Doctoral Thesis

Regulation of surface bound expression vs. secretion of IL-1α

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Regulation of surface bound expression vs. secretion of IL-1α

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for the degree of
DOCTOR OF SCIENCES

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Abstract

The Interleukin (IL)-1 superfamily consists of 11 members and belongs to the large family of interleukins within the cytokines. Most IL-1 members are very potent pro-inflammatory cytokines, and therefore tight regulation is required. Thus, every pro-inflammatory member has its anti-inflammatory antagonist.

IL-1α and IL-1β were the first described members of the IL-1 superfamily. Both pro-inflammatory cytokines are produced as an intracellular precursor. For IL-1β, proteolytical cleavage by caspase-1 into a mature and a secreted form is required for biological activity. Caspase-1 activity is regulated by the inflammasomes, an intracellular multiprotein platform. In contrast, IL-1α exists not only as a mature secreted form but also as a membrane-associated cell surface form. This cell surface form has hardly been studied and its biological relevance has been questioned. Furthermore, in contrast to IL-1β, neither the processing of IL-1α nor regulation of IL-1α secretion is understood. Although caspase-1 does not cleave IL-1α, the secretion is caspase-1 dependent.

In the main part of this thesis, we investigated the regulation of IL-1α surface expression and secretion with respect to the role of the inflammasome and caspase-1. Our results demonstrate that IL-1α surface expression depends on TLR mediated NFκB activation, whereas IL-1α secretion required additional activation of the inflammasome and caspase-1. We also showed that presence of IL-1β was required for IL-1α secretion and found that IL-1β acted as a shuttle for IL-1α secretion. Moreover, we characterized the surface form of IL-1α by mutation analysis of posttranslational modifications sites and found that lectin - carbohydrate interactions were the most promising candidates to mediate surface anchoring.

IL-1α and IL-1β, both very potent pro-inflammatory cytokines, are involved in many important diseases, such as rheumatoid arthritis, diabetes, atherosclerosis or Alzheimer’s. Our data on regulation of IL-1α surface expression and secretion helps to better understand the underlying mechanisms of such diseases and has direct impact on selective treatment modalities of inflammatory diseases. We
demonstrate that the cell surface form of IL-1α and the secreted form follow different pathways and also are differentially regulated. This may allow a selective therapeutic blockade of either one of the IL-1α forms. Further research must therefore characterize the relative importance of these two IL-1α forms in antimicrobial protection and in disease.

In several side projects of this thesis, we were interested in vaccine related issues. Firstly, we evaluated the role of IL-1α during cancer progression. We found IL-1α to be involved in tumour growth and tumour angiogenesis. In another project we aimed to improve immunogenicity of the tuberculosis vaccine BCG by using a mutant strain. A third side project evaluated and characterized a T-cell independent vaccine suitable for vaccination against amyloid-β in Alzheimer’s disease. In yet another side project, we addressed the side effects of a commonly used antihistamine on the innate immune system.
Zusammenfassung

Die Interleukin (IL)-1 Superfamilie besteht aus 11 Mitgliedern und gehört innerhalb der Zytokinen zu der großen Familie der Interleukine. Die meisten IL-1 Mitglieder sind sehr potente pro-inflammatorische Zytokine, die daher einer strengen Regulation unterliegen. Demzufolge hat jedes pro-inflammatorische Mitglied seinen anti-inflammatorischen Antagonist.

IL-1α und IL-1β sind die ersten beschriebenen Mitglieder der IL-1 Superfamilie. Beide pro-inflammatorische Zytokine werden zunächst als Pro-Form produziert. Für IL-1β ist die proteolytische Spaltung durch caspase-1 in die mature und sekretierte Form Voraussetzung um die biologische Aktivität zu erreichen. Caspase-1 Aktivität wird von der intrazellulären Multiproteinplattform den Inflammasomen reguliert. Im Gegensatz dazu existiert das IL-1α nicht nur als mature sekretierte Form sondern auch als Membran-assozierte Zelloberflächenform. Diese Zelloberflächenform wurde nur wenig untersucht und seine biologische Relevanz ist fraglich. Zudem ist im Gegensatz zu IL-1β weder die Prozessierung noch die Regulation von der IL-1α verstanden. Obwohl Caspase-1 IL-1α nicht schneidet, so ist die Sekretion doch Caspase-1 abhängig.

Im Hauptteil dieser Thesis untersuchen wir die Regulation der IL-1α Oberflächen Expression und Sekretion im Hinblick auf die Rolle des Inflammasomes und der Caspase-1. Unsere Resultate demonstrieren, dass die IL-1α Oberflächenexpression von der TLR vermittelten NFκB Aktivierung abhängig ist, wohingegen die IL-1α Sekretion zusätzliche Aktivierung des Inflammasomes und der Caspase-1 benötigte. Zudem haben wir gezeigt, dass die IL-1α Sekretion die Präsenz von IL-1β benötigt und haben daraufhin gefunden, dass IL-1β als Shuttle für die IL-1α Sekretion fungiert. Des Weiteren haben wir die Oberflächenform von IL-1α durch Mutationenanalysen der posttranslationalen Modifikationsstellen charakterisiert und gefunden, dass die Lektin-Karbohydratinteraktionen der vielversprechendste Kandidat für die Vermittlung der Oberflächenverankerung war. IL-1α und IL-1β, beide sehr potente pro-inflammatorische Zytokine, sind in viele wichtige Krankheiten wie Rheumatoide Arthritis, Diabetes, Arteriosklerose oder
Alzheimer involviert. Unsere Daten zur IL-1\(\alpha\) Oberflächenexpressions- und Sekretions-Regulation hilft es die zugrundeliegenden Mechanismen solcher Krankheiten besser zu verstehen und hat direkten Einfluss auf selektive Behandlungsmodalitäten entzündlicher Krankheiten. Wir zeigen, dass die Oberflächenform und die sekretierte Form von IL-1\(\alpha\) verschiedene Expressionswege haben und somit unterschiedlich reguliert sind. Dies erlaubt eventuell eine selektivere therapeutische Blockade von der einen oder der anderen Form von IL-1\(\alpha\). Jedoch bedarf es dazu weitere Charakterisierung der relativen Wichtigkeit beider Formen von IL-1\(\alpha\) in Bezug auf antimikrobiellen Schutz und in Krankheit.

In verschiedenen Nebenprojekten haben wir uns für Vaccine-verwandte Fragestellungen interessiert. Im ersten Nebenprojekt dieser Thesis haben wir die Rolle von IL-1\(\alpha\) in der Krebsprogression untersucht. Wir haben gefunden, dass IL-1\(\alpha\) in das Tumorwachstum und die Tumorangiogenese involviert war. Ein anderes Projekt hatte das Ziel die Immunogenizität der Tuberculose-Vakzinierung BCG mit einem mutierten Stamm zu verbessern. Ein drittes Nebenprojekt evaluierte und charakterisierte eine T-Zell unabhängige Vakzine die für die Vakzinierung gegen das Alzheimer Protein Amyloid-\(\beta\) geeignet ist. Ein weiteres Nebenprojekt adressierte die Nebenwirkung eines gegenwärtig üblichen Antihistamins auf das angeborene Immunsystem.
## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Beta amyloid</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>AIM-2</td>
<td>Absent-in-melanoma-2 (AIM2) inflammasome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a caspase recruitment domain</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette Guerin</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BmDC</td>
<td>Bone-marrow derived dendritic cells</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domains</td>
</tr>
<tr>
<td>CD</td>
<td>Clustering of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CoIP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>ΔT</td>
<td>Delta T mutant, deletion of the type IV secretion system</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>H1R</td>
<td>Histamin 1 Receptor</td>
</tr>
<tr>
<td>HAT</td>
<td>Histon acetyl transferase</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group 1 protein</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1 converting enzyme (= caspase-1)</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunglobulin</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase-complex</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>Interleukin-1 receptor accessory protein</td>
</tr>
</tbody>
</table>
i.n. Intra nasal
iNOS Inducible nitric oxide synthase
IRAK Interleuin-1 receptor-associated kinase 4
JNK C-Jun N-terminal kinase
Ko Knockout
LM *Listeria monocytogenes*
LPS Lipopolysaccharide
MAPK Mitogen-activated protein kinase
MHC Major histocompatibility complex
MIP-1 Macrophage inflammatory protein-1
MMP Matrix metalloproteases
MOI Multiplicity of Infection
MOMA-1 Monoclonal antibody to metallophilic macrophages
MPLA Monophosphoryl lipid A
MSU Monosodium urate
*Mtb* *Mycobacterium tuberculosis*
MyD88 Myeloid differentiation primary response gene 88
MΦ Macrophages
NFκB Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK Natural killer
NLR Nod-like receptor
NLS Nuclear localization side
NO Nitric oxide
Lpn *Legionella pneumophila*
LRR Leucine rich repeat
PAMP Pathogen associated molecular pattern
PBMC Peripheral blood mononuclear cell
PGE2 Prostaglandin E2
PKC Protein kinase C
PNA Peanut agglutinin
PRR Pattern receptor recognition
PS Polysaccharide
PYD Pyrin domain
P2X7R Purinergic receptor P2X, ligand-gated ion channel, 7
RANTES Regulated upon Activation, Normal T-cell, and Secreted
RIGI Retinoid-inducible gene 1
RLH RIG-like helicases
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single immunoglobulin IL-1R-related molecule</td>
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<tr>
<td>sIL-1RIIAcP</td>
<td>Soluble IL-1RIIAcP</td>
</tr>
<tr>
<td>sIL-1RII</td>
<td>Soluble IL1RII</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β activated kinase 1</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T-cell/thymus dependent</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TI</td>
<td>T-cell/thymus independent</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR (Toll/interleukin-1 receptor)-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>up-LPS</td>
<td>Ultra-pure LPS</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Zmp</td>
<td>Zink metalloprotease</td>
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Chapter 1

Introduction
1.1 Dendritic cells and the immune system

The immune system has evolved to protect the host against bacteria, viruses, parasites, and cancer. Through the interplay of innate and adaptive immunity, responses are efficient, fast and highly specific, discriminating foreign and danger from healthy self. The immune system eliminates pathogens usually without much tissue damage. This requires a well-coordinated interplay between the adaptive and innate immune system. DCs play a central role as they are part of the innate immune system and instruct T cells of the adaptive immune system towards the kind of response that must be developed in order to find a balance between tissue damage and efficient pathogen clearance. The communication between immune cells is mainly mediated via cytokines. Cytokines are a diverse group of low molecular weight proteins that mediate communication among all leukocytes. The largest group of cytokines are the Interleukins (IL) with 37 members (IL-1 to IL-37). Other important members are tumour necrosis factor (TNF-α), transforming growth factor (TGF)-β and the interferons (IFN), i.e. IFNα, IFNβ, and IFNγ. Cytokines act either locally or systemically and have a short half-life. They regulate both innate and adaptive immunity. Due to their regulatory and central function in the immune system, they are involved in a variety of inflammatory and infectious diseases.

1.2 Innate immunity and pro-inflammatory cytokines

The innate immune system is the first line of defence that provides not only help for the adaptive immune cells but also controls many pathogens during the initial phase of infection, i.e. before the adaptive immune system could proliferate antigen-specific T- and B-cells. Innate immune cells are mainly derived from myeloid progenitors and include DCs, monocytes, macrophages (MΦ), natural killer (NK) cells, mastcells and granulocytes (neutrophils, eosinophils and basophils) (Fig.1.1). DCs have a central function in orchestrating important immune regulatory mechanisms of innate and adaptive immunity. This regulation is in part mediated via cytokines, which can be either pro-
inflammatory or anti-inflammatory. With respect to adaptive immunity, naïve CD4+ cells can differentiate into Th1-, Th2-, Th9-, Th17-, Th22- effector T cells, depending on the cytokine milieu given by a DC as an antigen-presenting cell (APC) (1). A milieu of IL-1, IL-6, TNFα, IFNγ, IL-2 and IL-12, i.e. pro-inflammatory cytokines will favour an efficient Th1 response, whereas IL-4, IL-5, IL-10 and TGFβ are anti-inflammatory and will favour a Th2 response (Fig. 1.2).

