Hanau, Germany
Incubator-Cell safe	Integra Biosciences, Baar, Switzerland
Kryostat Machine, Frigocut E 2800	Reichert Jung, Nussloch, Germany
Laminair Flow HB 2448- Sterile Bank	Heraeus, Hanau, Germany
Microscope, Model CK2	Olympus, Hamburg, Germany
Microtiter plates	Falcon, Heidelberg, Germany
Neubauer counting chamber	Brand, Giessen, Germany
PCR Thermocycler	Perkin Elmer, Shelton, USA
Tissue Culture Dish (100 x 20 mm)	Becton Dickinson, Heidelberg, Germany
UV chamber (254 nm and 366 nm)	Bachofer, Reutlingen, Germany

Weighing machines: Sartorius R160P	Sartorius, Gottingen, Germany
Sartorius L2200S	Sartorius, Gottingen, Germany
Whatman Filter paper	Laborcenter, Nuremberg, Germany

2.3 Kits

ECL-Western Blot Detection Kit (Nowa)	MoBiTec, Gottingen, Germany
Mycoplasm Detection Kit	Roche Diagnostics, Mannheim, Germany
QIAshredder	Qiagen, Hilden, Germany

2.4 Molecular Weight Standards

DNA standards (II, III, X, XIV)	Roche Diagnostics, Mannheim
Protein Molecular Weight, SDS PAGE	BioRad, Munich, Germany

2.5 Antibodies and Enzymes

2.5.1 Antibodies

FACS:

Rat anti-mouse CD3-PE	Becton Dickinson, Heidelberg, Germany
Rat anti-mouse CD3-FITC	Becton Dickinson, Heidelberg, Germany
Rat anti-mouse B220-PerCp	Becton Dickinson, Heidelberg, Germany
LTβR Ig fusion protein	See Section 3.2.1
Hamster anti-mouse LTβ	Becton Dickinson, Heidelberg, Germany
Hamster IgG_2 λ1, isotype control	Becton Dickinson, Heidelberg, Germany
Biotinylated anti-hamster IgG	Becton Dickinson, Heidelberg, Germany
Streptavidin-FITC	Becton Dickinson, Heidelberg, Germany
Human IgG	Sigma-Aldrich, Munich, Germany
Donkey anti-human IgG Fc γ PE	Dianova, Hamburg, Germany
Rat $IgG_{2a,}$ κ FITC, Isotype control	Becton Dickinson, Heidelberg, Germany
Rat $IgG_{2a,}$ κ PE, Isotype control	Becton Dickinson, Heidelberg, Germany
Rat anti-mouse FcγRII/III (Clone 2.4G2)	Home made E 1.3, 14/05/03
mouse anti V5 monoclonal antibody	Invitrogen, Karlsruhe, Germany

goat anti mouse IgG peroxidase	Sigma-Aldrich, Munich, Germany

2.5.2 Enzymes

Taq-DNA-Polymerase	Roche, Mannheim, Germany

2.6 Primers

Every primer was ordered from Metabion (Martinsried, Germany).

LTβR7: 5'-TGT CAG CCG GGG ATG TCC TG- 3'

LTβR4: 5'-CTG GTA TGG GGT TGA CAG CG- 3'

HSVTK: 5'- ATT CGC CAA GAC GCT GG- 3'

2.7 Buffers

Ethidium Bromide	10 mg/ml ethidium bromide in H_2O
Imidazole elution buffer	500 mM Imidazole in native washing buffer pH 6.0 for protein purification
PBS	150 mM NaCl 8 mM K_2HPO_4 2 mM KH_2PO_4 pH 7.3
0.5 % Triton X-100 Buffer	0.5 % Triton X-100 in H_20
Trypan Blue buffer	0.15 % Trypan Blue 150 mM NaCl
Washing buffer for native protein	500 mM NaCl 20 mM NaP pH 6.0

2.7.1 Buffers for DNA experiments

Lysis buffer for mouse tails	0.1 M Tris HCl 0.2 M NaCl 5 mM EDTA 1 % (w/v) SDS pH 8

Proteinase K stock solution	10 mg/ml
Pronase E stock solution	10 mg/ml 10 µl of 1 M Tris HCl (pH 8.0) 2 µl of 5 M NaCl

2.7.2 Buffers for protein experiments

4 X Laemmli loading buffer	250 mM Tris-HCl 4 % SDS 40 % glycerol 4 % β-mercaptoethanol pH 6.8
Stacking Gel	0.85 ml acrylamide 1.5 ml 0.5 M Tris-HCl pH 6.8 3.75 ml H_2O 60 µl 10 % SDS 5 µl TEMED 50 µl 10 % APS
Resolving Gel	6.25 ml acrylamide 3.75 ml 1.5 M Tris-HCl pH 8.8 5 ml H_2O 150 µl 10 % SDS 10 µl TEMED 100 µl 10 % APS
5 X SDS-PAGE running buffer	120 mM Tris-HCl 950 mM glycine 0.5 % SDS
Western Blot Buffer A	300 mM Tris-HCl 10 % methanol pH 10.4

Western Blot Buffer B	25 mM Tris-HCl 10 % methanol pH 10.4
Western Blot Buffer C	25 mM Tris-HCl 40 mM 6-amino-n-capronic acid 10 % methanol pH 9.4
TBS	10 mM Tris-HCl 140 mM NaCl 1.5 mM NaN_3 pH 7.4
TBS/Tw (washing buffer)	TBS with 0.05 % Tween 20 (v/v)
Coomassie Blue Staining	10 % glacial acetic acid 40 % methanol 0.2 % Coomassie Brilliant Blue R250 filter before use
Coomassie destaining	10 % glacial acetic acid 40 % methanol

2.7.3 DAPI staining solutions

DAPI Stock solution	0.1 mg/ml in distilled water and stored in aliquots of 200 µl at –20° C.
DAPI working solution	Stock solution diluted 1 in 100 in methanol (they can be stored for 6 months)

2.8 Cells

2.8.1 Cell lines and their culture medium

A20	Tumor Mouse B cell line. Complete RPMI medium: RPMI 1640, 10 % FCS, Gentamycin (50 µg/ml), glutamine, 20 µM 2-ME, and 10 mM HEPES buffer, pH 7.4
DS-2	Drosophila melanogaster Schneider line 2. BioWithtaker Insect-Xpress, L-Glutamine, 100 mg/L Kanamycin

2.8.2 Primary cells

Lymph nodes	From C57Bl/6, $WBB6F_1^{+/+}$ and $Kit^W/Kit^{W\text{-}v}$ mice

2.9 Animals

C57BL/6	Central Animal Facility, Specific Pathogen Free (SPF) University of Regensburg, Germany Charles River Laboratories, Inc, Sulzfeld, Germany Harlan Winkelmann GmbH, Borchen, Germany
C57BL/6 $LT\beta R^{-/-}$ C57BL/6 $LIGHT^{-/-}$	Prof. Dr. K. Pfeffer, Institute of Medical Microbiology, University of Dusseldorf, Germany
$WBB6F_1^{+/+}$ ($Kit^{+/+}$) $Kit^W/Kit^{W\text{-}v}$ ($Kit^{-/-}$)	Dr. L. Hültner, GSF-Institut für Klinische und Molekularbiologie Tumorgenetik, Munich, Germany

3 Methods

3.1 DNA experiments

3.1.1 Genotyping of LTβR-deficient mice

3.1.1.1 Isolation of genomic DNA

Pronase E was preactivated by incubating 25 µl for 1 hour at 37° C. For genotyping approximately 0.6 cm of mouse tail was lysed with 500 µl of lysis buffer, 10 µl of proteinase K and 25 µl of pronase E and digested overnight at 56° C. After a centrifugation step at 13,000 rpm for 5 minutes the upper phase of the supernatant was transferred to a new eppendorf cup, 500 µl of phenol were added and the cup was vortexed. Again a centrifugation step at 13,000 rpm was performed for 5 minutes and the upper phase of the supernatant was transferred to a new eppendorf cup, 500 µl of phenol were added and the cup was vortexed. The centrifugation step mentioned before was repeated again twice with phenol/chloroform and twice with chloroform alone instead of phenol alone. After the final centrifugation step the upper phase of the supernatant was removed carefully and the DNA precipitated with ethanol. For this, 2 times the volume of 100 % ethanol and 1 in 10 volume of 3M sodium acetate pH 5.2 were added. Samples were incubated at –20° C for at least 20 minutes and centrifugated again at 13,000 rpm for 30 minutes. The pellet was washed with 1 ml of 70 % ethanol. The pellet was air dried and resuspended in 50 µl of DNAse-free water.

3.1.1.2 Genotyping of LTβR-deficient mice by PCR

The precipitated DNA from every single mouse was used for 2 PCR reactions, one for screening for the wild type gene and one for the insert. For the wild type mice the expected size of the band was 640 bp which contained the LTβR sequence and for the LTβR-deficient mice the expected size of the band was 200 bp which contained the neo cassette sequence.

Each PCR reaction for screening of the wild type mouse contained:

1 µl dNTP-mix (10 mM)
5 µl 10 x PCR reaction buffer
1 µl 0.5 U Taq-polymerase
1 µl DNA (0.2 µg/µl)
5 µl Primer 1 (1:10), stock at 100 pmol/µl
5 µl Primer 2 (1:10), stock at 100 pmol/µl
H_2O to a final volume of 50 µl

Each PCR reaction for screening of the LTβR-deficient contained:

1 µl dNTP-mix (10 mM)
5 µl 10 x PCR reaction buffer
1 µl 0.5 U Taq-polymerase
1 µl DNA (0.2 µg/µl)
5 µl Primer 2 (1:10), stock at 100 pmol/µl
5 µl Primer 3 (1:10), stock at 100 pmol/µl
H_2O to a final volume of 50 µl

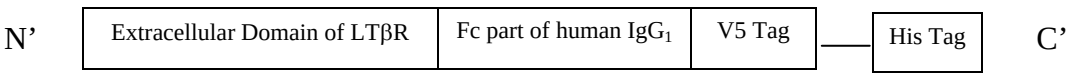
Primers used for wild type and LTβR deficient mice:
Primer 1, LTβR7: 5'-TGT CAG CCG GGG ATG TCC TG- 3'
Primer 2, LTβR4: 5'-CTG GTA TGG GGT TGA CAG CG- 3'
Primer 3, HSVTK: 5'- ATT CGC CAA GAC GCT GG- 3'

For amplification of the template the following PCR program was performed: PCR starts with a single step at 94° C for 5 minutes for denaturation of the genomic DNA, then thirty cycles (of denaturation at 94° C for 1 minute, annealing step at 62° C for 30 seconds and elongation at 72° C for 1 minute and 30 seconds) were performed. Finally the PCR products were separated in a 2 % agarose gel.

3.2 Protein methods

3.2.1 LTβR-Ig fusion protein

The LTβR-Ig fusion protein construct was previously cloned and stably transfected in DS-2 cells by the company BASF. Expression and purification of the LTβR-Ig fusion protein was performed in our laboratory. Briefly, the LTβR-Ig fusion protein is a type I membrane protein, which consists of the mouse LTβR extracellular domain at the N-terminus, followed by the Fc part of the human IgG_1 at the C terminus in the intracellular domain.



In stably transfected DS-2 cells the expression of LTβR was induced with $CuSO_4$, the recombinant protein purified on a chelating Sepharose column and analysed by Western Blot and FACS analysis.

3.3 Cell culture

Every cell culture work was performed under sterile conditions (Laminair Flow). The A20 cell line was incubated at 37º C with 5 % CO_2 and 95 % humidity. The cells were split 1 in 5 three times per week with fresh medium. DS-2 cells were incubated at 28ºC and split 1 in 5 three times per week.

3.3.2 Freezing and thawing of cells

To freeze cells, they were pelleted at 1200 rpm and redissolved to a concentration of $1x10^6$ cells/ml with complete RPMI medium. 500 µl of the cell suspension were diluted with the same amount of freezing medium (40 % RPMI 1640, 50 % FCS and 10 % DMSO) in cryo tubes. Cells were stored at -80° C for 2 days and then transferred to the liquid nitrogen tank. After thawing cells, were washed 3 times with complete medium and taken in culture.

The freezing medium of DS-2 cells contained 45 % of conditional medium, 45 % of fresh medium (without hygromycin) and 10 % DMSO.

3.3.3 Mycoplasm test

Cells were split 2 times without antibiotics before DAPI (4'-6-Diamidino-2-phenylindole) staining. 10^4 cells/ml were fixed on glass slides by centrifugation for 5 minutes at 800 rpm (cytospin). Slides were air dried, incubated with DAPI for 15 minutes in the dark, washed 5 minutes with running tap water and finally rinsed with distilled water. The slides were covered with mounting medium for fluorescence.

3.4 FACS analysis

3.4.1 Lymph node cell staining of $LT\alpha_1\beta_2$ and LIGHT expression with LTβR-Ig fusion protein

Mice were sensitised with oxazolone as described in section 3.5.1. Four days later the braquial and axillary lymph nodes were removed and cells were isolated by mechanical disruption. Cells ($1x10^6$) were washed three times with PBS and 5 % FCS (washing buffer). To block Fc receptors cells were incubated for 20 minutes with 1 μg/ml of anti-mouse FcγRII/III (Clone 2.4G2) diluted in PBS and 10 % FCS (antibody dilution buffer) before cells were incubated for 20 minutes with 10 μg/ml of LTβR-Ig fusion protein. Specificity of staining was confirmed by incubation with an isotype-matched control (human IgG). After 3 washes cells were incubated for 20 minutes in 50 μl of a 1/100 dilution of PE-conjugated donkey anti-human IgG antibody, washed three times, resuspended in 0.5 ml washing buffer and stored on ice in the dark until flow cytometric analysis. For dead cell discrimination PI was added at a final concentration of 2 μg/ml, so that PI negative or viable cells could be analyzed. All incubations were carried out on ice.

Viable lymph node cells were gated and analysed for staining of $LT\alpha_1\beta_2$ and LIGHT. As a positive control, A20 cells were stimulated with 50 ng/ml of PMA for 5 hours to induce the expression of LTβ (Browning *et al.*, 1997). Quadrant markers were set to distinguish positive and negative staining according to isotype controls.

3.4.2 Lymph node cell staining for LTβ expression with monoclonal anti-mouse LTβ antibodies.

Lymph node cells were treated as described in section 3.4.1. After blocking, cells were incubated for 20 minutes with 0.06 μg/10^6 cells of hamster anti-mouse LTβ antibodies. Specificity of staining was confirmed by incubation of cells with isotype-matched control antibodies (hamster IgG2 λ1). All incubations were carried out on ice. Cells were washed three times with washing buffer, incubated for 20 minutes in 50 μl of biotinylated goat anti-hamster IgG at a concentration of 1 μg/10^6 cells, washed three times, followed by incubation for 20 minutes with 0.015 μg/10^6 cells of FITC-conjugated streptavidin. After washing the cells three times they were resuspended in 0.5 ml of washing buffer and stored on ice in the dark until flow cytometric analysis. Propidium iodide was added at a final concentration of 2 μg/ml to each FACS tube, 1 minute prior to cell acquisition for the identification and analysis of viable cells.

3.4.3 Acquisition and analysis of flow cytometric data

Flow cytometric analysis was carried out using a FACSCalibur flow cytometer equipped with 488 nm blue and 633 red diode lasers. FITC-positive cells were detected through the FL-1 channel (530 nm), PE stained cells were detected through the FL-2 channel (585 nm) and the PI and PerCP fluorescence signal was detected through the FL-3 channel (682 nm). Data analysis was carried out using CellQuest software and data were analysed with WinMDI Version 2.8.

3.5 Animal experiments

C57Bl/6 mice were bred in a Specific Pathogen Free (SPF) animal facility of the University of Regensburg, or were purchased from Charles River Laboratories and Harlan Winkelmann GmbH. The LTβR-deficient (LTβR$^{-/-}$) and LIGHT-deficient mice (LIGHT $^{-/-}$) breeding pairs were kindly provided by Prof. Dr. Klaus Pfeffer. These mice were continously genotyped and were kept in individually ventilated cages (IVC). mast cell-deficient mice (Kit^{W}/Kit$^{W\text{-}v}$) and congenic normal WBB6F1$^{+/+}$ (Kit$^{+/+}$) mice were kindly provided by Dr. L. Hültner, Munich. All mice were 5 to 12 weeks of age and sex matched. The study was performed in accordance with German federal regulations of

animal experimentation. Interestingly, measurements on the left and right pinnae were consistently different with the swelling being more pronounced on the left ear. Therefore, only the left pinna swelling value was used throughout this work.

3.5.1 Contact hypersensitivity experiment

Contact hypersensitivity experiments were performed as described (Rennert *et al*., 2001). Briefly, the amount of hapten was initially titered to find a dose that induced the maximal CHS response and to reduce the possibility that excess hapten application could cause penetration beyond the epidermis, or induce other unanticipated responses, such as hapten tolerance (Grabbe *et al*., 1998). Mice were sensitised with 100 µl of 0.3 % oxazolone dissolved in ethanol on their shaved backs and four days later they were challenged with 10 µl of 0.3 % oxazolone on both pinnae. As a negative control, mice were treated with ethanol and four days later challenged with oxazolone. Contact hypersensitivity experiments were performed on C57BL/6, mast cell-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice, congenic normal $WBB6F_1^{+/+}$ ($Kit^{+/+}$) mice, LIGHT-deficient and LTβR-deficient mice. Caliper measurements of the pinnae were taken just before challenge and at several time points afterwards. Specific pinna swelling was calculated by pinna swelling minus pinna thickness before challenge.

3.5.2 Experiments to investigate the elicitation phase of contact hypersensitivity

3.5.2.1 Transfer of lymph node cells

Mice were sensitised with 100 µl of 0.3 % oxazolone, four days later the brachial and axillary lymph nodes were removed and cells were isolated by mechanical disruption. Lymph node cells isolated from C57BL/6 mice treated with ethanol (vehicle) were used as negative control. Isolated cells were washed 3 times with RPMI medium (with 5 % FCS and 0.1 % Gentamycin) and $2.5x10^7$ cells resuspended in 200 µl of washing medium were transferred intravenously by tail vein injection into C57BL/6 mice. Immediately after, pinna thickness was measured and mice were challenged with 10 µl of 0.3 % oxazolone on both pinnae.

3.5.2.2 Treatment with LTβR Ig fusion protein in contact hypersensitivity model

C57BL/6 mice were sensitised as described above (3.5.1). One day before and just before challenge mice were treated intraperitoneally with 100 μg of LTβR-Ig fusion protein diluted in 200 μl of PBS. As a negative control for LTβR-Ig treatment, mice were treated with 100 μg of human IgG. Negative controls and pinnae swelling measurements was performed as described above (3.5.1).

3.5.3 Histological score

Two days (48 hours) after challenge mice were sacrificed and the pinnae were removed. Pinnae were fixed in 10 % formalin (in PBS) overnight and washed with increasing concentrations of ethanol before embedding in paraffin. Sections of 3 μm thickness were cut at intervals of 100 μm distance and stained with hematoxylin and eosin. For the score lymphocytes, eosinophils, neutrophils and mast cells were counted. The mean cell number of the individual cell type was determined from sections of five mice per group, from two independent experiments. The score was performed as shown below:

Score	Infiltration
0	Low number of cells
1	Moderate number of cells
2	High number of cells

3.5.4 Mast cell staining

Paraffin sections of pinnae tissue were rinsed for 10 seconds each in decreasing concentrations of ethanol (100 %, 96 %, and 70 %) and finally in distilled water. Then staining with Giemsa (diluted 1:10 with distilled water) for thirty minutes and rinsing in 1% of acetic acid. Sections were rinsed for 10 seconds each in increasing concentrations of ethanol (96 % and 100 %), washed with xylol twice for 2 minutes and covered.

3.5.5 Statistical Analysis

Specific ear swelling was calculated as follows:

$$\Delta \text{ Ear Swelling} = \text{ear swelling} - \text{ear thickness before challenge}$$

Differences of specific ear swelling were determined using a non parametric test. The Global test, Kruskal-Wallis-Test, was used to compare 3 or 4 groups and when this test was significant, and then Mann-Whitney-Test was used to determine the difference between two groups. Both tests were considered significant at $P<0.05$.

4. Results

4.1 CH model to oxazolone in C57BL/6 mice

Contact hypersensitivity response (CHR) is a cellular immune response challenged by application of antigen to the skin of sensitised mice. In a regular CHR to oxazolone, absolute pinnae swelling was measured at several time points after elicitation. Mice sensitised and challenged with oxazolone (regular CHR) developed significantly higher pinna swelling from 19.5 to 51.5 hours after challenge compared to that of non-sensitised mice (Fig. 4.1 and 4.2). Lack of an antigen-specific response can be demonstrated when mice were treated with the vehicle (ethanol) and 4 days later were challenged with the antigen (Fig. 1).

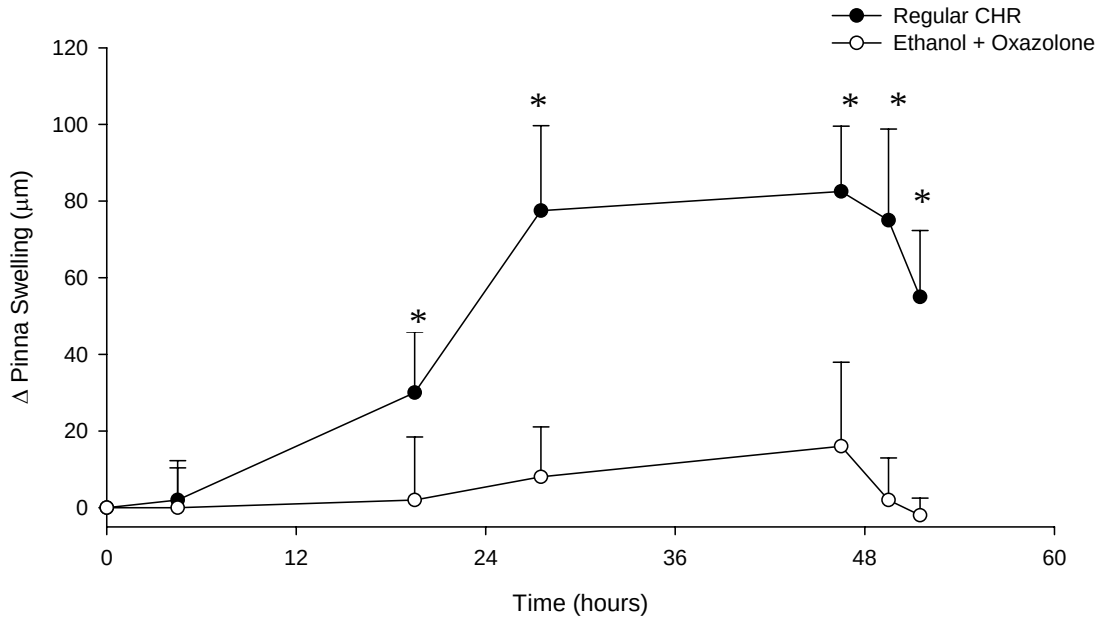


Figure 4.1 CHR experiment to oxazolone is antigen specific. Mice sensitised and challenged with oxazolone (●) (n = 5). Mice treated with ethanol and challenged with oxazolone (O) (n = 5). Statistical analysis was calculated by Kruskal Wallis and Mann Whitney Test and was considered significant when $p < 0.05$ (*).

This antigen-specific response induces the infiltration of inflammatory cells into the site where the antigen was applied for elicitation. As expected in CHR to oxazolone, 48 hours after elicitation the pinna swelling was due to a strong infiltrate of inflammatory cells (Fig. 4.2). Mice treated with ethanol and challenged with oxazolone had comparable

pinna thickness as naive mice. In addition, the infiltrating cells responsible for the pinna swelling were analysed by histology. In CHR to oxazolone at 24 hours and 48 hours, the number of lymphocytes, neutrophils and eosinophils was highly increased compared to naive and ethanol-treated mice. While in naive mice some lymphocytes were detected, eosinophils and neutrophils were completely absent, but neutrophils were present in ethanol-treated mice. Mast cells, as expected, were present in naive and ethanol-treated mice and slightly increased in mice with regular CHR (Fig. 4.3). Moreover, the number of mast cells at 48 hours after elicitation was slightly higher than at 24 hours.

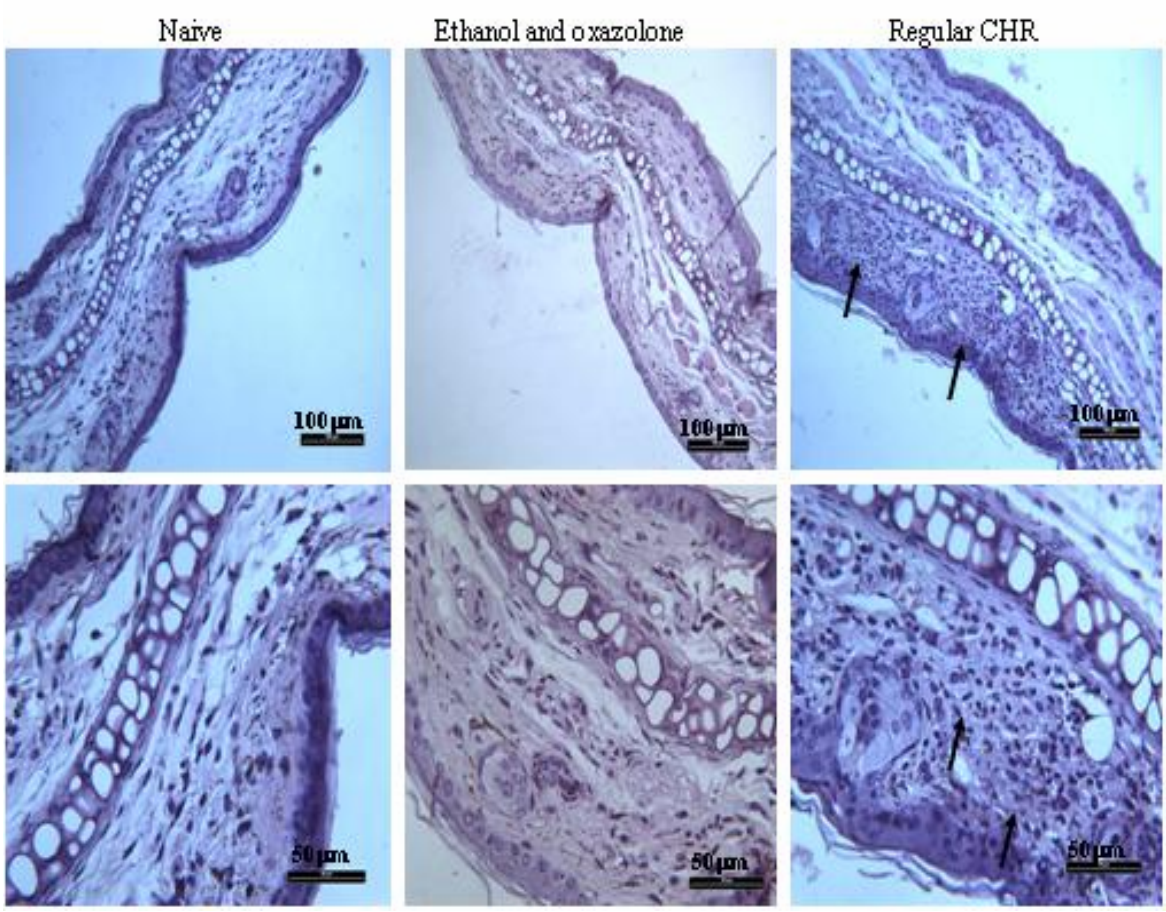


Figure 4.2 Contact hypersensitivity response to oxazolone is antigen-specific. Photographs of hematoxylin and eosin stained pinnae sections of CHR in wild type mice at 48 hours after elicitation. Arrows point to inflammatory infiltrating cells. The photographs are a representation of 5 pinnae in each group. Original magnification x 160 (top row) and x 400 (bottom row).