Figure 1.1 | Haematopoiesis. Immune cells are derived from a hematopoietic stem cell in the bone marrow. The pluripotent stem cell further differentiates into either a myeloid progenitor cell or a lymphoid progenitor cell. Myeloid progenitor cells will finally give rise to dendritic cells, neutrophils, monocytes, macrophages, eosinophils, mastcells, basophils, platelets and erythrocytes. Lymphoid progenitor cells give rise to dendritic cells, NK cells, T-cells and B-cells. (adapted from (2)).
Chapter 1 - Introduction

Dysregulation of the cytokine milieu can contribute to tissue damage, autoimmunity and chronic inflammation. In chronic inflammation pro-inflammatory cytokines dominate and will thereby contribute to a Th1 response leading to tissue damage. However, in order to be able to stimulate T-cells the APC requires stimulation itself. This stimulation is initiated via the innate pattern recognition receptors (PRR), a large family of germ-line encoded receptors recognizing repetitive and evolutionarily conserved elements. These receptors enable a rapid first line of defence against microbial invaders. This includes phagocytosis and inflammation. The PRR family can be subdivided into four subgroups, the Toll-like receptors (TLRs), C-type lectin receptors (CTLs), RIG-like helicases (RLHs) and the NOD-like receptors (NLRs). PRR are predominantly expressed by DCs, monocytes, MΦ, neutrophils and epithelial cells. Stimulation of such PRRs by ligand binding stimulates a signalling cascade often involving NFκB and AP-1 transcription factors and mediate expression of a specific set of cytokines that are released and generate the so-called signal 3 for the T-cells (3).
1.2.1 Sensing and signalling of innate immunity

The large diversity of PRR expressed on APCs sense a large variety of molecules derived from foreign pathogens including host-derived danger-associated molecules. More specifically, these receptors sense conserved microbial patterns, which are called pathogen-associated molecular patterns (PAMPs) (4), and additionally endogenous danger signals or alarmins released from stressed or necrotic cells after tissue injury. The latter danger signals are called danger-associated molecular patterns (DAMPs) (5). Although DAMPs can be released in sterile conditions as they are host-derived molecules, additionally immune responses against pathogens can lead to release of DAMPs by infected cells. Activation of the innate immune system via PRRs initiates an inflammatory response leading to secretion of cytokines, chemokines and induction of adhesion and costimulatory molecules, which altogether further recruit immune cells.

The first discovered group of PRRs are the TLRs (TLR1-11), which are membrane-bound sensors that recognise microbial products and lead to gene expression of pro-inflammatory molecules induced via NFκB signalling after TLR dimerization. The receptors are either cell surface associated or located in endosomal membranes. Therefore, they recognise pathogens in the extracellular milieu as well as in endosomal compartments. TLRs sense microbial products such as lipopolysaccharide (LPS), peptidoglycans, lipopeptides, microbial DNA (unmethylated CpG motifs), bacterial flagella, and double-stranded viral RNA (6). Similarly, membrane-bound CTLs (Dectin-1, -2, FcgRIII) scan the extracellular milieu and endosomal compartments for PAMPs such as β-glucans and zymosan (7). Receptors that detect microbial products exclusively in the cytosol are RLHs and NLRs, which consist of soluble proteins. RLHs (RIGI, MDA-5, DAI and AIM2) recognize intracellular nucleic acid (DNA and RNA) (8, 9). NLRs are composed of NODs, NALP- (or NLRP-), and IPAF (or NAIP)-inflammasomes, which sense intracellular PAMPs and DAMPs (10).
1.2.2 The inflammasomes

Inflammasomes (Fig. 1.3) belong to the group of NLRs and are intracellularly located multi-protein complexes able to activate caspase-1. Inflammasomes have a characteristic organization of three domains: the ligand-sensing leucine-rich repeat (LRR), the central NACHT domain which is essential for oligomerization and the effector domain consisting of a PYD, CARD or BIR domain. The LRR is involved in autoregulation and sensing of ligands, which are either of microbial origin or danger-associated self-molecules that are released from stressed, injured or necrotic cells. After sensing and oligomerization of the inflammasome the N-terminal effector domain, which can differ between the types of inflammasomes, can mediate a signal leading to expression of downstream targets and the activation of inflammatory caspase-1. Activated cleaved caspase-1 proteolytically cleaves and therefore activates the pro-inflammatory cytokines IL-1β and IL-18. IL-1β is one of the key molecules in acute inflammatory processes and in chronic inflammation (10-12). Inflammasome activation is tightly regulated and often interferes with other signalling pathways. For example, IL-1β secretion mediated by caspase-1 requires earlier activation of TLR signalling that lead to expression of pro-IL-1β and is called signal 1.

Figure 1.3 | The inflammasomes. The NLR family including NALPs, IPAF/NAIP, and NODs.(10)

The best characterized inflammasome is the NALP3 inflammasome. Many different, structurally unrelated PAMPs and DAMPs can activate the NALP3 inflammasome (Fig. 1.4).
NALP3 inflammasome activation. NALP3 inflammasome senses different PAMPs and DAMPs that induce oligomerization of NALP3 and further activation of caspase-1 resulting in an acute or chronic inflammatory response.

1.2.3 The IL-1 family of cytokines

IL-1α and IL-1β, the first two IL-1 family members discovered, were also the first two cytokines cloned in 1984 by Charles A. Dinarello. Today, the IL-1 family of cytokines consists of 11 members: IL-1α, IL-1β, IL-1RA, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36RA, IL-37 and IL-38 respectively (Fig 1.5).
IL-1α and IL-1β share low sequence homology (20-30%) but high 3-dimensional similarity and therefore both bind the same receptors. IL-1 receptor (IL-1R) type I signalling involves the adapter protein MyD88, which is shared with TLRs, and which recruits IL-1R-associated kinase 4 (IRAK4). IRAK4 recruits and phosphorylates IRAK1, which allows transient TRAF6 recruitment. Activated TRAF6 then associates with a complex including TAK1. TAK1 phosphorylates the inhibitor of kB (IKK) complex and several MAPKKs, finally leading to the activation of NFkB, p38 and JNK. This results in transcription of genes encoding cytokines, chemokines, acute phase proteins, cell adhesion proteins, degradation proteases and other enzymes (14). For IL-1 binding to induce a signalling cascade including MyD88 recruitment, IL-1RI has to associate with its IL-1R accessory protein (IL-1RacP). This IL-1RacP is shared by all signalling IL-1 receptors of the family. Once IL-1α or -β induce IL-1R signalling, a positive feedback mechanism triggers their own expression and the expression of other pro-inflammatory cytokines like IL-6. Therefore it is very important to stop this positive feedback loop and control excess of IL-1α/-β. Already minute and barely detectable amounts
of serum IL-1 have severe effects for the whole body. Injection of 1-3 ng/kg of IL-1 leads to dramatic reactions with fever, rashes, neutrophilia and elevated IL-6 and ACTH levels (15). Hence, several mechanisms have been evolved to tightly control IL-1: i) an IL-1 receptor antagonist (IL-1RA), which competes for binding to IL-1RI and thus prevents binding of IL-1α/β to IL-1RI, ii) a decoy receptor IL-1RII that lacks intracellular signalling domain, and iii) a soluble IL-1RII (sIL-1RII) that binds serum or secreted IL-1α/β together with a soluble form of IL-1RAcP (sIL-1RAcP). Single Ig IL-1-related receptor (SIGIRR) also contributes by inhibiting IL-1 signalling (Fig. 1.6).

**Figure 1.6 | Receptors and inhibitors of IL-1α and -β.** All IL-1 receptors possess three immunoglobulin (Ig)-like domains in the extracellular region. Only the signalling IL-1 receptors, being IL-1RI for IL-1α/β, posses a Toll-IL-1R (TIR) signalling domain leading to NFκB activation. Enabling activation of IL-1RI association of IL-1RAcP is necessary. The decoy receptor IL-RII lacks the signalling TIR domain. IL-1RA blocks binding of IL-1α/β to IL-1RI to encounter inflammation. SIL-1RII and sIL-1RAcP also prevent binding of IL-1α/β to IL-1RI by intercepting IL-1 in the serum.

Taken together the IL-1 system includes pro-inflammatory members such as IL-1α/β and IL-1RI but also anti-inflammatory members that trap the pro-inflammatory molecules. These anti-inflammatory members are IL-1RA, IL-1RII, sIL-1RII and SIGIRR. When IL-1α/β expression is induced IL-1RA is expressed in parallel. The importance of the IL-1/IL-1RA balance in the serum can be observed in mice and humans lacking IL-1RA. IL-1RA deficient mice develop spontaneous inflammatory diseases such as arthritis and psoriatic-like skin eruption. They also develop aggressive tumours after exposure to carcinogens. In humans, infants with non-functional IL-1RA soon after birth develop severe systemic and local inflammation with sepsis-like multi organ failure, although everything is sterile. Injections of IL-1RA can cure the symptoms (15).
1.2.4 Clinical relevance of IL-1

The IL-1 family is associated with acute and chronic inflammation. IL-1R and TLRs share a common TIR domain with similar functions. Very early after discovery of IL-1, it had been shown that small amounts of IL-1 were able to protect against bacterial infections (16). IL-1α and -β have also been shown to be involved in several diseases. IL-1α expressed on the surface of thrombocytes has recently been shown to play an important role in murine models of rheumatoid arthritis (17). In atherosclerosis cholesterol crystals have been demonstrated to be activators of the NALP3 inflammasome and therefore to drive inflammation of the blood vessel walls via IL-1 (18). In mice and rabbits colitis was demonstrated to strongly depend on IL-1 (19), and dysregulation of the IL-1/IL-1RA balance was found in ulcerative colitis and Crohn’s disease of the human (20). In gout uric acid crystals have been identified as activators of the NALP3 inflammasome (21). Similarly, the NALP3 inflammasome has been shown to regulate inflammation of the central nervous system (22). IL-1 also was found to be an important driver of insultitis in diabetes in human and mice (23), as well as fatty liver degeneration in mice (24). Interestingly, multiple types of cancers have been found to express IL-1α as well as IL-1β which both drive angiogenesis (25) and extracellular matrix breakdown (26, 27). Both processes are important in cancer progression. Finally, a growing number of so called ‘autoinflammatory diseases’ including Familial Mediterranean fever, adult-onset Still’s disease, Schnitzler’s syndrome, Muckle-Wells syndrome and other rare diseases (15) have been found to be caused by a dysregulated inflammasome and IL-1 system. For example, Muckle-Wells syndrome is caused by a mutation in the CIAS1 gene, leading to increased activation of NALP3 and uncontrolled IL-1β secretion, manifesting as episodic fever, chills and painful joints (28). The term ‘autoinflammatory diseases’ refers to diseases with dysregulated myeloid cells which are treatable with anti-IL-1 therapies (15). The IL-1RA (anakinra, Kineret©) that prevents binding of IL-1α or IL-1β to the IL-1R is approved to treat patients with rheumatoid arthritis, gout, systemic-onset juvenile idiopathic arthritis, and refractory adult Still’s disease. Another similar drug, an IL-1R fused to an Fc domain (IL-1 Trap, rilonacept, Arcalyst©) that interferes with IL-1α or IL-1β binding to IL-1R, is approved for ‘autoinflammatory diseases’ such as familial cold-induced autoinflammatory syndrome and Muckle-Wells syndrome.
(29). Just recently, an anti-IL-1β monoclonal antibody (canakinumab, Ilaris®) entered the market and has also demonstrated efficacy in autoinflammatory diseases, such as the cryopyrin associated periodic syndromes (CAPS) (30). However not only the above mentioned classical inflammatory diseases can be treated by IL-1 blockade, but also a double-blind randomised placebo controlled study showed that Anakinra improved insulin secretion from beta-cells and reduce markers of systemic inflammation in patients with type 2 diabetes mellitus. However, in most diseases the relative contribution of IL-1α and IL-1β has not yet been elucidated. To better understand the contribution of IL-1α and IL-1β in disease it is important to understand the regulation of IL-1α and IL-1β expression.

1.2.5 IL-1β

The main IL-1 producers are monocytes, MΦ, DCs, neutrophils, epithelial cells, endothelial cells, B-cells and platelets. In contrast to IL-1α, the secretion mechanism of IL-1β is very well understood. Upon NFκB stimulation, for example by a TLR agonist such as LPS (TLR4 antagonist), an intracellular pro-form of IL-α and IL-1β is produced. In the case of IL-1β a second stimulation of the cell is required to induce the proteolytical cleavage into a mature and secreted form. This second stimulation is induced by inflammasome activation i.e. by ATP or monosodium urate (MSU). The inflammasome activation and assembly will than lead to further cleavage and activation of pro-caspase-1 into active caspase-1 and active caspase-1 will cleave the 31 kDa pro-IL-1β into the 14 kDa N-terminal propiece and the 17 kDa mature and biologically active secreted IL-1β. Due to its role in IL-1β maturation, caspase-1 was previously known as IL-1 converting enzyme (ICE) (Fig. 1.7).
**Figure 1.7 | IL-1β secretion.** A TLR agonist provides signal 1 and leads to NFκB activation and expression of pro-IL-1β. Inflammasome activation provides signal 2 leading to caspase-1 activation and cleavage of pro-IL-1β into mature secreted IL-1β.

The secretion of IL-1β is dependent on caspase-1 and independent of the ER/Golgi pathway, the classical secretory pathway. This caspase-1-dependent secretion has been named unconventional protein secretion pathway and is still poorly understood (31-33).

IL-1α does not contain a caspase-1 cleavage site, however, in 1995 Kuida et al. first described that caspase-1 deficient mice do not only show reduced IL-1β secretion, but surprisingly also decreased IL-1α secretion (34). Later it has been suggested that caspase-1 binds IL-1α and other unconventionally secreted proteins and thus enables secretion (31).