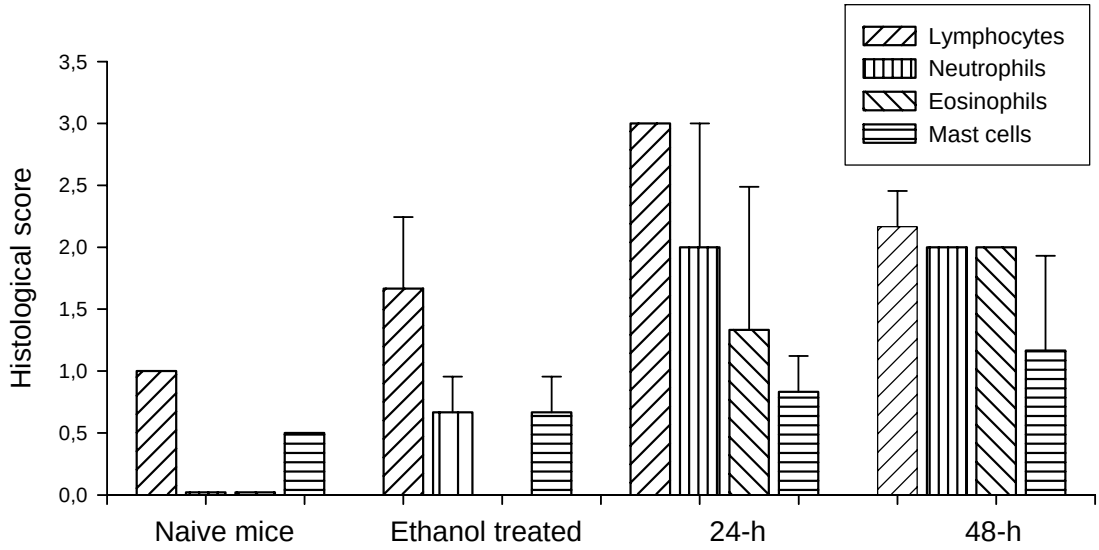


Figure 4.3 Cellular inflammatory infiltrate in CHR to oxazolone at 24 and 48 hours after challenge. Numbers of lymphocytes, neutrophils, eosinophils and mast cells were highly increased in mice with regular CHR at 24 (n = 3) and 48 hours (n = 5) compared to naive (n = 3) and ethanol-treated mice (n = 5).

4.2 Role of mast cells in contact hypersensitivity

4.2.1 Mast cell-deficient mice do not mount a CHR

Mast cell-deficient mice exhibited 50 to 70 % reduced tissue swelling in a regular CHR compared to wild type mice (Biedermann, *et al*., 2000), (Askenase *et al*., 1983). In addition, chemokines released by mast cells (i.e. MIP-2 and TNF) are essential for the recruitment of PMNs in a regular CHR (Biedermann, *et al*., 2000). Therefore, I wanted to investigate whether the role of mast cells could be demonstrated by applying a relatively low concentration of oxazolone as described earlier (Rennert, *et al*., 2001). Absolute pinnae swelling was measured at several time points after challenge. Regular CHR in mast cell-deficient mice was significantly decreased to background levels when measured from 24 until 74 hours after challenge (Fig. 4.4A). In contrast to the results of Biedermann and co-workers (Biedermann, *et al*., 2000), which reduced 50 to 70 % pinna swelling, the CHR in mast cell-deficient mice measured both at 24 and 48 hours after challenge was reduced to background levels compared to wild type mice (Fig. 4.4B).

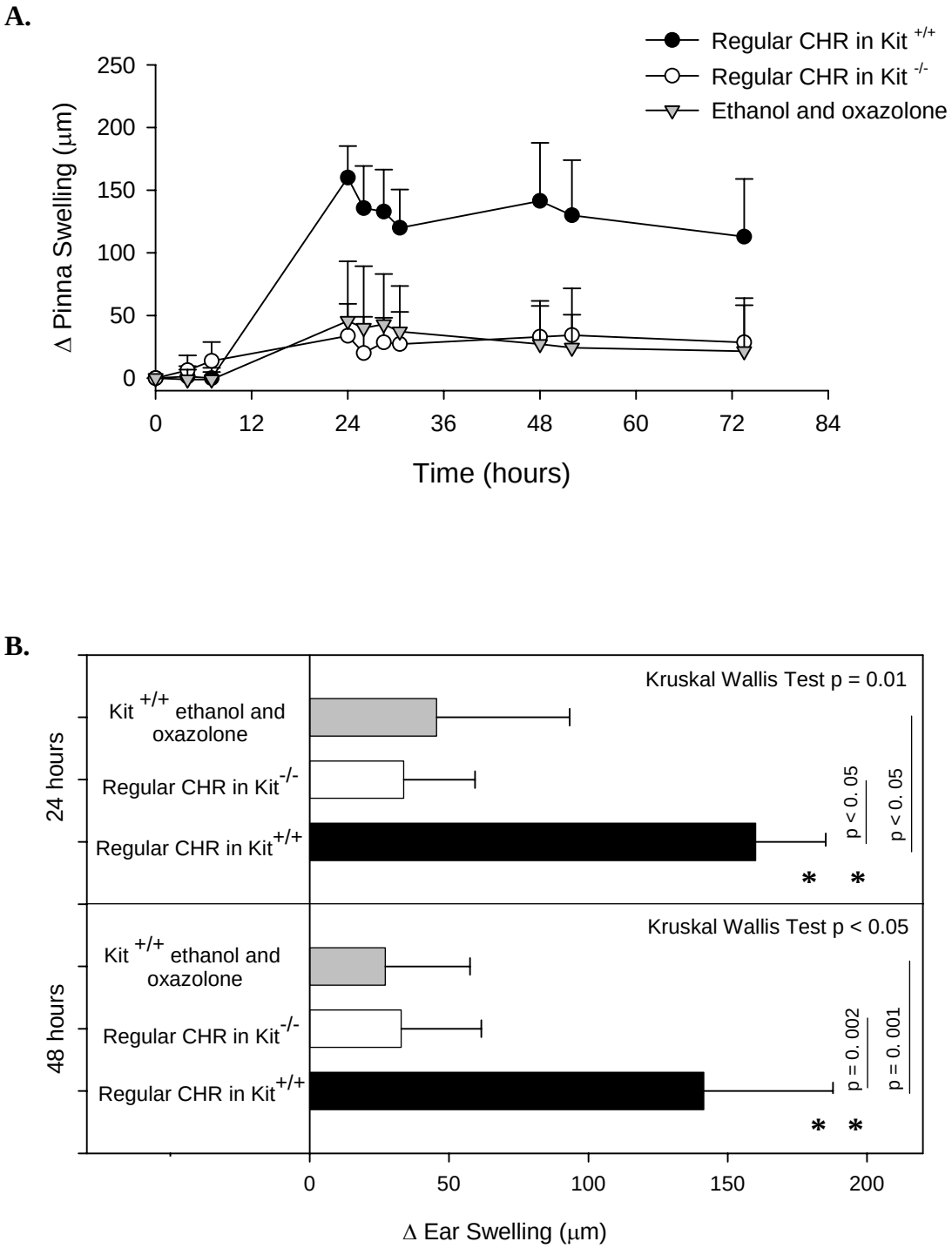


Figure 4.4 Kit^{W}/Kit^{W-v} mice do not mount a regular CHR. Kit^{W}/Kit^{W-v} ($Kit^{-/-}$) mice (n = 8) and congenic wild type ($Kit^{+/+}$) mice (n = 9) were sensitised and 4 days later challenged with oxazolone. As negative control mice were treated with ethanol and challenged with oxazolone (n= 9). A) CHR at several time points after elicitation. B) CHR 24 and 48 hours after challenge. Statistical analysis was calculated by Kruskal Wallis and Mann Whitney Test and was considered significant when $p < 0.05$ (*).

4.2.2 Mast cells reside in close proximity to lymphocytes in allergic reactions

The mast cell is known to be an essential effector cell in allergic diseases and contributes to chronic inflammatory processes. Mast cells were found to reside in close proximity to lymphocytes in inflamed tissues and allergic reactions (Fig. 4.5). In *in vitro* experiments, coculture of mast cells with hapten-specific type 1 T cells resulted in significant MIP-2 production, suggesting that T cell-mast cell interactions lead not only to mast cell degranulation (Inamura *et al*., 1998) but also to cytokine and chemokine production (Biedermann *et al*., 2000). This close apposition between mast cells and lymphocytes has led investigators to propose a functional relationship between these two cell populations that might facilitate elicitation of an immune response.

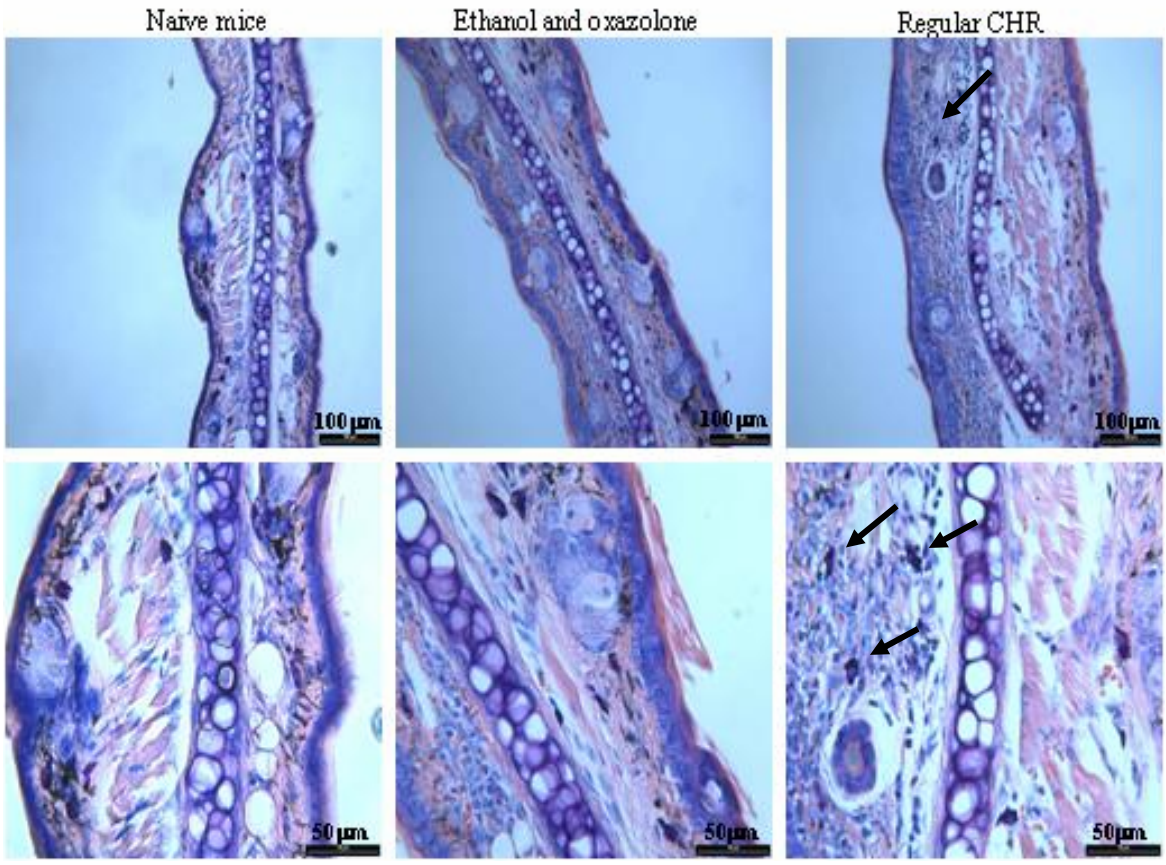


Figure 4.5 In CHR inflammatory cells are in close proximity to mast cells. Photographs of Giemsa stained pinnae sections of regular CHR in wild type mice at 48 hours after challenge. The arrows indicate stained mast cells in close proximity to lymphocytes, eosinophils and neutrophils. The photographs are a representation of 5 pinnae. Original magnification x 160 (top row) and x 400 (bottom row).

4.3 Role of LTβR in contact hypersensitivity

4.3.1 LTβR- and LIGHT-deficient mice do not mount a CHS response when measured at 24 hours but when measured at 48 hours after challenge.

$LT\alpha_1\beta_2$ and LIGHT are expressed on activated lymphocytes and are capable of signalling through the LTβR which is expressed on stromal cells, macrophages and mast cells (Stopfer *et al*., 2004). This hints to the possibility that the LTβR-LT/LIGHT interaction may represent a means of communication for activated lymphocytes with their neighbouring receptor-positive mast cells in CHR. Therefore, I investigated the role of LTβR in CHR using LTβR–deficient mice. Absolute pinnae swelling was measured at several time points after challenge (Fig. 4.6A). In C57BL/6 mice, the regular CHR to oxazolone shows a significant pinnae swelling when measured both at 24 and 48 hours after challenge compared to background levels (Fig. 4.6B). In LTβR–deficient mice, the CHR to oxazolone measured at 24 hours after challenge was significantly reduced compared to that of wild type mice. However, 48 hours after challenge LTβR–deficient mice were able to respond to oxazolone even significantly stronger than wild type mice. In LIGHT –deficient mice, the CHR was very similar to that of LTβR–deficient mice (Fig. 4.6B). Thus, LTβR and LIGHT seem to be involved in the CHR to oxazolone when measured 24 hours after challenge. The delay of the response in these mice might influence the 48 hour response, which is significantly higher in LTβR–deficient and slightly higher in LIGHT–deficient mice compared to wild type mice. Note that at 48 hours LIGHT–deficient was not significantly different compared to negative control this might be due to few mice in this group.

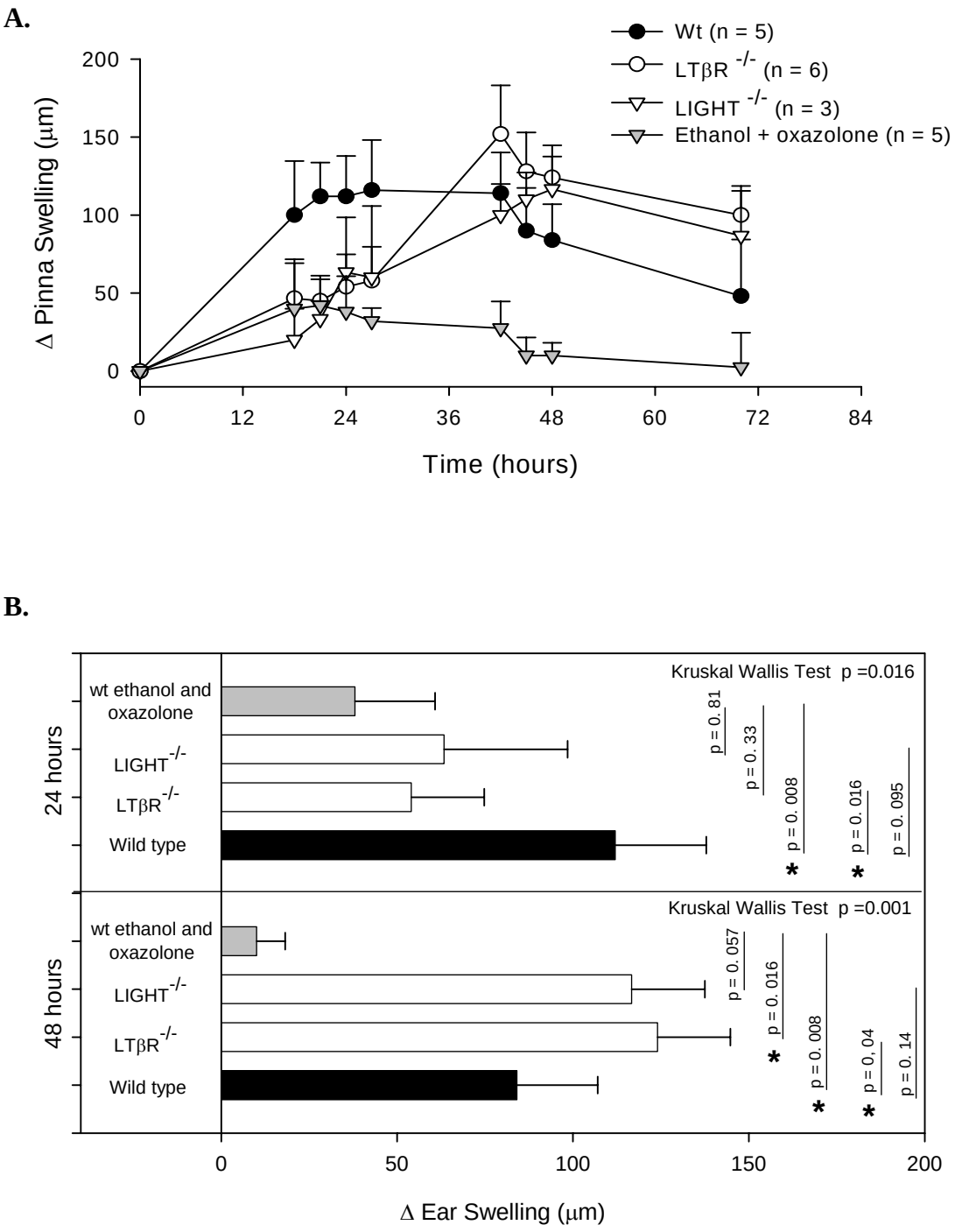


Figure 4.6 LTβR–deficient and LIGHT–deficient mice do not mount a CHR when measured at 24 hours after challenge but were able to respond 48 hours after challenge. A) Regular CHR to oxazolone at several time points after challenge. B) CHR to oxazolone 24 and 48 hours after challenge. Statistical significance was analysed by Kruskal Wallis and Mann Whitney Test and was considered significant when $p < 0.05$ (*). Abbreviation: wt, wild type; n, number of mice.

4.3.2 Blocking the ligands of the LTβR with a LTβR-Ig fusion protein in C57BL/6 mice had no effect on pinnae swelling in our CHS model

To test whether LTβR expression is necessary to mount a regular CHR 24 hours after challenge, mice were treated with LTβR-Ig to block the LTβR-LT/LIGHT interaction. Therefore, mice were treated twice with 100 μg/ml of LTβR-Ig-fusion protein at both, one day before and immediately before challenge. The CHR to oxazolone was measured at several time points after challenge (Fig. 4.7A). LTβR-Ig-treated mice were able to mount a CHR at 24 and 47 hours after challenge (Fig 4.7B). The pinnae swelling of LTβR-Ig-treated mice was not significantly different compared to that of wild type mice or human IgG-treated mice measured at 24 and 47 hours after challenge. In contrast to the regular CHR in LTβR-deficient mice, treatment with LTβR-Ig fusion protein before challenge did not demonstrate and essential role for LTβR in CHR after 24 hours.

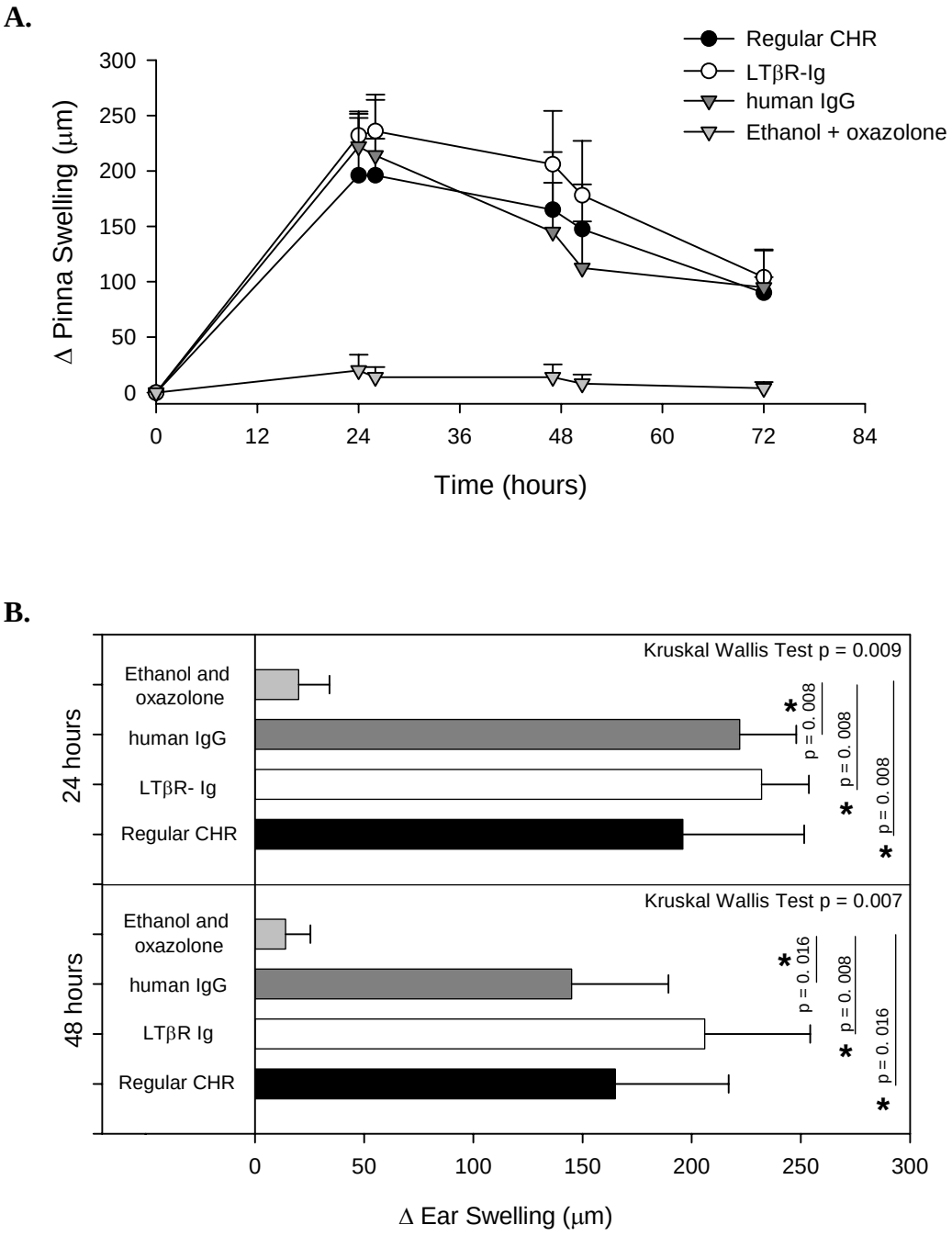


Figure 4.7 LTβR-Ig-treated mice were able to mount a regular CHR to oxazolone at 24 and 48 hours after challenge. In the CHR experiment, NMRI mice were treated one day before and immediately before challenge with 100 μg/ml of LTβR-Ig fusion protein or human IgG. A) Regular CHR to oxazolone at several time points after challenge (n = 5). B) CHR to oxazolone 24 and 48 hours after challenge. Statistical analysis was calculated by Kruskal Wallis and Mann Whitney Test and was considered significant when $p < 0.05$ (*).

4.4 LTβR-Ig fusion protein binds to $LT\alpha_1\beta_2$/LIGHT on activated lymphocytes

4.4.1 Staining of lymph node cells with monoclonal antibodies against mouse LTβ

The expression of LTβ on sensitised and non-sensitised lymph node cells was demonstrated by FACS analysis. As a positive control staining with the monoclonal antibodies against mouse LTβ was positive on activated A20 cells. Four days after sensitisation with oxazolone the braquial and axillary lymph nodes from sensitised and non-sensitised mice were removed and the cells analysed by FACS analysis. 4.7 % of non-sensitised lymph node cells were found to be LTβ-positive. Of these 4.7 % positive cells, 0.7% was expressed on T cells and 4 % on B cells. In sensitised lymph node the LTβ-positive cell number decreased to 3.7 % compared to non-sensitised lymph nodes. In sensitised lymph node cells only B cells stained positive for LTβ (Fig. 4.8). Therefore, LTβ was expressed on the surface of sensitised and non-sensitised lymph node cells and mostly expressed on B cells (Table 4.1).

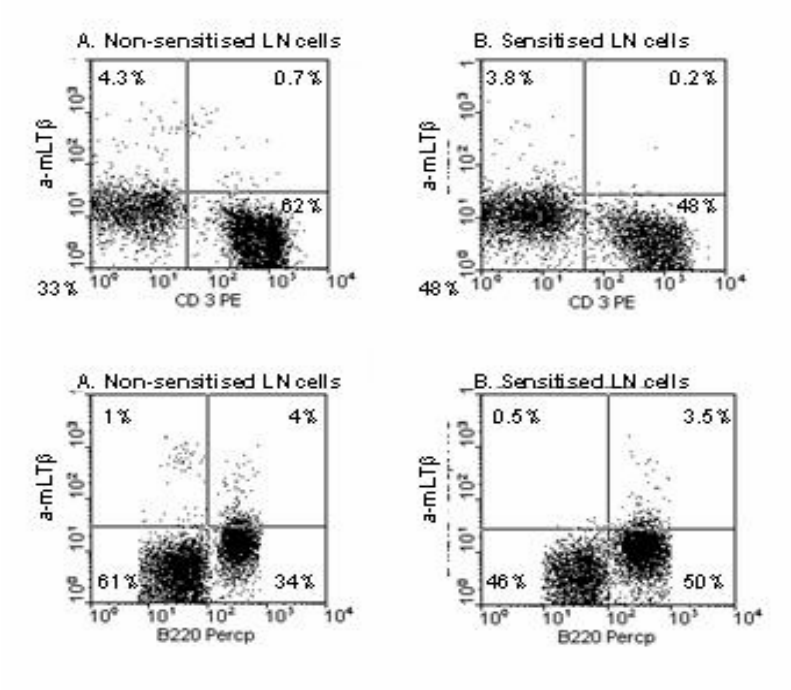


Figure 4.8 LTβ was expressed in sensitised and non-sensitised lymph node cells. Braquial and axillary lymph nodes from A) non-sensitised and B) sensitised mice were removed 4 days after sensitisation and the cells were analysed by FACS analysis. Anti-CD3 PE was used as marker for T cells and anti-B220 PerCp as marker for B cells.

	T cells	B cells	Total
Non-sensitised LN cells	0.7 %	4 %	4.7 %
Sensitised LN cells	0.2 %	3.5 %	3.7 %

Table 4.1 LTβ expression on sensitised and non-sensitised lymph node cells. Lymph node cells were analyzed by FACS analysis 4 days after sensitisation. The percentages are 1 selected out of 2 independent experiments. Anti-CD3 PE was used as marker for T cells and anti-B220 PerCp as marker for B cells. Abbreviation: LN: lymph node.

4.4.2 Staining of lymph node cells with LTβR-Ig fusion protein

To block the LTβR-LT/LIGHT pathway the LTβR-Ig fusion protein was used without changing the CHR (Section 4.3.2). To test the functional blocking activity the binding of LTβR-Ig fusion protein to sensitised and non-sensitised lymph node cells was tested four days after sensitisation. As a positive control, A20 cells (B cell lineage) were activated with 50 ng/ml PMA for 5 hours (Browning *et al*., 1997). The LTβR-Ig fusion protein binding was positive on these activated cells. Non-sensitised lymph node cells showed positive staining (5.4 %) and increased to 6.3 % on sensitised lymph node cells (Fig. 4.9). In addition, the positive staining was mainly on B lymphocytes for both non-sensitised and sensitised lymph node cells (Table 4.2). These results show that the LTβR-Ig preparation used in the experiments was able to bind to $LT\alpha_1\beta_2$ on B lymphocytes, demonstrating that the LTβR-Ig in principle was able to block the LTβR/LT pathway.