### 1.2.6 IL-1α

As previously mentioned, similar to IL-1β, TLR stimulation induces 31 kDa pro-IL-1α by activating NFκB. In contrast to IL-1β, however, the pro-IL-1α is already biologically active, although much less than maturate IL-1α. Moreover, the cleaved-off 14 kDa propiece contains a nuclear localization site.
(NLS) and is therefore able to modulate transcription of itself. In addition to the 17 kDa mature soluble form of IL-1α, there also exists a membrane-bound surface IL-1α form. The regulation of IL-1α surface expression, maturation and secretion, however, remains elusive. Pro-IL-1α is thought to be myristoylated and translocated to the cell membrane where it associates with cell surface components by an unknown mechanism (35, 36). It has been speculated that phosphorylation of pro-IL-1α induces a conformational change leading to surface anchoring via lectin binding (37, 38). Except for lectin binding sites, IL-1α contains no transmembrane regions, GPI anchors or other hydrophobic regions allowing surface anchoring. Secretion of IL-1α requires cleavage of pro-IL-1α into a pro-piece and a 17 kDa C-terminal mature soluble form. This cleavage can be mediated by calpains, Ca^{2+} dependent proteases present at the cytosolic side of the cell membrane (39) (Fig. 1.8).

**Figure 1.8 | IL-1α surface expression and secretion.** Stimulation of a TLR leads to expression of pro-IL-1α. Pro-IL-1α can be myristoylated and translocated to the cell membrane. At the intracellular site of the membrane, calpains can cleave pro-IL-1α into mature secreted IL-1α.
1.3 Side projects

1.3.1 Vaccination strategies to modulate the immune system

The name vaccination was introduced by Edward Jenner in the 18th century using cowpox virus to immunize humans against smallpox. Hence the name vaccine, i.e. from the cow, lat “vacca”. Nowadays we are confronted with vaccinations from very early on in life and are accompanied during the whole life. Apart from classic prophylactic vaccination against infectious diseases or toxins, in recent years research also focussed on therapeutic vaccination, e.g. in chronic infectious diseases, allergies or cancer.

1.3.1.1 Tumour vaccination using virus-like-particles (VLPs): role of IL-1α in tumour progression

IL-1α is involved in many events that are prominently associated with tumour progression i.e. proliferation, invasion and angiogenesis (40). Furthermore, cell death of tumour-infiltrating inflammatory cells lead to the release of pro-inflammatory mediators like IL-1α, which is thought to additionally contribute to cancer progression. This will be further illustrated in chapter 2.3. Therefore IL-1α may be a promising target in tumour therapy. However, vaccinations against self-proteins like L-1α require a vaccination strategy that can overcome tolerance to self. VLPs present antigens in a highly repetitive manner which efficiently crosslink B cell receptors, leading to immunoglobulin production without T cell help. VLPs are known to induce high and long lasting antibody titers (41). Also, VLPs can be manufactured to contain CpG oligodeoxynucleotides, i.e. potent TLR-9 stimulators, which further enhance the antibody response. We investigated whether an anti-IL-1α vaccination using VLPs engineered to express IL-1α on their surface could reduce tumour progression with respect to tumour growth and angiogenesis.
1.3.1.3 Vaccination against a bacterial pathogen: modified Bacillus Calmette-Guérin (BCG) with enhanced antigen presentation capacity

BCG is derived from a *Mycobacterium bovis* isolate that was attenuated by laboratory passaging. This non-pathogenic strain is used for vaccination against infections with *Mycobacterium tuberculosis* (Mtb). However, this vaccination provides very limited protection and therefore improved vaccines are required (42). A major drawback of immunization is the immune escape of *Mycobacteria* by arresting the phagolysosomal fusion and therefore interfering with the antigen-presentation machinery. This complicates the establishment of a long-lasting memory immune response towards Mtb. One common protein of BCG and Mtb, a zinc metalloprotease (zmp), is known to interfere with phagosome maturation of host cells and might thus influence immunogenicity of this bacterium (43). In order to enhance immunogenicity of the current vaccine against Mtb, we compared the BCG wt with the BCG zmp1 mutant in terms of antigen-presentation *in-vitro* and *in-vivo*.

1.3.1.4 T-cell independent vaccination using peptide and TLR agonist containing liposomes targeting Alzheimer’s disease

T-cell activation during vaccination can on one hand lead to effective immunization but on the other hand it could also lead to immunopathology by tissue destruction by infiltrating T-cells. In Alzheimer’s disease vaccines target pathogenic peptides located within the brain. Vaccination induced T-cell-responses could therefore severely damage brain tissue and hence must be avoided. Usually vaccination-induced protective antibody responses are secreted by B-cells that are activated by the classical T-cell dependent (TD) pathway which requires T-cell help (44). However, few antigens can induce B-cells in a T-cell independent (TI) fashion (45). There are two subclasses of TI responses, firstly the TI type 1 (TI-1) antigens are polyclonal B-cell activators such as bacterial cell wall components such as polysaccharides and lipopolysaccharides and secondly the TI type 2 (TI-2) antigens typically consisting of repetitive biochemical structures (46). The liposomes we designed for
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our study consist of phospholipids that form a lipid bilayer with the potential to integrate antigens. We densely packed our antigen on the surface of these liposomes in order to generate TI-2 antigens. The antigen was a 15-mer peptide derived from the human beta-amyloid (Aβ) with no known T-cell epitopes was palmytoylated and mixed with phospholipids and monophosphoryl A (MPLA) to allow spontaneous formation of liposomes with densely arranged peptides on the outer surface (47). In order to validate the TI immune response upon liposome vaccination we compared the induction of long lasting IgG responses in wt and athymic mice, CD4-T-cell depleted mice, and mice deficient in major histocompatibility complex (MHC) class II, T cell receptor (TCR), CD40L and CD28.

1.3.2 Antihistamines

Antihistamines are common drugs to treat allergic symptoms. They block the effects of histamine, which are released from granules of mastcells and basophils upon allergen mediated cross-linkaging of membrane-bound IgE. Histamin release leads to hives, itching, pain, smooth muscle contraction and increased vascular permeability via the histamin 1 receptor (H1R). Antihistamines or H1R antagonists are widely used and are considered very safe. However, recent studies suggest that antihistamines may influence and impair innate and adaptive immunity (48-52). Thus, we examined the extent and by which potential mechanisms histamine 1-receptor (H1R) antagonists exert immune suppressive effects.

1.4. Aim of the thesis

IL-1 contributes to many different diseases and is a key inflammatory molecule. In order to better understand the contribution of IL-1 to disease initiation and disease progression, it is important to understand the regulation and secretion mechanisms. Although IL-1α is one of the first cytokines discovered, only very little about surface expression and secretion mechanisms is known. The surface
form of IL-1α was never clearly proven to be biologically active and to exist as a distinct expression form of IL-1α. It has been suggested that surface IL-1α is simply an artefact with mature secreted IL-1α bound to IL-1RI (53). This may be one of the reasons why the regulation and functionality of IL-1α surface expression has been barely investigated. Secreted form of IL-1α, however, has gained attention in recent years. The reason why secreted IL-1α was for a very long time underestimated is probably because IL-1β secretion and regulation was well characterized since early on and all the phenomenons of IL-1 were attributed to IL-1β. However, this thinking began to change when first deflating trials were performed using the newly developed anti-IL-1β antibody in comparison with the older and more promising IL-1RA treatment, inhibiting IL-1α and IL-1β. Astonishingly the first foundation for mechanisms in IL-1α secretion was found already in 1995 when Kuida et al. observed lower IL-1α levels in caspase-1 deficient mice (34), but then only in 2008 this finding could be transformed into a new mechanistic idea suggesting caspase-1 as a regulator of unconventionally secreted proteins like IL-1α (31).

The aim of this project was to explore the role of caspase-1 and the inflammasomes on the surface and secreted form of IL-1α. Moreover, we wanted to better understand the pathways leading to the surface expressed form of IL-1α.

Furthermore, I actively participated in different side projects, which were mostly not related to IL-1. My collaboration in these projects gave me the opportunity to participate in other immunology-related research projects and thus provided insight into additional techniques and topics.
Chapter 2

Results
Chapter 2.1

**IL-1β is a shuttle for IL-1α secretion, but not for IL-1α surface expression**

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2.1.1 Abstract

Interleukin-1α (IL-1α) and -β both bind to the same IL-1 receptor (IL-1R) and are potent pro-inflammatory cytokines. Production of pro-IL-1α and pro-IL-1β is induced by Toll like receptor (TLR)-mediated NFκB activation. Additional stimulus involving activation of the inflammasome and caspase-1 is required for proteolytic cleavage and secretion of mature IL-1β. The regulation of IL-1α maturation and secretion, however, remains elusive. IL-1α exists as a cell surface associated and as a mature secreted form. Here we show that both forms of IL-1α, the surface and secreted form, are differentially regulated. Surface IL-1α requires NFκB activation only, whereas secretion of mature IL-1α requires additional activation of the inflammasome and caspase-1. Surprisingly, secretion of IL-1α also required presence of IL-1β as demonstrated in IL-1β deficient mice. We further demonstrate that IL-1β directly binds IL-1α, thus identifying IL-1β as a shuttle for another proinflammatory cytokine. These results have direct impact on selective treatment modalities of inflammatory diseases.

2.1.2 Introduction

Pathogens activate the innate immune system and inflammatory responses via pattern-recognition receptors (PRRs), which include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like helicases (RLHs), and C-type lectin receptors (CLRs) (10, 12). Stimulation of TLRs leads to MyD88-dependent NFκB activation and induces expression of the 31 kDa precursors of both IL-1α and IL-1β. Both cytokines bind to the IL-1 receptor (IL-1R) and are potent pro-inflammatory cytokines, mainly produced by myeloid cells such as macrophages, monocytes and dendritic cells. IL-1α and IL-1β are processed into smaller biologically active forms, which are then secreted via the ER/Golgi-independent, but poorly understood unconventional protein secretion pathway (31, 54). The cleavage mechanism of pro-IL-1β into mature IL-1β is well characterised. Upon assembly of different types of inflammasomes, caspase-1 is activated and cleaves pro-IL-1β into a N-terminal 14 kDa inactive
propiece and a C-terminal 17 kDa mature and biologically active form of IL-1β. In contrast to IL-1β, IL-1α exists not only as a mature soluble form, but also as a cell surface protein. Pro-IL-1α is thought to be myristoylated and translocated to the cell membrane where it associates with cell surface components by an unknown mechanism (35, 36). It has been speculated that phosphorylation of pro-IL-1α induces a conformational change leading to surface anchoring via lectin binding (37, 38). Except for lectin binding sites, IL-1α contains no transmembrane regions, GPI anchors or other hydrophobic regions allowing surface anchoring. Processing of IL-1α induces the cleavage of pro-IL-1α into a pro-piece and a mature soluble form. This cleavage depends on calpains, Ca\(^{2+}\) dependent proteases present at the cytosolic side of the cell membrane (39). While pro-IL-1α does not contain any predicted cleavage site for caspase-1, there is evidence that it can bind to caspase-1, and that caspase-1 may be involved in the secretion of IL-1α (34, 55). Therefore, caspase-1 may act as a regulator of unconventional protein secretion (31). Here, we investigated the role of the inflammasome and caspase-1 on the expression of secreted and surface IL-1α and found that surface expression was caspase-1 independent, while secretion of mature IL-1α required activation of both inflammasome and caspase-1. Surprisingly, we also found a role for IL-1β in the secretion of IL-1α.

2.1.3 Results

2.1.3.1 IL-1α expressed on the cell surface is biologically functional and depends on TLR stimulation only, whereas secretion of mature IL-1α requires additional stimuli

To study the kinetics of IL-1α surface expression, we stimulated human monocytes with LPS. Maximal levels of the cell surface bound IL-1α were detected after 6 h, whereas intracellular IL-1α levels continuously increased even at later time points (Fig. 2.1.1A and Fig. S2.1.1). As it remains unknown how IL-1α is anchored to the cell membrane, it has been debated whether surface associated IL-1α is biologically active, or if it merely represents mature secreted IL-1α that is bound to its
receptor IL-1RI (53). However, IL-1α surface expression on murine bone-marrow derived dendritic cells (bmDCs) from IL-1R−/− mice was comparable to that in C57BL/6 wild type mice (wt), indicating that IL-1R is not required for cell surface expression of IL-1α (Fig. 2.1B). For unknown reasons, IL-1α surface expression was even consistently higher in IL-1RI−/− than in wt cells. Furthermore, to exclude that the IL-1α detected by surface staining was not IL-1α bound to the decoy receptor IL-1RII, we shedded the IL-1RII in IL-1RI−/− bmDCs using PMA/Ionomycin (56) and compared the amount of surface IL-1α by flow cytometry. Shedding of IL-1RII did not decrease levels of surface IL-1α (Fig. 2.1C), while shedding was confirmed by increased amounts of IL-1RII in the supernatant by Western blotting (Fig. 2.1D).