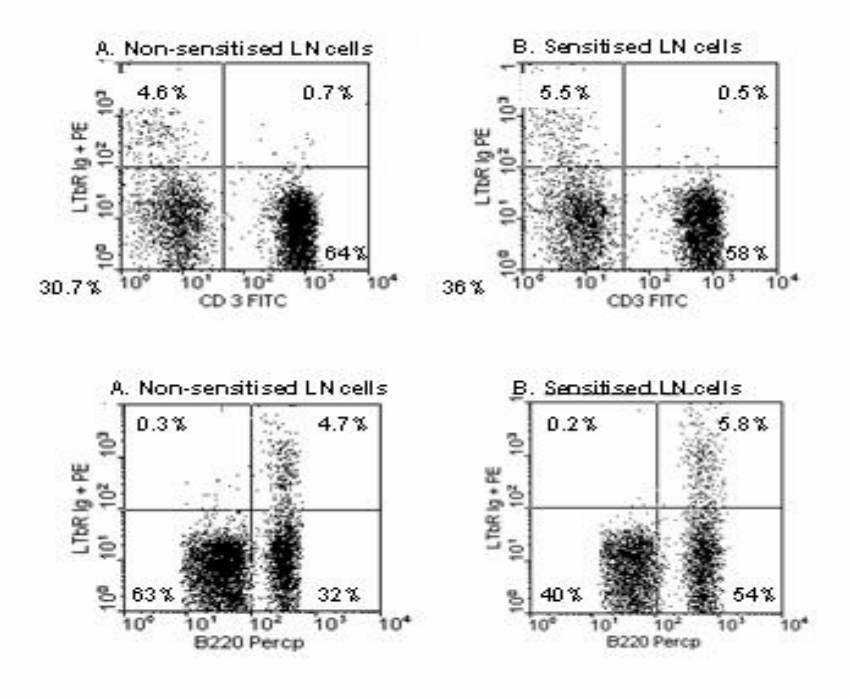


Figure 4.9 $LT\alpha_1\beta_2$ was expressed on sensitised and non-sensitised lymph node cells. Lymph node cells from A) non-sensitised and B) sensitised mice were removed 4 days after sensitisation and cells were analysed by FACS analysis. Anti-CD3 PE was used as marker for T cells and anti-B220 PerCp as marker for B cells.

	T cells	**B cells**	**Total**
Non-sensitised LN cells	0.7 %	4.7 %	5.4 %
Sensitised LN cells	0.5 %	5.8 %	6.3 %

Table 4.2 Staining of $LT\alpha_1\beta_2$ with LTβR-Ig fusion protein in sensitised and non-sensitised lymph node cells. Lymph node cells were analyzed by FACS analysis 4 days after sensitisation. The percentages are representative of 2 independent experiments. Anti -CD3 PE was used as marker for T cells and anti-B220 PerCp as marker for B cells. Abbreviation: LN: lymph node.

4.5 Lymphocyte transfer experiments

4.5.1 Titration of sensitised lymph node cells required for transfer a CHS response

To investigate whether the expression of LTβR on mast cells is necessary for the elicitation of CHR, sensitised lymph node cells were transferred into naive, mast cell-deficient ($Kit^{-/-}$), and LTβR-deficient mice. As a positive control C57BL/6 mice were sensitised and challenged and the CHR compared to the CHR after sensitised lymph node cells had been transferred into naive C57BL/6. To determine the optimal cell number for the transfer experiment, 2.5 $x10^7$, $1x10^7$, or $1x10^6$ cells were transferred into naive mice, and the elicitation of a CHR compared (Fig. 4.10B). The more sensitised lymph node cells were transferred the more pinnae swelling was measured after elicitation. Transfer of $2.5x10^7$ lymph node cells was capable of inducing CHR in naive mice (Fig. 4.10A) as already published earlier (Rennert *et al*., 2001). Transfer of $2.5x10^7$ cells induced about 30 % of the CHR measured in mice that had been sensitised and challenged in a regular CHR. Surprisingly, the transfer of sensitised lymph node cells did not induce a CHR at early time points after challenge, i.e. after 24 hours but only after late time points i.e. after 38 hours. As a negative control non-sensitised lymph node cells were transferred into naive mice which also lead to a slight swelling reaction after 38 hours.

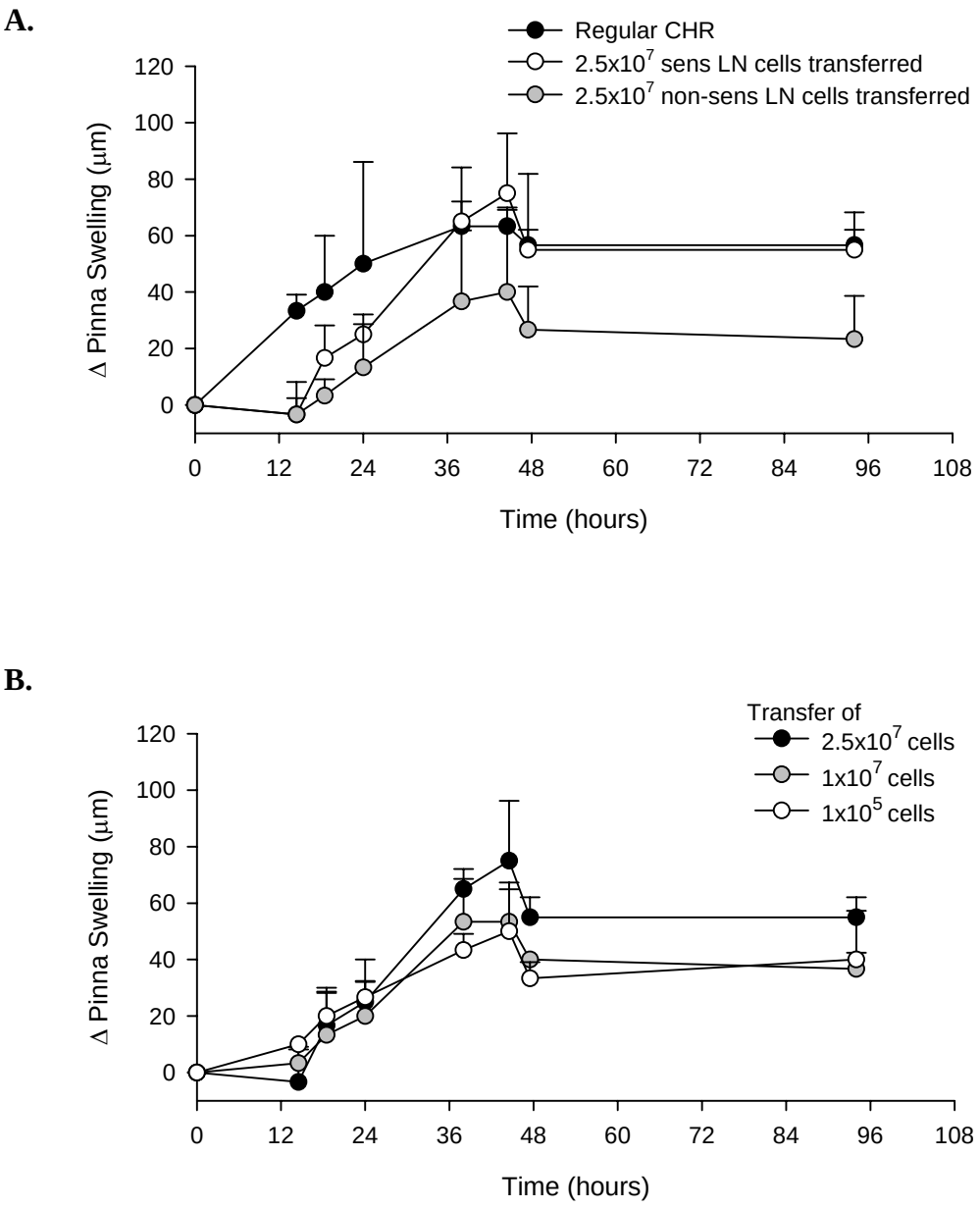


Figure 4.10 Titration of sensitised lymph node cells transferred into naive mice to determine the required cell number for transfer. Mice were sensitised and 4 days later the braquial and axillary lymph nodes were removed. A) Regular CHR in naive mice compared to sensitised and non-sensitised $2.5x10^7$ lymph node cells transferred into naive mice. B) Different numbers of lymph node cells from sensitised mice transferred into naive mice (n = 3). Abbreviation: LN, lymph node; sens, sensitised, CHR, contact hypersensitivity response.

4.5.2 Sensitised lymph node cells induce a response independently of the presence of mast cells at the late i.e. 40 to 48 hours elicitation phase

To investigate whether activated lymphocytes interact with mast cells in the elicitation phase of the CHR, sensitised lymph node cells were transferred into mast cell-deficient mice. Immediately after transfer of lymph node cells, mice were challenged and pinna swelling was measured at several time points (Fig. 4.11A). Again, the transfer of sensitised lymph node cells did not induce a CHR at 24 hours after challenge neither in wild type nor in mast cell-deficient mice. Transfer of sensitised lymph node cells to wild type and to mast cell-deficient mice was not significantly different in pinna swelling at 48 hours after challenge. Sensitised lymph node cells induced 30 % of the regular CHR in both wild type mice and mast cell-deficient mice when measured at 40 to 48 hours after challenge (Fig. 4.11B). Therefore, the 30 % of CHR was induced by sensitised lymph node cells independently of mast cells at 40 to 48 hours after challenge. As negative control, non-sensitised lymph node cells were transferred into wild type mice.

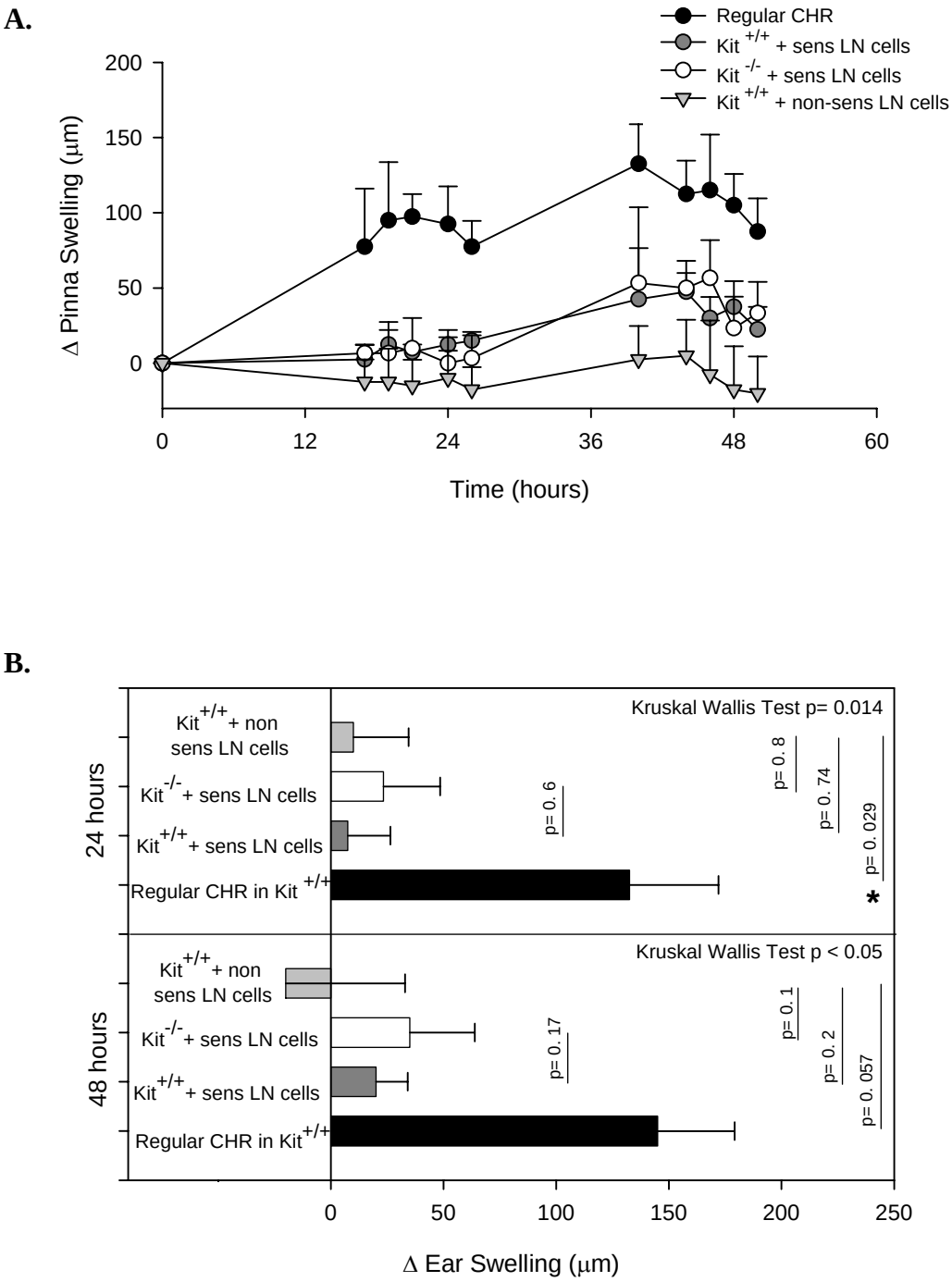
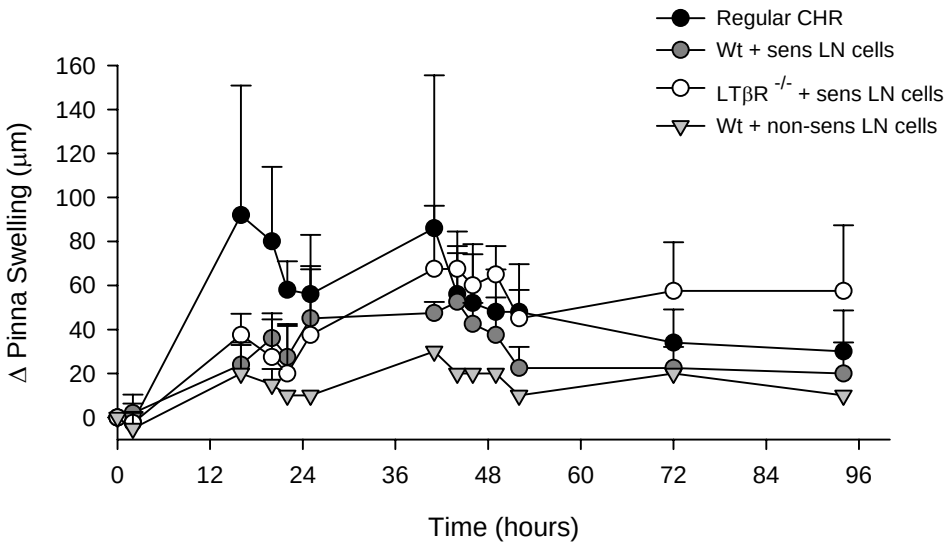


Figure 4.11 Sensitised lymph node cells induce CHR independently of the presence of mast cells in the late i.e. 40 to 48 hours elicitation phase. A) Pinna swelling was measured at several time points after challenge (n = 4). B) Transfer experiment response at 24 and 48 hours after challenge. Statistical analysis was calculated by Kruskal Wallis and Mann Whitney Tests and they were considered significant when $p < 0.05$ (*). Abbreviation: LN, lymph node; wt, wild type, sens, sensitised, CHR, contact hypersensitivity response.

4.5.3 LTβR is not involved at the late response of contact hypersensitivity measured 24 hours after challenge

The initial experiment in LTβR-deficient mice showed that the regular CHR to oxazolone was defective at early 24 hours but not at the late 48 hours phase. To assess whether the lack of the LTβR interaction with the LT ligands was responsible for the defective CHR 24 hours after challenge, sensitised lymph node cells of wild type mice were transferred to LTβR-deficient mice. After transfer of sensitised lymph node cells, pinna swelling was measured at several time points after challenge (Fig. 4.12A). As shown in Fig 4.12A, the CHR measurable at early time points after transfer and challenge was partially induced in LTβR-deficient and wild type mice when compared to a regular CHR in wild type mice. Transfer of sensitised lymph node cells in LTβR-deficient and wild type mice showed no difference in pinnae swelling at time points later than 24 hours after challenge (Fig. 4.12A and B). Therefore, the LTβR does not seem to be involved in the later elicitation phase of a CHR measured after 24 hours after challenge. The CHR of LTβR-deficient mice grafted with sensitised lymph node cells was always more pronounced than wild type mice grafted with sensitised lymph node cells (Fig. 4.12A and B). This results correlate to the observation in the regular CHR in LTβR-deficient mice versus wild type mice (Fig. 4.6A). As a negative control, non-sensitised lymph node cells were transferred into wild type mice (Fig. 4.12).

A.



B.

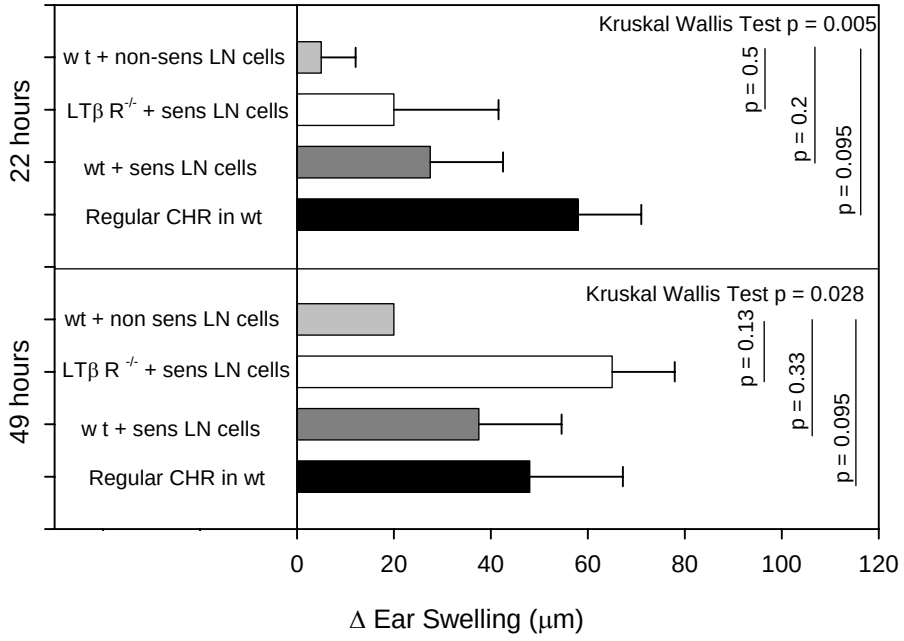


Figure 4.12 LTβR is not involved in the late elicitation phase of a CHR measured after 24 hours after challenge. A) Pinna swelling was measured at several time points after challenge (n = 5). B) Transfer experiment response at 22 and 49 hours after challenge. Statistical analysis was calculated by Kruskal Wallis test and was considered significant when $p < 0.05$. Abbreviation: LN, lymph node; wt, wild type, sens, sensitised, CHR, contact hypersensitivity response.

LTβR-deficient mice develop a late 48 hours CHR and the infiltrating inflammatory cells were analysed by histology. The score demonstrated a high number of lymphocytes, neutrophils, eosinophils, and mast cells. Moreover, the infiltrating cells in adoptive transfer experiments were also analysed. Transfer of sensitised lymph node cells to LTβR-deficient was able to induce a similar CHR as regular CHR in LTβR-deficient. Also the cellular infiltrate after transfer to LTβR-deficient was comparable to regular CHR in LTβR-deficient mice. In both cases the number of infiltrating cells was higher than after adoptive transfer of sensitised lymph node cells to C57BL/6 mice. Furthermore, the number of lymphocytes was the same in every group (Fig. 4.13).

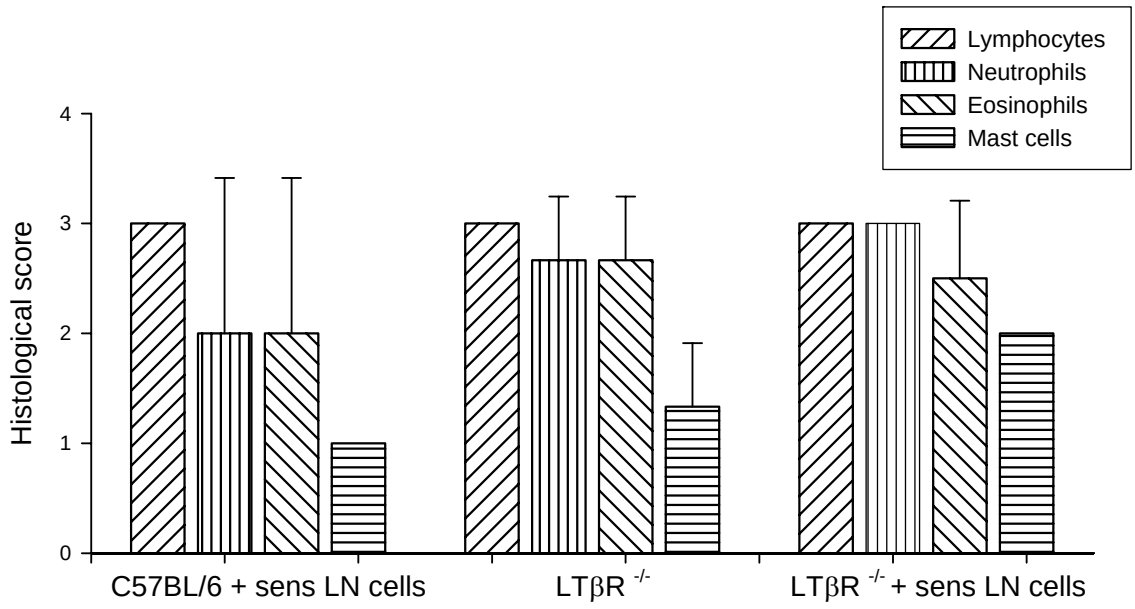


Figure 4.13 Cellular inflammatory infiltrate in a regular CHR in LTβR-deficient ($LT\beta R^{-/-}$) and after transfer of sensitised lymph node cells to C57BL/6 and LTβR-deficient mice 48 hours after challenge. Cellular inflammatory infiltrate in LTβR-deficient and in LTβR-deficient after transfer of sensitised lymph node cells demonstrated a higher cellular infiltrate compared to transfer into C57BL/6 (n = 3). Abbreviations: LN, lymph node; sens, sensitised.

5. Discussion

5.1 Early (24 hours) effector phase of contact hypersensitivity

Several cell types in the adaptive and the innate immune system contribute to the inflammatory process in CHS. Close apposition of T cells and mast cells in inflammation led to the investigation of physical interactions between these two cell populations. Activation of T cells leads to the expression of several proteins on the membrane such as $LT\alpha_1\beta_2$ or LIGHT, and since mast cells express LTβR on the membrane, this study examined the role of activated T cells interacting with mast cells via the LTβR-$LT\alpha_1\beta_2$ system during the elicitation phase of CHS. In order to see whether the LTβR or its ligands are involved, CHS experiments were performed in mice deficient in the LTβR or its ligand LIGHT. Both mouse strains were unable to develop a response 24 hours after challenge. This could indicate that activation of the LTβR plays an important role in CHS. This result is consistent with work by Rennert and co-workers, who showed that LTα-deficient mice were unable to respond to oxazolone, DNFB, and FITC 24 hours after challenge (Rennert *et al*., 2001). However, the developmental defects observed in these knockout mice may be the reason for their unresponsiveness at 24 hours, because signalling through the LTβR by the $LT\alpha_1\beta_2$ heterotrimer is critical during lymphoid development, and both LTα- and LTβ-deficient mice lack all or a subset of peripheral lymph nodes (Banks *et al*., 1995; De Togni *et al*., 1994). Mice deficient in the *Ltβr* gene exhibit a phenotype characterised by a failure to form lymph nodes and Peyer's patches and the absence of natural killer (NK) and NK-T cells. In the adult mice LTβR signalling is required for maintaining splenic architecture, the integrity of the marginal zone, and compartmentalisation of T and B cells, also they lack germinal centres and networks of follicular dendritic cells (Dejardin *et al*., 2002). Additionally, Rennert and other group, showed that lymph nodes are essential for inducing a contact hypersensitivity response (Macher *et al.*, 1969a; Macher *et al.*, 1969b; Rennert *et al*., 2001). The phenotype of LIGHT-deficient mice showed a full complement of lymph node, although LIGHT can contribute to lymph node development (Scheu *et al*., 2002), as revealed by LIGHT-deficient LTβ-deficient double knockout mice (Ware, 2005). However, the unresponsiveness of LIGHT at the early 24 hours CHS could be because LIGHT induces

T cell proliferation, NF-κB translocation, and secretion of IFN-γ *in vitro* (Chapoval *et al*., 2000), a lack of which may influence CHS responses in the LIGHT-deficient mouse.

Therefore, LTβR-Ig fusion protein was used to block both the $LT\alpha_1\beta_2$ and LIGHT pathways and to circumvent the developmental defects observed in the knockout mice. In contrast to CHS in knockout mice, mice treated with LTβR-Ig fusion protein did develop the early response. Mice were treated with 100 μg of LTβR-Ig fusion protein at day 1 and 3 (data not shown) and at day 3 and 4 (Fig. 4.7) after sensitisation, and both treatments failed to inhibit the early 24 hour response, indicating that signalling via the LTβR does not play a critical role during the early elicitation stage. Moreover, activated T cells have been shown to migrate to the skin within the first 2 hours after challenge (Hwang *et al*., 2004), thus LTβR-Ig treatment immediately before challenge should be able to block LIGHT or $LT\alpha_1\beta_2$ on circulating lymphocytes. To demonstrate that the LTβR-Ig fusion protein is able to interact with the ligands of the LTβR, the LTβR-Ig preparation used in the experiments was analysed by FACS analysis and was shown to be able to bind to $LT\alpha_1\beta_2$ on lymphocytes, demonstrating that the LTβR-Ig in principle was able to block the LTβR/LT pathway (Fig. 4.9). Interestingly, LTβ as well as $LT\alpha_1\beta_2$/LIGHT molecules were mainly expressed on activated and non activated B cells recovered from lymph node. While LTβ-positive cells were reduced in numbers after sensitisation, LTβR-Ig binding cells were slightly enhanced showing LIGHT expression on these cells.

To elucidate these contradictory results adoptive transfer of sensitised lymph node cells were transferred into LTβR-deficient mice. However, adoptive transfer of sensitised lymph node cells into wild type mice did not induce CHS at 24 hours, but only at 48 hours after challenge (Fig. 4.10). Rennert and co-workers demonstrated that LTα expression by lymphocytes does not play a role in CHS (Rennert *et al*., 2001). They transferred bone marrow cells from LTα-deficient mice into irradiated wild type mice, which were able to mount a CHS response at 24 hours. These results are consistent with the results after LTβR-Ig fusion protein treatment that demonstrated that the ligands of the LTβR are not involved in the early phase of CHS.