To assess whether cell surface associated IL-1α was biologically active, human monocytes were stimulated with LPS, washed to eliminate secreted IL-1α and fixed with paraformaldehyde to block further secretion. Serial dilutions of these monocytes were then co-incubated with an IL-1 reporter cell line which secretes IL-2 upon IL-1RI signalling. Monocytes were able to induce IL1-RI-dependent IL-2 secretion in a cell number dependent manner, and IL-2 secretion could be suppressed by an anti-IL-1α antibody (Fig. 2.1E).

To compare the kinetics of IL-1α surface expression with IL-1α secretion, monocytes were stimulated with LPS for different time periods. While surface expression of IL-1α was already detectable 3 h after LPS stimulation (Fig. 2.1F, S2.1) and reached its maximum after only 6 h, IL-1α secretion was low during the first 16 hours and continuously increased up to 24 h (Fig. 2.1G). Interestingly, we observed that stimulation of monocytes using a conventional, i.e. not highly purified LPS preparation stimulated both IL-1α surface expression and secretion, whereas an ultra-pure (up)-LPS preparation induced only surface expression but not secretion of IL-1α (Fig. 2.1F, G), suggesting that IL-1α secretion was induced by contaminations in the conventional LPS preparation which may stimulate additional pathways other than TLR4 and NFκB. It is known that muramylidipeptide (MPD) contaminated LPS activates the NALP3 inflammasome (57). To investigate whether such contaminants induced IL-1α secretion through inflammasome activation, a macrophage cell line was stimulated either with up-LPS or up-LPS in combination with the NALP3 inflammasome activator
monosodium urate (MSU) (57). Indeed, cells stimulated with ultra-pure LPS secreted the mature form of IL-1α only in the presence of MSU (Fig. 2.1.1H, I).

Figure 2.1.1 | IL-1α expressed on the cell surface is biologically functional and depends only on TLR stimulation whereas secretion of mature IL-1α requires additional stimuli. (A) Cell surface and intracellular expression of IL-1α in human monocytes after LPS stimulation at different time points was measured by flow cytometry. Surface expression (maximal after 6 h) and intracellular expression (maximal after 24 h) are shown. Representative stainings of three independent experiments are shown. (B) BmDCs derived from IL-1RI−/− mice (open bars) and wt mice (closed bars) were stimulated with up-LPS for 24 h and surface-associated IL-1α was measured by flow cytometry. Data shown are mean and standard deviation of duplicates. One representative of three independent experiments is shown. (C, D) BmDCs derived from IL-1RI−/− mice were stimulated with medium or up-LPS in presence (open bars) or absence (closed bars) of PMA/Ionomycin for surface IL-1RI shedding. Amounts of surface IL-1α were compared by flow cytometry (C). Shedding of IL-1RII was confirmed by an IL-1RII Western blot of precipitated supernatant (D). Lysates of cells were tested for intracellular IL-1RII and IL-1α expression by Western blot (D). Data shows mean and standard deviation of one representative of two independent experiments. (E) EL4-6.1 cells were incubated with paraformaldehyde fixed human monocytes expressing surface IL-1α after LPS stimulation in the presence (open squares) or absence (closed squares) of anti-IL-1α antibody. IL-2 production was quantified by ELISA. Mean and standard deviation of triplicates is shown. (F, G) Human monocytes were stimulated with up-LPS (open squares) or conventional LPS (closed squares) over the indicated time period. Surface IL-1α expression was measured by flow cytometry (F) and secreted IL-1α was quantified by ELISA (G). (H, I) IC-21 macrophages were treated with up-LPS with or without MSU during the last 6 h of a 24 h stimulation period. IL-1α secretion was measured by ELISA (H). Mean and standard deviation of 6 replicates is shown. * p < 0.05; ** p < 0.01; *** p < 0.001 (unpaired, 2-tailed Mann Whitney test). Pro-IL-1α and mature IL-1α were assessed in cell lysate and supernatant, respectively by Western blot (I). As control actin Western blot was performed.
Figure S2.1.1 | Kinetics of intracellular and surface IL-1α. Cell surface and intracellular expression of IL-1α in human monocytes after LPS stimulation at different time points was measured by flow cytometry. Data is representative of three independent experiments.

2.1.3.2 Inflammasome activation is crucial for IL-1α secretion, but not required for surface expression

To further confirm the role of inflammasome activation in IL-1α secretion, bmDCs from ASC-, NALP3-, P2X7R- and IPAF-deficient mice were stimulated using either up-LPS alone, or up-LPS plus ATP. ATP activates the ASC-dependent NALP3 (also known as cryopyrin, CIAS1 or NLRP3) inflammasome via P2X7 receptor (purinoceptor 7) which leads to pannexin pore formation and efflux of K⁺ (58, 59). ATP does not activate the ICE-protease activating factor (IPAF) (also known as NLRC4) inflammasome (60-62). Several inflammasomes share the adapter protein ASC, which is able to link the PYD domain of an inflammasome sensor to the CARD domain of caspase-1 (10, 12). As expected, secretion of IL-1β was impaired in ASC deficient bmDCs when treated with up-LPS and
ATP. In line with the above data, the same was observed for IL-1α secretion, which was also impaired in ASC<sup>−/−</sup> bmDCs. In contrast, IL-1α surface expression was not affected in ASC<sup>−/−</sup> bmDCs (Fig. 2.1.2A-C). Consistent with our observations in the ASC<sup>−/−</sup> bmDCs, cells from NALP3<sup>−/−</sup> and P2X7R<sup>−/−</sup> mice expressed normal levels of cell surface IL-1α (Fig. 2.1.2A), but were not able to secrete IL-1α and IL-1β upon stimulation with up-LPS and ATP (Fig. 2.1.2B, C). Furthermore, the reduction of IL-1α secretion in the ASC<sup>−/−</sup>, NALP3<sup>−/−</sup> and P2X7R<sup>−/−</sup> cells was not due to a missing feedback-loop of secreted IL-1β signalling via IL-1RI, since we found comparable levels of IL-1α secretion in bmDCs from IL-1RI<sup>−/−</sup> and wt mice (Fig. 2.1.2A-C). Expectedly, IPAF-deficiency did not have an impact on IL-1α and IL-1β secretion using up-LPS and ATP stimulation (Fig. 2.1.2B, C). Induction of intracellular pro-IL-1α and pro-IL-1β by up-LPS was comparable in all the above tested bmDCs and therefore to excluded transcriptional differences (Fig. 2.1.2D).

Figure 2.1.2 | Inflammasome activation is crucial for IL-1α secretion, but not required for surface IL-1α expression. (A-C) Human monocytes were stimulated with LPS in presence or absence of the pan-caspase inhibitor z-VAD fmk. IL-1α surface expression was analysed by flow cytometry after 6 h of stimulation (A), and IL-1α and -β secretion was quantified by ELISA after 24 h of stimulation (B, C). Control samples were treated with the DMSO containing solvent (closed bars). Mean and standard deviation of 6 replicates is shown. * p < 0.05; ** p< 0.01; *** p < 0.001 (unpaired, 2-tailed Mann Whitney test). One representative of two independent experiments is shown. (D-F) BmDCs derived from caspase-1-deficient (open bars) and wt control mice (closed bars) were stimulated with up-LPS in presence or absence of ATP during the last 30 min of a 24 h stimulation period. Surface IL-1α expression was analysed by flow cytometry (D), IL-1α (E) and -β (F) secretion were quantified by ELISA. Data shows mean and standard deviation of duplicates and one representative of three independent experiments. (G) Pro-IL-1α and -β were measured by Western blot in lysates prepared from bmDCs stimulated with up-LPS. (H, I) IL-1α (H) and -β (I) levels in the BAL of Lpn infected caspase-1-deficient and C57BL/6 control mice were quantified by ELISA. Mean and standard deviation of n=3 and one representative of three independent experiments is shown. * p < 0.05; ** p< 0.01; *** p < 0.001 (unpaired, 2-tailed Mann Whitney test).
tailed t-test). \( (J, K) \) IL-1\( \alpha \) (J) and IL-1\( \beta \) (K) levels were measured in the BAL of wt mice infected with wt JR32 \( Lpn \) or \( \Delta T Lpn \). Mean and standard deviation of \( n=2 \) or \( n=3 \) and one representative of three independent experiments is shown. * \( p < 0.05 \); ** \( p< 0.01 \); *** \( p < 0.001 \) (unpaired, 2-tailed t-test).

### 2.1.3.3 In vitro and in vivo secretion of IL-1\( \alpha \) is caspase-1-dependent

The importance of inflammasome activation for IL-1\( \alpha \) secretion was confirmed by inhibiting caspases with the pan-caspase inhibitor \( z \)-VAD fmk. This resulted in decreased IL-1\( \alpha \) and -\( \beta \) secretion in LPS pulsed human monocytes. In contrast, IL-1\( \alpha \) surface expression was even enhanced (Fig. 2.1.3A-C).

The role of caspase-1 in IL-1\( \alpha \) and -\( \beta \) secretion was confirmed by stimulation of caspase1\( ^{-/-} \) bmDCs with up-LPS and ATP, which resulted in decreased IL-1\( \alpha \) and IL-1\( \beta \) secretion (Fig. 2.1.3E, F) while the intracellular pro-forms of both cytokines were induced at normal levels (Fig. 2.1.3G). In line with the above findings, caspase-1\( ^{-/-} \) bmDCs were able to express cell surface IL-1\( \alpha \) under all experimental conditions (Fig. 2.1.3D), confirming that IL-1\( \alpha \) surface expression is independent of the inflammasome and caspase-1. The validity of the above in vitro observations was tested in vivo in an airway infection model. Caspase-1\( ^{-/-} \) and wt mice were infected intranasally (i.n.) with the Gram-negative bacterium \( Legionella pneumophila \) (\( Lpn \)), and the secretion of IL-1\( \alpha \) and IL-1\( \beta \) was measured in bronchoalveolar lavage (BAL). Indeed, caspase-1\( ^{-/-} \) mice exhibited both impaired IL-1\( \alpha \) and IL-1\( \beta \) secretion (Fig. 2.1.3H, I). Similar results were obtained in wt mice infected with either wild type \( Lpn \) or \( \Delta T Lpn \), the latter lacking the type IV secretion system (T4SS) and therefore being unable to activate caspase-1 (63, 64) (Fig. 2.1.3J, K).
Figure 2.1.3 | *In vitro and in vivo secretion of IL-1α is caspase-1-dependent.* (A–C) Human monocytes were stimulated with LPS in presence or absence of the pan-caspase inhibitor z-VAD fmk. IL-1α surface expression was analysed by flow cytometry after 6 h of stimulation (A), and IL-1α and -β secretion was quantified by ELISA after 24 h of stimulation (B, C). Control samples were treated with the DMSO containing solvent (closed bars). Mean and standard deviation of 6 replicates is shown. * p < 0.05; ** p< 0.01; *** p < 0.001 (unpaired, 2-tailed t-test). (D–F) BmDCs derived from caspase-1-deficient (open bars) and wt control mice (closed bars) were stimulated with up-LPS in presence or absence of ATP during the last 30 min of a 24 h stimulation period. Surface IL-1α expression was analysed by flow cytometry (D) IL-1α and -β secretion in the supernatant was quantified by ELISA (E, F). Data shows mean and standard deviation of duplicates and one representative of three independent experiments. (G) Pro-IL-1α and -β were measured by Western blot in lysates prepared from bmDCs stimulated with up-LPS. H, I, IL-1α and -β levels in the BAL of Lpn infected caspase-1-deficient and C57BL/6 control mice were quantified by ELISA. (J, K) IL-1α and IL-1β levels were measured in the BAL of wt mice infected with wt JR32 Lpn or ΔT Lpn. Mean and standard deviation of n=2 or n=3 and one representative of three independent experiments is shown. * p < 0.05; ** p< 0.01; *** p < 0.001 (unpaired, 2-tailed t-test).

2.1.3.4 IL-1α secretion is IL-1β mediated and uses a distinct pathway from IL-1α surface expression

The reason why IL-1α secretion is caspase-1 dependent although it is not a direct substrate for caspase-1 was previously attributed to the binding of IL-1α to caspase-1, thereby facilitating unconventional protein secretion (31). Surprisingly, co-immunoprecipitation (Co-IP) experiments using lysates of up-LPS primed murine bmDCs revealed a weak band for IL-1β after an anti-IL-1α pulldown, suggesting an intracellular interaction between IL-1α and IL-1β (Fig. 2.1.4A). To confirm this interaction between IL-1α and IL-1β, we over-expressed human pro-IL-1α (myc-tagged) and pro-IL-1β (HA-tagged) in Cos cells. Precipitation of pro-IL-1α by using an anti-myc antibody led to co-precipitation of pro-IL-1β, detected using an anti-HA antibody (Fig. 2.1.4B). Furthermore, the endogenous association of IL-1α and IL-1β could be demonstrated in lysates of differently stimulated bmDCs using a modified sandwich-ELISA with an IL-1α capture antibody and a non-cross-reactive IL-1β detection antibody (Fig. 2.1.4C). While up-LPS stimulated cells showed a strong signal for IL-1α-bound IL-1β, this signal was reduced when cells were additionally treated with ATP, most likely due to reduced intracellular availability as a result of secretion and thereby confirming the co-IP data of the endogenous protein. The requirement of IL-1β for secretion of IL-1α was confirmed in a functional assay showing that IL-1β-deficient cells were unable to secrete IL-1α (Fig. 2.1.4D),
whereas the level of intracellular pro-IL-1α were comparable in IL-1β+/− and IL-1β−/− bmDCs (Fig. 2.1.4E). Therefore, the IL-1β dependence of IL-1α secretion was not explained by differences in transcription, as previously reported (65). In contrast to that, IL-1β secretion was independent of IL-1α secretion shown in IL-1α deficient mice (Fig. S2.1.2). Furthermore, the inflammasome activity, measured by the unconventionally secreted caspase-1 p10 subunit in the supernatant, was not impaired in IL-1β-deficient cells when stimulating with up-LPS and ATP (Fig. 2.1.4E). Consistent with the inflammasome-independent presentation of IL-1α on the cell surface, cell-bound IL-1α was readily detected in IL-1β deficient bmDCs upon stimulation (Fig. 2.1.4F).

Note that upon stimulation of bmDCs with up-LPS and ATP, with time no secretion of IL-1α was detected by ELISA in IL-1β−/− mice (Fig. 2.4G). However, a release of significant amounts of pro-IL-1α was detectable in the supernatant by Western blotting (Fig. 2.1.4H). This indicates that the ELISA only detected the mature form of IL-1α. IL-1β deficient bmDCs release pro-IL-1α, but were not able to actively secrete intracellularly processed mature IL-1α (Fig. 2.1.4G, H). Pro-IL-1α has lower biological activity than mature IL-1α, but could be further cleaved by other extracellular proteases, i.e. cathepsin G, elastase, proteinase-3 and chymase in vivo.

This and the previous experiments suggest the presence of two distinct pathways for IL-1α surface expression and IL-1α secretion. This interpretation was further supported by cell surface protein biotinylation of up-LPS pulsed and therefore surface IL-1α expressing bmDCs. Following biotinylation of surface proteins including IL-1α (Fig. 2.1.4I), cells were treated with the inflammasome activator ATP to induced IL-1α secretion. Quantitative ELISA demonstrate that the secreted IL-1α was almost entirely composed of non-biotinylated IL-1α, indicating that secreted IL-1α was not cleaved-off from the surface but rather released from intracellular compartments (Fig. 2.1.4J).
Figure 2.1.4 | IL-1α interacts with IL-1β for secretion. (A, B) Co-IP of bmDCs stimulated with medium or up-LPS in the presence or absence of ATP as indicated during the last 10 or 30 minutes of a 24 hour stimulation using an anti-IL-1α pulldown or an isotype control antibody (A) and co-IP of Cos cells transfected with either pro-IL-1α-myc or pro-IL-1β-HA alone or in combination of both using an anti-myc pulldown antibody (B). (C) Sandwich-ELISA of lysed bmDCs stimulated with up-LPS in the absence or presence of ATP during the last 10 or 30 min of a 24 h stimulation using IL-1α capture antibody and IL-1β detection antibody of undiluted (closed bars), 1:1 (grey bars) and 1:10 (open bars) diluted bmDCs lysates stimulated as indicated. Mean and standard deviation of n=4 and one representative of two independent experiments is shown. (D) BmDCs derived from IL-1β-deficient (open bars) or wt mice (closed bars) were stimulated with up-LPS in presence or absence of ATP as indicated during the last 30 min of a 24 h stimulation period. IL-1α secretion in the supernatant was quantified by ELISA (D), intracellular pro-IL-1α and -β and actin control in lysates as well as secreted caspase-1 p10 subunit in the supernatant were analysed by western blot (E), and surface IL-1α expression was analysed by flow cytometry (F). Mean and standard deviation of n=2 and one representative of two independent experiments are shown. * p < 0.05; ** p< 0.01; *** p < 0.001 (unpaired, 2-tailed Mann Whitney test).

Figure S2.1.2 | IL-1β secretion is unimpaired in IL-1α-deficient mice. (A, B) BmDCs derived from IL-1α-deficient (open bars) or wt mice (closed bars) were stimulated with up-LPS in presence or absence of ATP as indicated during the last 30 min of a 24 h stimulation period. IL-1β secretion in the supernatant was quantified by ELISA. Mean and standard deviation of duplicates and one representative of two independent experiments is shown. (A). Intracellular pro-IL-1α and -β and actin control in the lysates were analysed by western blot (B).
2.1.4 Discussion

Our data demonstrate for the first time that IL-1α surface expression and secretion can be dissected into two independent pathways. While TLR stimulation alone is sufficient for the production of IL-1α determined for the cell surface, additional activation of the inflammasome is required for the production of secreted and soluble IL-1α. This study further demonstrates that, against former notion, secretion of IL-1α is not the result of cleavage of cell surface bound IL-1α, but occurs via a distinct pathway dependent on the inflammasome, caspase-1 and IL-1β. Since IL-1α does not have a caspase-1 cleavage site, the observed inflammasome and caspase-1 dependency appears to be explained by binding of IL-1α to IL-1β and piggy-backing on IL-1β during transfer from the cytoplasm to the extracellular space via caspase-1 and probably also via calpains, which are relevant for cleavage of IL-1α. The finding that bmDCs from IL-1β⁻/⁻ mice are also deficient in secretion of mature IL-1α suggests that certain findings in IL-1β⁻/⁻ mice should be revisited. Susceptibility to *M. tuberculosis* infection may be one such example. IL-1β⁻/⁻ mice were found to be more susceptible to *M. tuberculosis* infection than wt mice (66), but when wt mice were depleted with IL-1α or IL-1β antibodies, it was IL-1α but not IL-1β which determined susceptibility to infection (67). Recently, the Mtb susceptibility was also confirmed in IL-1α⁻/⁻ mice (68). Moreover, our data suggests a new role for IL-1β in unconventional protein secretion. It remains to be resolved whether interaction with IL-1β is also a prerequisite for the secretion of other unconventionally secreted proteins, such as FGF-2 and Bid.

Our observation that IL-1β deficient mice do not secrete mature IL-1α, but may still release significant amounts of pro-IL-1α, is likely due to cell damage upon massive stimulation. Cell damage and inflammasome activation may explain why another group detected IL-1α in the serum of LPS infected IL-1β deficient mice (69). This maybe either pro-IL-1α, which is biologically less active, or pro-IL-1α matured by extracellular proteases, i.e. cathepsin G, elastase, proteinase-3 and chymase. Alternatively, cells other than macrophages and dendritic cells studied by us may have been the source of IL-1α after LPS injection.
We have tested three cell types, bmDCs, macrophages and monocytes, in regards to signalling requirements for secretion of mature IL-1α. While others have shown that monocytes do not require additional inflammasome activation in order to secrete mature IL-1, explained by the observation that monocytes express a constitutively active caspase-1 (70), in our hands two separate stimuli were required also in monocytes.

The identification of distinct pathways, i.e., for IL-1α surface expression and IL-1α secretion, suggests that the two forms of IL-1α may exert different biological functions. Indeed, a differential role of surface-bound and secreted IL-1α in different inflammatory situations has recently been suggested. Surface-bound but not secreted IL-1α has been demonstrated to trigger senescence-associated secretion of IL-6/IL-8 (71) and surface-bound IL-1α caused inflammatory destruction of cartilage during arthritis (72, 73). Thus, the presented data on the regulation of cell surface and secreted IL-1α may offer the possibility to selectively modulate the expression of the two forms of IL-1α. Such regulation may furthermore allow dissecting protection against pathogens from inflammatory diseases. Insight into such regulation of IL-1α may furthermore allow to better understand its role in protection against pathogens and in mediating inflammatory diseases. Consequently, this will allow to design new and improved treatment options in such diseases.

2.1.5 Outlook and preliminary results

2.1.5.1 Calpain-inhibitor effects IL-1α and IL-1β secretion

Calpains are intracellular calcium-dependent non-lysosomal cystein proteases that are located in proximity to the cell membrane and are known to cleave pro-IL-1α into its mature form (36, 39, 74). Stimulated cells additionally treated with a calpain inhibitor (ZLLY) showed an increase in surface IL-1α and had reduced capability of secretion of IL-1α and IL-1β (Fig. 2.1.5). This supports the idea of crosstalk between the surface and secretion pathway. If calpains are inhibited and therefore not able to
cleave pro-IL-1α to generate the soluble mature form, there might be a shift towards higher expression of IL-1α on the cell surface. However, at this stage we do not understand why not only IL-1α but also IL-1β secretion is dependent on calpains. It is known that the raise of intracellular calcium is important during inflammasome activation, which is important for cleavage and secretion of IL-1β (21). Another explanation may be that according to our data that IL-1β forms an intracellular complex with pro-IL-1α in order to shuttle the secretion of IL-1α. However, without cleavage of IL-1α by calpains, the complex could be stuck inside of the cell and may not be secreted. In order to answer these questions the effect and contribution of calpains and intracellular calcium levels on IL-1β secretion would need to be further characterized.

**Figure 2.1.5 | Calpain inhibition increases IL-1α surface expression and decreases IL-1α and IL-1β secretion.** Peritoneal macrophages were stimulated with up-LPS/ATP in the presence or absence of ZLLY, a calpain inhibitor. (A) IL-1α surface staining on macrophages (CD11b+) analysed by flow cytometry. (B) IL-1α and IL-1β secretion quantified by ELISA. Mean and standard deviation of one representative of duplicates of three independent experiments are shown.

### 2.1.5.2 Role of surface IL-1α during *Listeria monocytogenes* infection

*Listeria monocytogenes* is a gram positive bacterium and infects monocyctic cells including macrophages (75). The innate immune system and in particular IL-1 have been characterized as crucial players for an efficient pathogen clearance. Mice vaccinated against IL-1α, IL-1β, or IL-1α and -β demonstrate the importance of IL-1α and the low contribution of IL-1β in pathogen clearance (67) (Figure 2.1.6A). Caspase-1 deficient mice, which secrete reduced levels of mature IL-1α and IL-1β but express similar levels of surface IL-1α (Fig. 2.1.4D, F), were found to have decreased bacterial
clearance (Fig. 2.6B). However, this needs to be further evaluated by comparing infected IL-1α-surface-expressing IL-1β deficient mice and IL-1α/β double knockout (ko) mice.

**Figure 2.1.6** | Caspase-1 and IL-1α but not IL-1β are crucial players during *Listeria monocytogenes* infection. Intraperitoneal challenge with $10^5$ cfu Listeria monocytogenes. Bacterial burden in spleen analysed at day three. (A) Bacterial burden in spleens of IL-1α, IL-1β, and IL-1α and -β subcutaneously vaccinated mice using protein coupled VLPs were challenged with Listeria. (B) Bacterial burden in spleens of caspase-1 and heterozygous littermates. Mean and standard deviation of one representative of duplicates of three independent experiments are shown.

### 2.1.6 Materials and Methods

#### 2.1.6.1 Mice

Female 6-10 week-old C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). *Caspase-1*<sup>−/−</sup> and *IpaF*<sup>−/−</sup> mice were kindly provided by Wolf-Dietrich Hardt, Institute of Microbiology, ETH Zürich, Switzerland. *Nalp3*<sup>−/−</sup> mice were provided by Jürg Tschopp, Department of Biochemistry, University of Lausanne, Switzerland. *Asc*<sup>−/−</sup> mice obtained from Genentech (San Francisco, USA). *IL-1rF*<sup>−/−</sup> and *P2x7r*<sup>−/−</sup> mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All wild type control mice were either littermates of knockout mice or age and sex-matched wild type mice. Mice were kept under specified pathogen-free (SPF) conditions. All animal experimentation was approved by Swiss Veterinary authorities.

#### 2.1.6.2 Bacteria and infection

*Legionella pneumophila* (*Lpn*) strains used in this study were JR32 (wild type Philadelphia-1) (76) and GS3011 (∆T, *icmT* deletion lacking a functional Icm/Dot T4SS) (77). *Lpn* was grown for 3 d on
charcoal yeast extract agar plates. Mice were infected intranasally (i.n.) with 5x10^6 cfu *Lpn* in 20 µl PBS. Four h post-infection, the BAL was collected by flushing the airways with 1 ml PBS after sublethal anesthesia. Cells were recovered from the BAL by centrifugation and the cell-cleared BAL fluid was stored at -20°C for further analysis.

Mice were infected with 100’000 CFU *Listeria monocytogenes* intraperitoneally (a gift from Reinhard Zbinden, Institute for Medical Microbiology, University of Zürich, Switzerland). The inocula were prepared from overnight log-phase cultures in trypticase soy broth, and washed in buffered saline prior to injection. Three days later, spleens were isolated, and single cell splenocytes suspensions were homogenized and lysed in 0.5% saponin for 1 h. The numbers of viable bacteria were determined by plating serial dilutions of the homogenates on tryptic soy agar plates and counting the bacterial colonies after 24 h incubation at 37 °C.