C57BL/6 wild type recipient mice of sensitised lymph node cells did not develop an early effector phase of CHS. Ozaki and co-workers (Ozaki *et al*., 2003) showed similar results with transfer of antigen specific T cells into naive rats, in which the early phase reaction was absent. The authors discussed that the early phase might be absent because the B cell compartment is not primed at the sensitisation stage and therefore antigen-specific IgE is not produced. An immediate allergic reaction is characterised by elevated serum IgE levels, production of allergen-specific IgE, and the release of inflammatory mediators from mast cells and granulocytes. Moreover, immunoglobulin production is not restricted to IgE, also allergen-specific IgG_1, IgG_{2a} and IgM are produced in allergic reactions (Jarman *et al*., 1999). Consecutively, mast cell activation can be mediated by cross-linking of surface FcεRI (IgE) and FcγRIII (IgG) molecules (Alber *et al*., 1992; Katz *et al*., 1992), and augmented by immune complex-mediated complement fixation. Activated mast cells produce TNF and MIP-2, which are essential for PMN recruitment during TNCB-induced CHS responses (Biedermann *et al*., 2000). Therefore, a lack of allergen-specific immunoglobulins leads to absence of immune complexes, complement and mast cells are not activated, and PMNs are not recruited to the site of challenge, which leads to a lack of the early elicitation stage in CHS response.

In addition, the early phase could also be absent because the sensitised lymph node cells used for transfer experiments lack T_H1 cells. For transfer of sensitised lymph node cells, the draining lymph nodes were removed 4 days after sensitisation. Mohler and co-workers (Mohler *et al*., 1990), showed that lymph node cells sensitised with picryl chloride had detectable IL-2 mRNA on day 1 to 3 and IL-4 mRNA on days 3 to 5. Thus, lymph node cells isolated in this model at day 4 produced IL-4, which is a typical cytokine produced by T_H2 cells. This is consistent with the data by Weigmann and co-workers, that suggested that T_H1/Tc1 cells mediated the early 24 hours and that T_H2/Tc2 cells mediated the late 48 to 72 hours effector phase of CHS (Weigmann *et al*., 1997). Therefore, if sensitised lymph node cells of the T_H2 subtype are transferred into naive mice, the early phase cannot be induced while a late effector phase can be induced in the elicitation stage of CHS.

In contrast, Ptak and co-workers showed that they can induce the early effector phase of CHS after transfer and they confirmed that the response is antigen specific (Ptak *et al*., 1991). They sensitised mice with 3 % of oxazolone on the 4 paws and on the shaved abdomen. Four days later 5 to 7 x 10^7 sensitised cells (splenocytes and lymph node cells) were transferred by i.v. injection (in 1 ml) via the retroorbital plexus into recipient mice which were challenged 18 to 24 hours after transfer with 0.8 % oxazolone. Interestingly, they used Balb/c and CBA/J mice. Rennert and co-workers were also able to induce the early CHS response in adoptive transfer experiments by using a similar protocol, as was used in this thesis (Rennert *et al*., 2001). Pregnant female Balb/c mice treated with the LTβR-Ig fusion protein lack all peripheral lymph nodes but retain mesenteric lymph nodes and spleen, were sensitised with 0.3 % of oxazolone. Four days later mesenteric lymph node cells and splenocytes were isolated and transferred into naive Balb/c mice which were then challenged with 0.3 % oxazolone. Transfer of lymph node cells only, and not splenocytes, induced an increase of 30 % in pinna thickness similar to positive control mice. The mouse strain used by Ptak and co-workers and by Rennert and co-workers was Balb/c, which may be an explanation for the early CHS response observed after transfer of sensitised cells. Other experiments showed that C57BL/6 mice reacted with a reduced pinna swelling compared to Balb/c mice (Berg *et al*., 1995; Weigmann *et al*., 1997). The authors discussed that Balb/c mice were sensitised with DNFB which is a more potent allergen than oxazolone, which was the allergen used for C57BL/6 mice. Another explanation could be that C57BL/6 mice have a point mutation in the gene encoding murine mast cell protease 7, which prevents these mice from producing this mast cell protease (Hunt *et al*., 1996). C57BL/6 mice are also deficient in the phospholipase A_2, which has been implicated in facilitating exocytosis in mast cells by generating lysophospholipids for fusion of the perigranular and plasma membrane and in providing arachidonic acid used for eicosanoid biosynthesis (Drazen *et al*., 1996). Therefore, these mutation and deficiencies in C57BL/6 mice perhaps reduce CHS response or may influence the early response after adoptive transfer into recipient mice.

Mast cell-deficient mice ($Kit^W/Kit^{W\text{-}v}$) showed no response during the early and late phase of the elicitation stage (Fig 4.4). Askenase and co-workers also showed that two different strains of mast cell-deficient mice (W/W^v and SI/Sid) developed no response at

the early and late phase of CHS (Askenase *et al*., 1983). The Kit^{W}/Kit^{W-v} mouse was shown to be deficient in serotonin-containing mast cells at skin sites preferred for elicitation of DTH in normal mice, such as the ear or footpad. As both strains of mast cell-deficient mice with completely different genetic defects developed an impaired CHR, mast cell deficiency is probably the cause of their inability to manifest normal CHR. Biedermann and co-workers showed that CHR was 50 to 70 % reduced in Kit^{W}/Kit^{W-v} compared to wild type mice (Biedermann *et al*., 2000). They used 2 % of TNCB for sensitisation and 1 % of TNCB for challenge and probably this high hapten concentration led to an unspecific response. An alternative explanation for a defective CHR in Kit^{W}/Kit^{W-v} mice is the phenotype of the knockout. The Kit^{W}/Kit^{W-v} mice have multiple defects in their hematopoietic system (Galli *et al*., 1996), profound defects in T cell responses, and altered migration through vascular walls. On the other hand, it was shown by Biedermann and co-workers that hapten-specific proliferation and IFN-γ production in T cells were comparable in wild type and Kit^{W}/Kit^{W-v} mice (Biedermann *et al*., 2000).

To investigate the function of the LTβR on mast cells in the elicitation phase of CHS, adoptive transfer of sensitised lymph node cells from wild type mice were transferred into Kit^{W}/Kit^{W-v} mice. Sensitised lymphocytes express the ligands for LTβR, and their transfer into Kit^{W}/Kit^{W-v} should give a clear result for a possible interaction between mast cells and lymphocytes via the LTβR-$LT\alpha_1\beta_2$/LIGHT pathway. However, the adoptive transfer, as mentioned before, in our model failed to induce the early phase of the elicitation stage in CHS (Fig. 4.11B). Thus, no conclusion can be drawn from this experiment for the requirement of LTβR activation on mast cell for the early effector phase of CHS.

Various groups that have examined CHS in mast cell-deficient mice reported different results. Some groups found similar pinna swelling responses in wild type and mast cell-deficient mice (Asada *et al*., 1997; Galli *et al*., 1984). Others found a strongly reduced response in mast cell deficient mice (Askenase *et al*., 1983) or an attenuated response (Biedermann *et al*., 2000). These different requirements of mast cells during T cell-mediated immune responses suggest that the requirement for mast cells depends on the

strength of these immune responses under investigation. The immune response is influenced by various factors such as the concentration of allergen at the sensitisation and elicitation stage, the solvent used, the housing conditions, which will determine the activation of memory cells and APCs.

5.2 Late (48 hours) effector phase of contact hypersensitivity

CHR in LTβR- and LIGHT-deficient mice was observed at 48 hours after challenge. This is a surprising result considering that Rennert and co-workers showed that lymph nodes are essential for inducing a CHS response (Rennert *et al*., 2001), and LTβR-deficient mice lack all or a subset of peripheral lymph nodes (Banks *et al*., 1995; De Togni *et al*., 1994). Also, LIGHT is involved in inducing T cell proliferation, NF-κB translocation, and secretion of IFN-γ *in vitro* (Tamada *et al*., 2000b), which may influence CHS responses. Obviously, the phenotype of the deficient mice influences the early phase and this early phase may influence the late phase of CHS, since the late phase in CHR in LTβR–deficient mice was significantly more pronounced than in wild type mice (Fig. 4.6B). This result is consistent with the late phase of CHR in LIGHT–deficient mice which also showed a more pronounced response than wild type (Fig. 4.6B). Furthermore, also when LTβR-Ig fusion protein was used to block both the $LT\alpha_1\beta_2$ and LIGHT pathways the mice showed a slight enhancement of the late phase response compared to controls (Fig. 4.7B). Moreover, transfer of sensitised lymph node cells into LTβR-deficient showed an enhanced response at the late phase of CHS (Fig. 4.12). These results suggest that LTβR activation leads to a downregulatory effect at the late phase of CHS. Another study showed that mice treated with LTβR-Ig fusion protein in acute colitis showed enhanced inflammation compared to controls (unpublished data). Therefore, the LTβR activation might exert a downregulatory role in acute inflammation. Additional molecules influence CHS response. Interestingly, other group showed that mice deficient in IFNγ, its receptor, or the TNF receptor are all able to mount a DTH response and the reaction is stronger than footpad swelling measured in wild type littermates. The pinna swelling response of TCR δ-deficient mice, which lack TCR $\gamma\delta^+$ T cells, was increased compared to control mice, suggesting also a downregulatory role of $\gamma\delta^+$ T cells in CHR (Weigmann *et al*., 1997). These data was consistent with previous findings by Sullivan

and co-workers, which demonstrated a down-regulatory role of Thy-I^+ epidermal cells in CHS (Sullivan *et al*., 1986).

To investigate the function of the LTβR on mast cells in the elicitation phase of CHS, sensitised lymph node cells from $Kit^{+/+}$ were transferred to Kit^W/Kit $^{W\text{-}v}$ mice and induced 30 % of the response at the late phase (48 hours) compared to a regular CHR in wild type. Since, $Kit^{+/+}$ and Kit^W/Kit $^{W\text{-}v}$ showed similar responses at the late phase of CHS after lymph node cell transfer, it is suggested that mast cells are not involved in the late phase or not involved within the 30 % response induced by transfer (Fig. 4.11B). Transfer of lymph node cells is not sufficient to induce the complete late phase response in Kit^W/$Kit^{W\text{-}v}$ mice. Probably a higher number of lymph node cells is required for the induction of the entire late effector phase in this mouse strain. In addition, independently of sensitised lymph node cells, the lack of mast cells in the skin may also influence the infiltration of inflammatory cells like PMNs (Biedermann *et al*., 2000).

Further experiments could clarify the interaction between mast cells and T cells in CHS. For example, CHS could be performed in a conditional knockout mouse that does not express LTβR specifically on mast cells. It would also be interesting to use gene deficient mice with a different genetic background, as C57BL/6 showed a reduced response compared to other strains (discussed earlier).

5.3 Expression of LTβR ligands in sensitised lymph node cells

LTβR-Ig fusion protein and monoclonal antibodies to LTβ are very useful tools to investigate the expression of $LT\alpha_1\beta_2$ and LIGHT on draining lymph node cells. Four days after sensitisation, LTβ expression on lymphocytes decreased by 1 % on sensitised compared to non sensitised lymph node cells. From this 1 % decrease, expression on B lymphocytes decreased by 0.5 % and by 0.5 % on T lymphocytes (Fig. 4.8). This decrease could be because activated lymphocytes recirculate to encounter antigen. T_H1 cells produce LT, and Mohler and Butler showed that from day 1 to 3 after sensitisation mRNA for the T_H1 cytokine IL-2 was detectable while at days 3 to 5 mRNA for the T_H2 cytokine IL-4 was detectable (Mohler and Butler, 1990). Therefore, less expression of

LTβ at day 4 after sensitisation could be due to migration of T_H1 cells into the circulation. Moreover, LTβ was mostly expressed on B lymphocytes, which is consistent with expression of T_H2 cytokines at day 4 after sensitisation, because T_H2 cells are specialised for B-cell activation and they secrete the B cell growth factors IL-4 and IL-5 (Janeway C. *et al*., 2001). Determination of intracellular cytokines by FACS in our system would clarify this point.

Although contact sensitisation is considered to be a T cell-mediated immune response, mice exposed to allergens show increases in B lymphocytes in the draining lymph nodes. The phenotype of cells in lymph nodes following exposure to 0.3 % of oxazolone consisted of a 24 % increase in B cells and a 20.3 % decrease in T cells in sensitised compared to non-sensitised lymph node cells at day 4 after sensitisation (Table 4.1). In addition, Gerberick and co-workers showed that mice treated specifically with allergens but not irritants show an increase in I-A^{κ} (MHC class II) and CD86 on $B220^+$ or IgG/IgM^+ B cells (Gerberick *et al*., 1999). Manetz and workers also reported that the $B220^+$ population becomes significantly elevated in draining lymph node following exposure to allergen (Manetz *et al*., 1999). Both B cell and T cells are important for CHR. Another group showed that the phenotype of cells in lymph nodes consisted of 44.7 % $B220^+$, 37.8 % $CD4^+$, and 16.6 % $CD8^+$ cells following exposure to 1% of oxazolone at day 4 after sensitisation (Kuhn *et al*., 1995).

	Exp 1	**Exp 2**	**Exp 3**	**mean**
T cells	20 % decrease	14 % decrease	27 % decrease	20.3 % decrease
B cells	25 % increase	16 % increase	31 % increase	24 % increase

Table 4.1 T cells decrease and B cells increase in sensitised lymph node cells compared to non sensitised lymph node cells at day 4 after sensitisation. Anti-CD3 PE was used as marker for T cells and anti-B220 PerCp as marker for B cells.

Staining with the LTβR-Ig fusion protein demonstrates the expression of LIGHT on T cells and of $LT\alpha_1\beta_2$ on T- and B-cells. In contrast to LTβ expression, the LTβR-Ig positive staining showed a 0.2 % decrease on T cells and a 1.1 % increase on B cells

when comparing sensitised to non-sensitised lymph node cells. Although the expression of the ligands on T cells decreases by 0.2 %, the decrease was smaller than the 0.5 % decrease of the LTβ staining, probably because LIGHT was detected in addition. In contrast, expression of the ligands on B cells increased by 1.1 % on sensitised compared to non-sensitised lymph node cells.