### 2.1.6.3 In vitro stimulation experiments

Cells were stimulated with up-LPS from *E. coli* 0111:B4 (InvivoGen, San Diego, USA) or LPS from *E. coli* 0111:B4 (Sigma-Aldrich, Buchs, Switzerland) at 1 µg/ml. Up-LPS stimulated cells were stimulated additionally using either 5 mM ATP (Sigma-Aldrich, Buchs, Switzerland) for 30 min or 150 µg/ml MSU (Alexis, Farmingdale, USA) for 6 h. Cells were treated with the caspase inhibitor 2.5 µM z-VAD fmk (Alexis, Farmingdale, USA), 5 µM z-LLY fmk (BioVision, Mountain View, CA, USA) or solvent only (DMSO) with a final DMSO content of 0.1% 1 h before stimulation with up-LPS or LPS. IC-21 macrophages were plated at a density of 5x10^5 cells in a 6 well plate, and bmDCs in a density of 2x10^6 cells in a 6 well plate or 7.5x10^4 cells in a 96 well plate. Human monocytes were plated at a density of 2x10^6 cells per 3 ml in a 6 well plate, and peritoneal macrophages in a density of 2.5x10^5 cells in a 6 well plate.
2.1.6.4 Bone-marrow derived dendritic cells (bmDCs) and peritoneal macrophages

Murine bmDCs were prepared as previously described (78). Briefly, bone marrow cells were cultured in RPMI supplemented with 10% FCS, Glutamine, Sodium pyruvate, Penicillin and Streptomycin for 6 to 8 days in the presence of GM-CSF containing X-63 cell supernatant. For collection of peritoneal macrophages 6 ml of DMEM medium containing 10% FCS was injected intraperitoneally, and the total peritoneal liquid was collected.

2.1.6.5 Cell lines

The IL-1 reporter cell line EL4-6.1 was kindly provided by Prof. Robsen McDonald (Institute of Developmental Immunology, Ludwig Institute for Cancer Research, Lausanne, Switzerland) and grown in DMEM supplemented with 5% FCS. The IC-21 macrophage cell line was grown in RPMI supplemented with 10% FCS. Cos cells were grown in RPMI supplemented with 10% FCS.

2.1.6.6 IL-1RII shedding

Shedding of IL-1RII was induced upon 0.1 µM and 1 µm Ionomycin treatment for 24 hours during medium or up-LPS incubation. IL-1RII was detected using a goat anti-mouse IL-1RII (R&D Systems, Abingdon, UK).

2.1.6.7 Human monocytes and IL-2 assay

PBMCs from human buffy coats were isolated by Ficoll. In order to separate monocytes/macrophages from PBMCs, all white blood cells were plated on 10 cm culture dishes at 5.8x10^6 cells/ml and in RPMI 10% FCS so that monocytes and macrophages could attach. After 1 h dishes were washed to remove all non-adherent cells. Monocytes and macrophages were stimulated with 1 µg/ml LPS over night. Monocytes and macrophages were detached by incubation with 2 mM EDTA for 10 min at 4°C,
then washed with PBS and fixed in 1% paraformaldehyde for 10 min at 20°C. Finally cells were washed twice with glycin buffer (150 mM glycin, 75 mM NaCl, pH 7.4) and resuspended in 5% DMEM. Different monocyte/macrophage numbers (10'000; 5'000; 1'000; 500; 100; 10; 0) were transferred into 96-well plates in triplicates with or without anti-IL-1α antibody kindly provided by John Simard (XBiotech, Austin, TX, USA). Cells and antibody were incubated for 30 min at 37°C. Meanwhile EL4-6.1 cells (5 x 10^5 cells/ml) were stimulated with 2 µg/ml ionomycin in DMSO. Activated EL4-6.1 cells were then added to wells and incubated for 24 h. Supernatants were tested for IL-2 by ELISA (R&D Systems, Abingdon, UK).

2.1.6.8 Flow cytometry

Cells were incubated with an anti-Fc-R blocking antibody (mouse: 1:200, human: 1:20) for 5 min before staining with anti CD11C-APC (mouse, 1:200; human, 1:20), anti IL-1α-PE antibody (mouse, 1:100; human, 1:100) and incubated for 30 min on ice. Antibodies were purchased from BD Bioscience (Heidelberg, Germany), eBioscience (San Diego, USA) or Miltenyi Biotech (Bergisch Gladbach, Germany). Cells were fixed in 2% paraformaldehyde (PFA) and acquired using a BD FACS Canto and analysed with the FlowJo software 8.5.2 (Tree Star, Ashland, USA).

2.1.6.9 Western Blot

5x10^5 (IC-21) or 2x10^6 (bmDCs) cells were lysed in RIPA buffer (20mM TrisHCl, pH7.5, 150 mM NaCl, 5mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 1 small tablet / 10 ml protease inhibitor (Sigma-Aldrich, Buchs, Switzerland), 1 ml / 100 ml phosphatase inhibitor (Sigma-Aldrich, Buchs, Switzerland)). Supernatants of cells were precipitated with Tricholroacetic acid (TCA) (1 volume 50% TCA / 2 volumes sample). Proteins were separated on a NuPAGE 4-12% or 10% Bis-Tris gel (Invitrogen, Carlsbad, USA) under denaturing and reducing conditions followed by transfer onto a nitrocellulose membrane (Invitrogen, Carlsbad, USA). Proteins were detected with anti-mouse IL-1α or anti-IL1β antibody diluted 1:1000 (R&D Systems, Abingdon, UK), anti-human IL-1α diluted 1:500
or anti-caspase-1 p10 diluted 1:200 (Santa Cruz Biotechnology, Santa Cruz, USA) or anti-human cleaved IL-1β diluted 1:500 (Cell Signalling Technology, Danvers, USA), and GAPDH diluted 1:5000 (Abcam, Cambridge, UK), or anti-actin antibody diluted 1:5000 (Santa Cruz Biotechnology, Santa Cruz, USA) in 1% milk/PBST or 1% BSA/PBST overnight at 4°C. HRP-conjugated secondary goat-anti-mouse IgG (Zymed Laboratories, San Francisco, USA), rabbit-anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, USA) or anti-rat IgG (GE Healthcare, Buckinghamshire, UK) antibodies were diluted 1:10’000. ECL-detection was performed by a chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

2.1.6.10 Transfection of Cos cells

Cos cells were grown up to 80% confluence in 6 cm dishes and transfected with Lipofectamine (Invitrogen, Carlsbad, USA) using 5 µg DNA. We used tagged human IL-1α and -β for the CoIP.

2.1.6.11 Co-immunoprecipitation (Co-IP)

Cos cells were grown in 6 cm dishes (80% confluence) and transfected with 5 µg of either human IL-1α-myc, or IL-1β-HA or both. Cells were lysed in RIPA buffer and incubated with 1 µg of anti-myc (mouse monoclonal, Clonetech Laboratories, Mountain View, USA) or 1 µg of anti-HA (rabbit, Santa Cruz Biotechnology, Santa Cruz, USA) and Protein G Dynabeads (Invitrogen, Carlsbad, USA) over night. For immunoblotting a hybridoma supernatant containing anti-myc antibodies (mouse) or the same anti-HA were used. To avoid crossreactivity problems of secondary antibodies, we always stained first for the CoIP product (IL-1β-HA) and than after stripping for the IP product (IL-1α-myc). BmDCs were grown in 6-well plates (2x10⁶ cells) and stimulated with either medium, ultra-pure LPS, or up-LPS in combination with ATP for 10 or 30 minutes. Cells were lysed in RIPA buffer and incubated with 10 µg of anti-mouse IL-1α antibody (goat, R&D Systems, UK or eBioscience, San Diego, USA) or an isotype control (goat IgG, R&D Systems, UK or eBioscience, San Diego, USA). For immunoblotting membranes were first stained for IL-1β with an rat IL-1β antibody (Santa Cruz
Biotechnology, Santa Cruz, USA), stripped and than stained for IL-1\(\alpha\) with an rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, USA) to check pulldown efficacy. To avoid crossreactivity problems of secondary antibodies, we always stained first for the CoIP product (IL-1\(\beta\)) and than after stripping for the IP product (IL-1\(\alpha\)).

2.1.6.12 Biotinylation assay

BmDCs were stimulated for 4 hours with either medium or up-LPS in order to express IL-1\(\alpha\) on their cell surface. 25x10\(^6\) of these pretreated cells were than biotinylated using Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, USA) according to the manufacturer instructions, the rest of the medium and ultra-pure treated cells were plated in 6-wells (2x10\(^6\) cells). After biotinylation the cells were also plated in 6-wells (2x10\(^6\) cells). Half of the wells for each condition were stimulated with ATP for 30 minutes. Supernatant was collected for ELISA for performance with (total IL-1\(\alpha\)) and without detection antibody (biotinylated IL-1\(\alpha\)). Biotin on the surface of bmDCs was measured by flow Cytometry using Streptavidin-FITC (1:200) and CD11c-APC (1:200).

2.1.6.13 ELISA

Cytokines were measured by ELISA according to the manufacturer instructions (R&D Systems, UK or eBioscience, San Diego, USA). For the biotinylation assay plates were coated with IL-1\(\alpha\) capture antibody. After 1 hour of blocking and 2 hours of sample incubation, ELISA was either continued as described in the instructions or plates were directly incubated without addition of the detection antibody with the SA-HRP as described in the instructions. In both approaches TMB substrate was incubated for 10 minutes before stop solution was added. For the modified sandwich ELISA using IL-1\(\alpha\) capture and IL-1\(\beta\) detection, plates were coated with IL-1\(\alpha\) capture antibody, incubated with samples and detected with IL-1\(\beta\) detection antibody as described in the manufacturer instructions.
2.1.6.14 Vaccination using IL-1α- and/or IL-1β-VLPs

Cytos Biotechnology AG (Schlieren) was kind enough to provide us with IL-1α and IL-1β displayed on VLP of the bacteriophage Qb (79). Mice were immunized in a bi-weekly fashion for 3 times using 5 µg VLPs. Antibody titer were followed by ELISA at several time points.

2.1.7 Acknowledgements

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2.1.8 Contribution

For this project I designed and performed all experiments with exception of the *Legionella pneumophila* infection that was designed and performed by Salomé LeibundGut-Landmann.
Chapter 2.2

How is IL-1α anchored to the cell surface?

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In preparation
2.2.1 Abstract

Interleukin-1α (IL-1α) belongs to the IL-1 family of cytokines and is a potent pro-inflammatory mediator. It is expressed as an intracellular pro-form and a biologically active cell surface and secreted form. This cell surface form of IL-1α has merely been studied. It still is unclear how IL-1α is anchored to the cell membrane, as it lacks a GPI anchor or any hydrophobic or transmembrane regions in its sequence. To investigate the possible involvement of different posttranslational modification sites, we deleted and exchanged amino acids, which might be functionally involved in cell surface association of IL-1α.

2.2.2 Introduction

IL-1α belongs to the IL-1 family of cytokines and is together with IL-1β the first cytokine described. Both of them are produced as an intracellular 31 kDa pro-form. In the case of IL-1β, the pro-IL-1β is not biological active and requires further maturation into a 14 kDa propiece and the biological relevant secreted 17 kDa form of IL-1β. In contrast, IL-1α exists in four biological active forms, the intracellular pro-form, a membrane-bound form, a 14 kDa cleaved-off propiece, which contains a nuclear localization site (NLS), and a 17 kDa secreted form (36, 54). Although pro-IL1α is already biological active, the matured form shows approx. 5-10 times increased activity (personal communication Seamus Martin, Ireland). Both secreted forms of IL-1α and IL-1β are maturated in different ways. For IL-1β, the intracellular proteolytical cleavage is performed by caspase-1, which itself requires activation by the inflammasome (10), however, for IL-1α the situation is much more ambiguous. The secretion of intracellular matured IL-1α is also dependent on the inflammasome-mediated caspase-1 activation, but additionally requires caspase-1 and IL-1β as a shuttle to be transported through the cell in order to be secreted (31). The intracellular cleavage is performed by calcium-dependent calpains. Pro-IL-1a and pro-IL-1β that are released from dying cells can be
matured outside of the cells by other proteases resulting in cleavage products with similar activity levels as compared to intracellular cleavage (personal communication Seamus Martin, Ireland).

The surface-associated form of IL-1α has first been described in 1985 (80). Nevertheless, it is not yet clear by which mechanism IL-1α gets translocated to the outer side of the cell membrane and how it is then anchored to the membrane. The problem is that the cDNA sequence does not predict any transmembrane regions, hydrophobic regions, or a GPI anchor. The most convincing hypothesis is the lectin binding theory. This theory postulates that pro-IL-1α is myristoylated and therefore translocated to the inner side of the cell membrane to provide an initial interaction with the lipid bilayer (35), then gets transported to the outer side of the membrane after a being phosphorylated, which induced a conformational change (37), and finally anchors there via lectins (38).

In order to address the involvement of the different posttranslational modification sites we performed transfection studies using site-specific sequence mutagenesis to delete or exchange specific amino acids thought to be involved in IL-1α surface expression. We produced a negative and a positive control construct. The positive control encodes for an artificial membrane IL-1α, with the mature form of IL-1α being fused to a transmembrane region. The negative control encodes for an artificial secreted IL-1α with a mature form being fused to a classical ER/Golgi secretion sequence. We further functionally tested our hypothesis in biological assays. Our study could exclude the phosphorylation site to be responsible for the surface anchoring. The myristoylation site was also unlikely to be involved, whereas the lectin bindings were the most promising candidate to mediate cell surface association.

2.2.3. Results

2.2.3.1 Allocation of posttranslational modification sites

Following transcription and translation, pro-IL-1α can get myristoylated (35) and phosphorylated (37). The role of these posttranslational modification sites in IL-1α surface expression and secretion are
unclear. It has been speculated that myristoylation and phosphorylation are required to induce the surface expression of IL-1α. However, this does not explain the surface anchorage. For that, membrane-bound lectins that can bind glycosylated proteins have been suggested to mediate this surface-association. This implies glycosylation sites of IL-1α and in fact these can be found in silico using a glycosylation predictor (http://comp.chem.nottingham.ac.uk/glyco/) (Fig. 2.2.1A) or determining the amino acid series A-X-S/T (Fig. 2.2.1B). Phosphorylation site (37), lectin-binding sites (38), and the clapain cleavage site (39) are known for the murine IL-1α sequence (Fig. 2.2.2A). However, the myristoylation sites is not known, but may be allocated based from the approx. 80% homologous human IL-1α sequence. In order to do so we used the NCBI sequence referenced in NM010554 for mouse and BC013142 for human IL-1α. The myristiolyation occurs on specific internal lysine residues within the pro-piece and we found two corresponding sites in the murine genome, Lys-85 and Lys-86 (Fig. 2.2.2C).
### Figure 2.2.1 | Prediction of glycosylation sites of IL-1α.

**A.** Prediction using the website http://comp.chem.nottingham.ac.uk/glyco/. G = residue is glycosylated, n = residue is not glycosylated, - = other residue.

**B.** Prediction according to amino acid sequence A-X-S/T (Asn – X – Ser/Thr) in red.

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### Table A

<table>
<thead>
<tr>
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<th>Mouse</th>
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</thead>
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| **Myristoylation site** | Lys-82 (bp 253-255)  
Lys-81 (bp 252-254) |
| **Phosphorylation site** | Ser-90 (bp 328-330)  |

### Table B

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<tr>
<td><strong>Phosphorylation site</strong></td>
<td>Ser-90 (bp 328-330)</td>
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### Table C

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
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</thead>
</table>
| **Myristoylation site** | Lys-82 (bp 253-255)  
Lys-81 (bp 252-254) |
| **Phosphorylation site** | Ser-90 (bp 328-330)  |

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MVKVPDLFED LKNCRYSENED YSSAIDHLS NLQKFDSAD YSLHECTDQ FVSLRTSETS KMS4FTFKES RTVSATSSN GKLKIIKRLS FSETETFEDIL QSYQAPFTQPSAPYTV QLQRYKLMV LVRQFVMDN SVQDYOIDY DHKYLSTTWL NDQOEQVDK MYAUYSGDDG SKYPTPLVSDSQDFVSAAG EDPQVLLKEI PETKPLITGS ETLILFWSK ISKNKYFSTA AYPFLFIATQ EQSRVHLARG LPSMTDFQIS
2.2.3.2 IL-1α surface expression after transfection does not depend on cell types

After transfection with a full-length murine IL-1α construct of HEK293T cells, Cos cells or of a murine macrophage cell line (Raw) membrane-associated surface IL-1α was comparably detectable on all three cell types (Fig. 2.2.3A). Furthermore, amounts of secreted IL-1α (Fig. 2.2.3B) were comparable. Although myeloid cells are the main natural source of IL-1α, it is still possible for other cell types like HEK293T or Cos cells to express IL-1α on their cell surface when transfected with the full-length DNA. Therefore the cell surface expression is not cell type restricted.

2.2.3.3 Different constructs of deleted posttranslational modifications sites and control vectors

Although it did not matter which cell type we chose for transfection, we continued with the macrophage cell line, being closest to the natural situation. In order to further investigate the role of the different posttranslational modification sites for IL-1α surface expression we prepared several constructs with amino acid deletions and replacements to test in our RAW transfection system. Our test constructs were full-length IL-1α pcDNA3 constructs with single amino acid replacements in the
myristoylation sites (Lys 85 → Ala 85, Lys 86 → Ala 86), the phosphorylation site (Ser 90 → Ala 90), or the lectin binding sites (Asn 64 → Ala 64, Asn 139 → Ala 139, Asn 143 → Ala 143) (Fig. 2.4A). The functional amino acids were replaced with Alanin. As negative control vectors we used the empty pcDNA3 vector and a pcDNA3 vector containing an IL-1α fusion product derived from mature IL-1α sequence together with the pre-placed classical ER/Golgi secretion sequence derived from the Ig κ-chain leader sequence (Fig. 2.2.4B). As a positive control for the surface expression we generated an IL-1α fusion product using mature IL-1α with a pre-placed transmembrane region derived from the low affinity IgE Fc receptor II (CD23) (Fig. 2.2.4C). Another positive control was generated by deletion of the calpain cleavage site (Fig. 2.2.4A). Calpain is the protease that intracellularly cleaves IL-1α into the pro-piece and its mature secreted form. Although, we previously showed that IL-1α secretion and IL-1α surface expression are regulated differently using two distinct pathways (Chapter 2.1), we think interfering or blocking one form or the other will have impact the second pathway. In order to confirm this hypothesis, we stimulated ultra-pure (up)-LPS/ATP treated peritoneal macrophages in presence or absence of a calpain inhibitor (z-LLL-fmk) and analysed for IL-1α surface expression by flow cytometry (Fig. 2.1.5A) and quantified secretion by ELISA (Fig. 2.1.5B). ZLLL treated cells displayed increased levels of surface IL-1α whereas IL-1α secretion was decreased indicating that a blockade of the secretion pathway leads to enhanced surface expression.

**Figure 2.2.4 | Different IL-1α constructs.** PcDNA3 vector backbone was used for all constructs (A) Exchange of myristoylation sites (Lys 85 → Ala 85, Lys 86 → Ala 86) within the full-length IL-1α; exchange of phosphorylation site (Ser 90 → Ala 90) within the full-length IL-1α; exchange of lectin binding sites (Asn 64 → Ala 64, Asn 139 → Ala 139, Asn 143 → Ala 143) within the full-length IL-1α; calpain cleavage site deleted on
full-length IL-1α; (B) Fusion of Ig κ-chain leader sequence with mature IL-1α. (C) Fusion of low affinity IgE Fc receptor II (CD23) transmembrane region with mature IL-1α.

2.2.3.4 Identification of responsible posttranslational modification site for surface-bound IL-1α

To identify the posttranslational modification site that is required for IL-1α surface expression, RAW macrophages were transfected with our different manipulated constructs described above and analysed for IL-1α surface expression and secretion. To our surprise, none of the modified constructs reduced IL-1α surface expression with the exception of our negative control, the mature IL-1α linked with the ER/Golgi secretion sequence (Fig. 2.2.6A). As expected, this secretion construct showed the highest levels for IL-1α secretion (Fig. 2.2.6B). Note that our positive control, the transmembrane region linked to mature IL-1α did not induce surface expression for unknown reasons (Fig. 3.6). Furthermore, the highest surface expression was obtained when using the construct with a deletion in the calpain cleavage site, confirming the above presented data on calpain inhibition. Hence, this set of experiments could not identify a posttranslational modification site, which is alone responsible for IL-1α surface expression.

However, as pointed out above there are several ways to predict lectin binding sites. In our transfection system we only generated a mutant according to amino acid sequence A-X-S/T (Asn – X – Ser/Thr). Although using a different glycosylation prediction method, more residues are found and our mutant might not be a complete N-glycosylation knockout. Therefore, if the mechanism by which IL-1α is bound to the cell surface is lectin dependent, it should be possible to reduce this binding by adding an extracellular sugar source. Thus, glycosylated IL-1α competes with the extracellular sugar for lectin binding sites. To address this question, RAW macrophages were stimulated with up-LPS for 6 hours. Subsequently, their medium was exchanged to either control medium (0.2% NaN₃/PBS), 0.5 M D-Mannose/PBS, or 0.5 M D-Fucose/PBS and incubated for one hour on ice. The amount of surface IL-1α was quantified by flow cytometry. Both sugar treatments could decrease the IL-1α
surface expression, hence, suggesting a competitive role for the glycosylated IL-1α lectin interaction (Fig. 2.2.6C).

Figure 2.2.6 | Extracellular sugar competes for IL-1α binding sites. (A, B) Transfection of vectors described in Figure 3.4 into RAW macrophages. (A) Surface expression was analysed by flow cytometry. (B) IL-1α secretion was quantified by ELISA. One representative of three independent experiments showing mean and standard deviation of duplicates. (C) Raw macrophages were up-LPS stimulated to induce IL-1α surface expression. Incubation with either control medium, 0.5 M D-Fucose, or 0.5 M D-Mannose for one hour on ice. Analysis of IL-1α surface expression by flow cytometry. One representative of three independent is experiments shown.

2.2.4 Discussion

IL-1α surface expression can be detected by cell surface staining and flow cytometry using an anti-IL-1α antibody. In contrast, IL-1β cannot be detected on the cell surface (81). Additionally, IL-1α has been found to be biological active (Chapter 2.1). However, the sequence of IL-1α lacks any site that would hint toward how it associates with the cell surface. Therefore, we tried to shed light on the surface expression and performed mutation analyses. However, our results are inconclusive and could not identify a responsible posttranslational modification site. However, we may not have deleted all potential lectin binding sites. Thus, lectin binding is still a potential mechanism, whereas our results make a role for myristoylation and phosphorylation unlikely. It must, however, be kept in mind that the myristoylation site of the mouse construct is not known and our mutant was designed based on
allocation of the human sequence. However, to further assess the role of lectin binding without mutating all the potential glycosylation sites, our sugar competition assay revealed a potential role for lectins in surface anchoring. Reduced levels of surface IL-1α were detected when adding the competing sugar.

In conclusion, these studies suggest that the glycosylation to lectin binding is involved in the expression of cell surface IL-1α. This is in line with the results of Brody et al (38). In contrast, the phosphorylation site seems unlikely to participate in the cell surface anchoring. We mutated the only known phosphorylation site and could still detect surface IL-1α after transfection. It is likely, however, that the phosphorylation site is required for IL-1α secretion. Very often proteins that are secreted are phosphorylated. It has been found that an IL-1β subpopulation localises within an endosomal subcompartment, which were positive for Lamp-1 and cathepsin D (82). Moreover, it has been speculated that upon inflammatory process these IL-1β containing vesicles undergo fusion with the plasma membrane in order to release their content. The endosomal localisation could also been shown for other unconventionally secreted proteins such as high mobility group 1 protein (HMGB1) and FGF-2 (83). In the case for IL-1α the phosphorylation might be a marker for secretion, which then might enable an endosomal-like localisation and finally leading to lysosomal secretion.

The myristoylation site seemed unlikely to be involved in surface anchoring, but requires further investigation for definitive exclusion. Moreover, it has been reported that both, IL-1α and IL-1β can be myristoylated to varying degrees (84). Since IL-1β does not exist as a cell surface form, it is rather unlikely that myristoylation of IL-1α is involved in this process. Therefore, the myristoylation could also be involved during the process of IL-1α secretion by targeting the pro-IL-1α to the calpains that are located at the membrane inside of the cell and will induce a proteolytical cleavage into secreted mature IL-1α.

Unfortunately, our positive control with a transmembrane sequence in front of the mature IL-1α did not work. The surface IL-1α is believed to anchor with the N-terminal end rather than with the C-terminal end (35). Therefore, the transmembrane sequence was taken from a type III transmembrane protein. Type III transmembrane proteins are intracellularly anchored with the N-terminus whereas the
C-terminus is on the outside of the cell. Maybe our transmembrane construct produces an IL-1α that is not correctly folded and therefore not recognised by our flow cytometry antibody.

2.2.5 Materials and Methods

2.2.5.1 Cell lines

Cos1 cells were grown in RPMI supplemented with 10% FCS. HEK293T cells were grown in DMEM supplemented with 10% FCS. The RAW macrophage cell line was grown in DMEM supplemented with 10% FCS.