These results demonstrate that lymph node cells transferred 4 days after sensitisation consisted mainly of B cells expressing $LT\alpha_1\beta_2$, and of a small population of T cells also expressing both ligands ($LT\alpha_1\beta_2$/LIGHT). Moreover, within the first 2 hours of challenge, lymphocytes seem to enter the circulation and migrate to the skin, thereby modifying the environment for further recruitment of inflammatory cells (Hwang *et al.*, 2004). Within this early 2 hours phase, possible interactions between mast cells and lymphocytes via the LTβR-$LT\alpha_1\beta_2$/LIGHT pathway may be crucial for further recruitment of inflammatory cells. Unfortunately, the adoptive transfer of lymph node cells 4 days after sensitisation did not induce the early effector phase of CHR (24 hours), thus it is difficult to conclude that the LTβR is involved in the early phase of CHS. Maybe transfer of lymph node cells of day 1 or 2 after sensitisation may induce the early effector phase, because sensitised lymph node cells showed a high increase in T cell numbers at day 1 after sensitisation and a lower increase in T cell numbers at day 2 after sensitisation (data not shown). In this case, we could conclude whether the early phase is mediated by the increase of T cells in draining lymph node produced at day 1 or 2 after sensitisation compared to non-sensitised lymph node cells. Because it has already been shown that the late phase is induced by an increase in B cells and a decrease of T cells of sensitised compared to non-sensitised lymph node cells at day 4 after sensitisation (Fig. 4.8, 4.9 and 4.10).

5.4 Infiltrating cells in the early and late phase of contact hypersensitivity

Cytokines are important for the recruitment of inflammatory cells to the site of challenge. Enk and Katz found that within the first hour of allergen application to the skin, expression of mRNA was enhanced for IL-1β, TNF, and IFN-γ (Enk *et al.*, 1995). The upregulation of these cytokines was followed by enhanced expression of IP-10 (Abe *et*

al., 1996), MIP-2 and IL-1α, as well as MHC class II, whereas β-actin signals used as a control remained unchanged. TNF and IFN-γ were also up-regulated non-specifically after application of the vehicle control to the skin. In contrast, signals for IL-1β, IL-1α, MIP-2, IP-10 and MHC class II molecule I-Aα were induced only after application of the allergen.

Histologically, pinna sections at the early and late effector phase of CHS showed high numbers of lymphocytes, neutrophils and eosinophils and a slight increase in mast cells. Many cell types, cytokines, and adhesion molecules are involved in the infiltration of inflammatory cells. TNF and IL-1 are potent inducers of endothelial cell adhesion molecules and they are secreted from many cell types and from macrophages in particular. Activation of keratinocytes and Langerhans' cells leads to the production of several cytokines important for CHR. Activated keratinocytes produce TNF, GM-CSF, IL-1, IL-3, IL-6, IL-8, M-CSF, TGFα, and TGFβ. IL-3 can enhance antigen presentation for T cell dependent responses, augment macrophage cytotoxicity and adhesion, and promote the function of eosinophils, basophils, and mast cells (Cannistra *et al*., 1988; Kimoto *et al*., 1988; Madden *et al*., 1991; Pulaski *et al*., 1996; Rothenberg *et al*., 1988). IL-3 can activate Langerhans' cells, co-stimulate proliferative responses and recruit mast cells. Activated keratinocytes, Langerhans' cells or skin macrophages also generate C5a, which activates local mast cells, resulting in degranulation and release of MIP-2, TNF and serotonin as well as activation of endothelial cells and upregulation of adhesion molecules such as β_2-integrins and P-selectin (Foreman *et al*., 1994; Jagels *et al*., 2000). Circulating hapten-specific T cells will bind to the activated endothelial cells and then migrate across the endothelium into the challenged site of the skin. Subsequently T_H1-cells interact with hapten-MHC II molecules on local APCs. T cells are induced to produce inflammatory cytokines such as TNF and IFN-γ. Recruitment of $CD4^+$ lymphocytes in the first 2 hours obviously modified the microenvironment to allow for the 24 hours recruitment of inflammatory cells. Expression of adhesion molecules like VCAM-1, the α4-integrin ligand, is elevated by 4 hours after challenge (McHale *et al.*, 1999b). Therefore, Hwang and co-workers suggested that at 24 hours after challenge leukocyte recruitment is α_4-integrin mediated (Hwang *et al*., 2004).

Degranulation and cytokine release by mast cells follows soon after contact with an allergen. Moreover, Biedermann and co-workers demonstrated that mast cells can determine the pattern of cells infiltrating into the sites of inflammation through the chemokines they produce (Biedermann *et al*., 2000). Mast cells are capable of producing high amounts of TNF and chemokines including MIP-2. It was shown that TNF and MIP-2 were essential for appropriate PMNs recruitment during CHR and that both were dependent on the presence of mast cells (Biedermann *et al*., 2000). In addition, TNF was not required for MIP-2 induction during CHR. The biological function of TNF seems to be the induction of expression of adhesion molecules required for PMN attachment to endothelial cells (McHale *et al.*, 1999a). The chemokine MIP-2 establishes a chemotactic gradient required for diapedesis and directed migration of PMNs into the site of inflammation (Feng *et al*., 1995; Hang *et al*., 1999).

A histological score of the cellular infiltrate during the late phase was also analysed in LTβR-deficient mice and after transfer of sensitised lymph node cells into wild type, and into LTβR-deficient mice. The cellular infiltrate of regular CHR in LTβR-deficient mice was comparable to that after transfer into LTβR-deficient mice. In both cases a higher cellular infiltrate could be seen compared to transfer of sensitised lymph node cells into wild type mice. Since the effect was enhanced when sensitised lymph node cells were transferred into LTβR-deficient mice, these results suggest that the LTβR activation mediates a downregulatory effect on the late effector phase of CHS.

The downregulatory effect of the LTβR activation could be mediated by interaction with either $LT\alpha_1\beta_2$ or LIGHT. In figure 4.6, regular CHS model in LIGHT-deficient mice showed comparable results to the LTβR-deficient mice. Both developed an enhanced response at the late phase of CHR. This result suggested that the downregulatory effect could be mediated via LTβR-LIGHT interaction. In addition, wild type mice treated with the LTβR-Ig fusion protein also induced an enhanced response, which suggested the role of both ligands $LT\alpha_1\beta_2$ and LIGHT. Moreover, transfer of sensitised lymph node cells into LTβR-deficient mice also induced an enhanced response at the late phase and FACS

analysis demonstrated that most of the cells that were transferred expressed $LT\alpha_1\beta_2$ on B cells (Fig. 4.8). Thus the activation of the LTβR could be mediated via both ligands $LT\alpha_1\beta_2$ and LIGHT in CHS.

LTβR activation induces different pattern of gene expression via two NF-κB pathways (Dejardin *et al*., 2002). The first pathway leads to the expression of inflammatory genes such as VCAM-1, MIP-1β and MIP-2. This pathway is contradictory to the result that suggested a downregulatory effect of the LTβR in CHS. However, the second pathway leads to transcription of genes which are implicated in secondary lymphoid organogenesis and homeostasis such as SLC, ELC, SDF-1α, and BAFF. The second pathway could be an explanation for the downregulatory effect of the LTβR in CHS because SDF-1 plays roles in the early B cell development and BAFF has a critical role in B cell developments, formation of germinal centres and in B cell survival (Batten *et al*., 2000; Egawa *et al*., 2001). A possible explanation for the downregulatory effect of the LTβR activation at the late phase in CHS is that leads to expression of SDF-1 and BAFF, which leads to B cells development and production of specific immunoglobulins against an allergen in CHS. Because as discussed previously, the early response was initiated by immunoglobulins produced by B cells, if the response at 24 hours is affected, this may affect the response at 48 hours, leading to an enhancement at the late 48 hours. Probably, LTβR-LT interactions are not involved at the early phase as demonstrated by the LTβR-Ig fusion protein treatment, but maybe is involved at late 48 hours response. Thus, lack of the LTβR activation leads to an enhancement of the late CHS response.

5.5 Mast cell and T cell interactions by other ligands

Molecules other than the LTβR have been suggested to be involved in cell interaction between mast cell and T cells. One group demonstrated that bone marrow derived mouse mast cells (BMMCs) required pre-treatment with IL-4 to secrete active exosomes, which were found to express molecules such as MHC class II, CD86, LFA-1 and ICAM-1 (Skokos *et al*., 2001). Therefore, mast cells can represent a critical component of the immunoregulatory network through secreted exosomes that display mitogenic activity on

B and T lymphocytes both *in vitro* and *in vivo*. Inamura and co-workers have shown that activated mast cells were able to form heterotypic aggregates with activated but not with resting T lymphocytes (Inamura *et al*., 1998). This coculture, which resulted in degranulation of mast cells, was adhesion-dependent since addition of anti-LFA-1 and anti-ICAM-1 monoclonal antibodies inhibited T-cell and mast cell interactions *in vitro*, inhibiting mast cell degranulation. Furthermore, interactions between mast cells and B cells have also been demonstrated (Gauchat *et al*., 1993). Human mast cell lines and basophilic cell lines as well as purified human mast cells and blood basophils have been shown to express the ligand for CD40, which provides the cell contact signals to B cells to produce immunoglobulins.

5.6 Treatments for allergy

Current research in the field of allergic contact dermatitis and other skin diseases should aim at the development of strategies for the blockade of T cell-mediated cytotoxicity and T cell homing to the skin, and of protocols to shift the balance between effector and T regulatory (T reg) cells. Thus, agents like rapamycin, cyclosporin A, FK506 or other immunosuppressants, which are successfully used as therapeutic agents in skin diseases, may act by preventing T cell activation and subsequent induction of keratinocyte apoptosis (Martin, 2004). Several approaches that interfere with T cell activation and homing are being tested. The application of neutralising monoclonal antibodies and drugs blocking the action of chemokines or adhesion molecules in animal studies reveal a successful suppression of CHS. T reg cells producing immunosuppressive cytokines can be induced in specific immunotherapy like hyposensitisation to allergens from cat or house dust mite following intradermal allergen injection, thus demonstrating their usefulness in therapy (Martin, 2004). IL-10 was reported to be essential for downregulation of CHS response (Berg *et al*., 1995). IL-10 mRNA was shown to be up-regulated in keratinocytes, but not in T cells or Langerhans' cells following epicutaneous contact allergen application (Enk *et al*., 1992b). Adhesion molecules are another target to prevent recruitment of inflammatory cells. Hwang and co-workers suggested that targeting either endothelial selectin alone will not suffice, and targeting selectins after challenge will not inhibit the late phase of CHS (Hwang *et al*., 2004). But targeted

prophylactically this may be a potent therapeutic intervention. Targeting of the H1 receptor is another successful approach used for allergies. H1 receptor is expressed on peripheral nerves, keratinocytes and endothelial cells. The characteristic features of H1 receptor activation in the skin are itching and increased vascular permeability. H1 receptor antagonists have been successfully used as drugs for treating allergies (Seike *et al.*, 2005).

In conclusion, this study shows that blocking LTβR activation might not be a useful strategy to prevent CHS reactions. However, promising new therapeutic strategies based on a better understanding of the immune mechanisms underlying allergen-induced pathogenesis are being developed. They will hopefully allow us to design more specific drugs and replace systemic immunosuppressants with all their unnecessary side effects (Martin, 2004). It therefore seems worthwhile to continue and intensify research in this field since targeting molecules on T cells, targeting T cells to a desired location in the body or prevention of T cell migration to a tissue where they cause allergy or autoimmunity seems to be a feasible therapeutic approach.

6 Summary

Close apposition of T cells and mast cells in inflammation led to the investigation of physical interactions between these two cell populations. Activation of T cells leads to the expression of lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) or LIGHT. Since mast cells express lymphotoxin-β receptor (LTβR) on the membrane, this study examined the role of activated T cells interacting with mast cells via the LTβR-$LT\alpha_1\beta_2$/LIGHT system during the elicitation phase of contact hypersensitivity.

In order to see whether the LTβR or its ligands are involved in the development of contact hypersensitivity, such experiments were performed in mice deficient in either the LTβR or its ligand LIGHT. Both deficient mouse strains were not capable of developing a response 24 hours after challenge. To circumvent the developmental defects observed in the knockout mice, mice treated with the LTβR-Ig fusion protein was used to block both ligands, $LT\alpha_1\beta_2$ and LIGHT. In contrast to the contact hypersensitivity response in LTβR or LIGHT knockout mice, mice treated with LTβR-Ig fusion protein were able to develop the early effector response. To elucidate these contradictory results adoptive transfer of sensitised lymph node cells into LTβR-deficient mice was performed. However, adoptive transfer of sensitised lymph node cells into wild type mice did not induce any contact hypersensitivity response at 24 hours, but only at 48 hours after challenge. Therefore, additional experiments are necessary to elucidate the role to the LTβR at the early phase in the contact hypersensitivity response.

On the other hand, LTβR seems to have a downregulatory effect at the late (48 hours) effector phase of contact hypersensitivity. Contact hypersensitivity experiments performed in LTβR- and LIGHT-deficient mice showed an enhanced response at the late effector phase. Transfer of sensitised lymph node cells into LTβR-deficient mice also induced an enhanced late response in contact hypersensitivity. A histological score of the cellular infiltrate was also analysed during the late phase of regular contact hypersensitivity reaction in LTβR-deficient mice as well as in LTβR-deficient after transfer of sensitised lymph node cells. The cellular infiltrate of the regular contact hypersensitivity response in LTβR-deficient mice was comparable to that after transfer of

sensitised lymphocytes into LTβR-deficient mice, with both having a higher cellular infiltrate compared to that after transfer of sensitised lymph node cells into wild type mice. These results are consistent with the contact hypersensitivity in wild type mice treated with the LTβR-Ig fusion protein which also showed an enhanced late effector phase response. In conclusion, these results suggest that the LTβR activation mediates a downregulatory effect on the late effector phase of contact hypersensitivity. However, additional experiments are necessary to demonstrate whether this effect is mediated by the activation of the LTβR on mast cells.

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10 List of publications

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