2.2.5.2 In vitro stimulation / treatment of cells

Cells were stimulated with up-LPS from *E. coli* 0111:B4 (InvivoGen, San Diego, USA). Up-LPS stimulated cells were stimulated additionally using either 5 mM ATP (Sigma-Aldrich, Buchs, Switzerland) for 30 min. Cells were treated with the calpain inhibitor z-LY-fmk (Biovision, Palo Alto, CA, USA) or solvent only (DMSO) with a final DMSO content of 0.1% 1 h before stimulation with up-LPS or LPS. RAW and peritoneal macrophages were plated at a density of 5x10^5 cells in a 6 well plate. For sugar treatment, cells were incubated for one hour on ice in 0.2% NaN₃/PBS containing either 0.5 M D-Fucose or D-Mannose.

2.2.5.3 Cloning

Murine IL-1α RNA was derived from up-LPS stimulated RAW macrophages. RNA was isolated by Trizol and purified with ethanol. CDNA was synthesized by 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche, Rotkreuz, Switzerland). Primers for amplification of full-length pro-IL-1α: forward: 5’ATGGCCAAAGTTCTGACTTG-3’ and reverse: 5’-
TTATGATATCTGGAAGTCTGTCA-3’. Primers for amplification of mature IL-1α: forward: 5’-ATGTCAGCACCTACCTACCAG-3’ and reverse: 5’-GCCTCGAGTTATGATATCTGGAAGTCTGTCA-3’ according to NCBI sequence referenced in NM010554. Sequence for transmembrane domain was taken from NM_013517, mus musculus, Fc receptor, IgE, low affinity II, alpha polypeptide (Fcer2a), amino acid 1-49 (bp 173-319). Sequence for secretion sequence was taken from Ig κ-chain leader sequence.

2.2.5.4 Side directed mutagenesis

Mutations (replacements or deletions) of single amino acids were performed using the Site-Directed Mutagenesis QuickChange Kit by Stratagne (Stratgene Agilent Technologies, Santa Clara, CA, USA).

2.2.5.5 Transfection

At about 80-90% confluence of cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) using 4 µg DNA in 6-well plates.

2.2.5.6 Flow cytometry

Cells were incubated with an anti-Fc-R blocking antibody (mouse: 1:200) for 5 min before staining with anti CD11b-APC (mouse, 1:500) and anti IL-1α-PE antibody (mouse, 1:100) and incubated for 30 min on ice. Antibodies were purchased from BD Bioscience (Heidelberg, Germany) or eBioscience (San Diego, USA). Cells were fixed in 2% paraformaldehyde (PFA) and acquired using a BD FACS Canto and analysed with the FlowJo software 8.5.2 (Tree Star, Ashland, USA).

2.2.5.7 ELISA

Cytokines were measured by ELISA according to the manufacturer instructions (R&D Systems, UK).
2.2.6 Acknowledgments

We thank Manfred Kopf, Martin Bachmann and members of Kündig, Oxenius and French group for discussion.

2.2.7 Contribution

I designed and performed this study.
Chapter 2.3

IL-1α in cancer progression

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* equal contribution

In preparation
2.3.1 Abstract

IL-1α is involved in many events that are prominently associated with tumour progression i.e. proliferation, invasion and angiogenesis. Furthermore, chronic inflammation induced cell death of tumour-associated immune cells leads to the release of pro-inflammatory mediators like IL-1α that are thought to additionally contribute to the pathogenesis and diseases progression. Therefore IL-1α could be a promising target in tumour therapy. We investigated whether an IL-1α vaccination using VLPs could reduce tumour progression with respect to tumour growth and angiogenesis.

2.3.2 Introduction

IL-1 is a pleiotropic cytokine that drives a wide range of inflammatory responses and regulates haematopoiesis and proliferation. Together with TNFα, IL-1 is the most potent pro-inflammatory cytokine and regarded as an “alarm cytokine”. IL-1 is produced by myeloid cells rapidly after confrontation with an inflammatory stimulus (85-88). IL-1 initiates inflammation and induces the expression of genes encoding pro-inflammatory and adhesion molecules. Of major importance are cyclooxigenase-2 (COX-2), IL-6, inducible nitric oxide synthase (iNOS), prostaglandin-E2 (PGE2), nitric oxide (NO), and other cytokines and chemokines (40). IL-1-induced expression of adhesion molecules on endothelial cells and leukocytes promotes leukocyte extravasation, i.e. migration from blood into tissues.

Excessive IL-1 production has been implied in the pathogenesis of acute and chronic inflammatory diseases and malignancies in humans. These include rheumatoid arthritis, osteoarthritis, atherosclerosis, Crohn’s disease, ulcerative colitis, multiple sclerosis, Alzheimer’s disease, and various cancers (14). There are a number of different approaches being used to target the IL-1 pathway. Biopharmaceutical drugs blocking the IL-1 receptor have shown clinical efficacy in patients with rheumatoid arthritis, juvenile arthritis, autoinflammatory diseases (89), gout (90) and even diabetes (23).
There are three receptors for IL-1α and -β (IL-1Rs), which belong to the immunoglobulin superfamily and are expressed on almost every cell type. IL-1RI with its accessory protein (AcP) is the only signalling receptor. In contrast, IL-1RII, which serves as a decoy receptor, and a soluble IL-1 receptor (sIL-1R) both have negative regulatory functions (36). The receptor antagonist of IL-1α and IL-1β has a signal peptide and is secreted via the ER-Golgi exocytic pathway. IL-1RA binds to the IL-1 receptors with comparable affinity, but without signal transduction. Thus, blocking IL-1α and IL-1β signalling by IL-1RA (Anakinra®) is successfully used to treat certain inflammatory diseases like juvenile arthritis (91), and was recently shown to reduce insulinitis in diabetes (23).

The association of cancer with chronic inflammation has been recognized for a while. The tumour microenvironment consists of tumour, stromal cells, immune- and inflammatory cells which all produce various cytokines, growth factors, and adhesion molecules. Depending on the net signalling outcome, which is determined by expression patterns and concentration of each single cytokine and its receptors, these molecules may promote tumour progression and metastasis (92).

IL-1α is abundant at tumour sites, being produced by the tumour environment and/or by the tumour itself. Tumour promoting effects mediated by IL-1α include induction of proteins such as histon acetyl transferases (HATs), VEGF, or metalloproteinases (MMP) that drive proliferation, invasion, metastasis and angiogenesis (40). Furthermore, chronic inflammation and cell death mediated by necrosis is thought to contribute to pathogenesis and diseases. In this context, IL-1α was shown to be required for the inflammatory response to dead cells and tissue injury (93). Recently, IL-1α release by necrotic hepatocytes was shown to promote compensatory proliferation and hepatocarcinogenesis (94).

As stated above, targeting IL-1α could be an interesting therapeutic approach to cancer because IL-1α is expressed by tumour infiltrating cells as well as on the surface of various cancer cells including pancreatic (95), prostate (96), breast (97), bladder (98), ovarian (99), colon cancer (100), B cell leukaemia (101), B-cell lymphomas in children, gastric cancer (102), liver cancer (94, 103), melanoma (104), renal cancer (105) and T cell leukaemia (106). Thus, this suggests a rather universal role for IL-1α. Moreover, IL-1α expression correlates with the grade of malignancy. IL-1α is over expressed in poorly differentiated, highly malignant tumours. In prostate cancer, for example, expression of IL-1α
strongly correlates with the clinical grade of malignancy (96, 98, 107, 108). IL-1α has been found to promote proliferation of certain tumour and non-tumour cells. The intracellular precursor of IL-1α induces proliferation of smooth muscle cells through interaction with the HATs, unwinding the DNA for replication (109, 110). Also, proliferation of a murine T cell lymphoma expressing IL-1RI (EL4-6.1) (111) upon stimulation with IL-1α has been demonstrated (112). Moreover, hepatocyte death, which results in IL-1α release and activation of IL-1RI signalling in Kupffer cells, leading to IL-6 production, stimulates compensatory proliferation and hepatocarcinogenesis (94). Furthermore, it has been shown that IL-1α promotes angiogenesis. IL-1α stimulated peripheral blood mononuclear cells (PBMCs) secrete VEGF in a dose dependent manner (25). Also, IL-1α knockout mice show reduced tumour neo-vascularization and reduced tumour growth (113). Finally, IL-1α induces matrix MMPs thereby enhancing extracellular matrix invasion. It up-regulates matrix metalloproteinase-9 (MMP-9) and MMP-13 in metastases (103, 114), thereby promoting tissue matrix breakdown and invasion of tumour cells. MMPs are important in disassembling components of the extracellular matrix. Further, there is evidence that IL-1α expression on the tumour induces MMP expression in surrounding tissue (26, 27). Also, IL-1α increased proliferation and invasion of pancreatic cancer cells by up-regulating α6β5-integrin and uPA/uPAR expression, thus resulting in enhanced aggressive behaviour (108, 115).

In this study we evaluated the possibility to use therapeutic antibodies and active anti-cytokine vaccination targeting IL-1α to reduce tumour growth and angiogenesis. IL-1α depleting therapies were tested also in a model tumour cell line that does not express IL-1α by itself, so that only the stroma and infiltrating immune cells would contribute to IL-1α expression. Our study shows that anti-IL-1α vaccination in mice reduced tumour growth and angiogenesis independent of IL-1α expression by the tumour itself.
2.3.3 Results

2.3.3.1 Surface IL-1α expressed on a representative tumour cell line can be targeted by antibody-dependent cell-mediated cytotoxicity (ADCC)

Antibodies against cell surface expressed proteins can induce antibody-dependent cell-mediated killing (ADCC) that is characterized by killing of target cells mediated through the Fcγ-receptor III (FcgRIII) of the effector cells most commonly being monocytes, macrophages and natural killer (NK) cells. To assess ADCC induced by an anti-IL-1α antibody directed against IL-1α expressed on the cell surface of tumour cells, we incubated IL-1α-positive MDA-MB231 human breast cancer cells (116) (Fig. 2.3.1A) with PBMCs in the presence or absence of an anti-human IL-1α antibody and found significant killing of tumour cells in the presence of an anti-IL-1α antibody (Fig. 2.3.1B). From this data an application of an anti IL-1α antibody or an IL-1α vaccination could be an effective treatment option against tumour progression associated tumour growth.

![Figure 2.3.1 ADCC of an IL-1α-positive tumour cell line.](image)

(A) MDA-MB231 express surface-associated IL-1α shown by flow cytometry. One representative of three independent experiments shown. (B) ADCC using MDA-MB231 target cells and PBMCs effector cells in the ratio 25/1 and 10/1. Mean and standard deviation and one representative of three independent experiments shown.
2.3.3.2 Anti-IL-1α vaccination in mice with an IL-1α expressing tumour reduced tumour growth and angiogenesis

In order to elucidate the efficacy of an anti-IL-1α vaccination against growth of an IL-1α expressing tumour we tested a mouse mammary carcinoma cell line DA/3 for the presence of surface-, intracellular-, and secreted- IL-1α, as well as IL-1RI. In cell culture conditions a high percentage of the cells were IL-1α-positive as well as IL-1RI positive (Fig. 2.3.2).

Efficient active anti-cytokine vaccination of mice was achieved by displaying IL-1α in a highly ordered fashion on the surface of virus-like particles (VLP) (41). Thus, the induction of a strong and long lasting autoantibody response can be achieved without the need of additional adjuvants (117). Such immunization with VLP-Qb-IL-1α or with VLP-Qb-IL-1β induced robust anti-IL-1α and anti-IL-1β autoantibody responses. Antibody responses were of high titer, specific, and persistent over several months (79) (Fig. 2.3.3A). We established a neutralization assay by using an IL-1 reporter cell line that secreted IL-2 upon stimulation with recombinant IL-1α in a dose dependent fashion (Fig. 2.3.3B). Our mouse sera were tested in this biological assay and were found to be strongly neutralizing (Fig. 2.3.3C).
Figure 2.3.3 | Representative example of anti-IL-1 autoantibody response. (A) Female BALB/c mice were immunized three times using 5 mg VLPs per s.c. injection. Mean serum antibody titer and standard deviation are shown of one representative of four independent experiments. (B) IL-2 response of the IL-1 reporter cell line EL4-6.1 upon titrated IL-1α stimulation. Mean and standard deviation shown in one representative of three independent experiments. (C) Neutralization of IL-1α induced IL-2 secretion of EL4-6.1 cells by serum of VLP-Qb-IL-1α vaccinated mice.

After having found high and neutralizing antibody titers in vaccinated mice, IL-1α and control immunized mice were challenged with 5x10^5 IL-1α expressing DA/3 mammary carcinoma cells s.c. into the flank. Tumour growth was recorded weekly. As shown in Fig. 4.4A, tumour growth in mice vaccinated against IL-1α was significantly reduced when compared to control animals.

Figure 2.3.4 | Anti-IL1α vaccination reduced tumour growth and correlated with reduced blood vessel density. (A) Female BALB/c mice (n = 8 per group) were vaccinated against IL-1α or with control VLPs and then subcutaneously challenged with 5 x 10^5 DA/3 mammary carcinoma cells. Tumour growth was followed for 7 weeks. (B) Cross sections of s.c. implanted DA/3 tumours on day 30 were stained for van Willebrand factor (vWF). (C) Blood vessels in 5 visual fields of sections from 4 tumours were counted and displayed as mean vessel density (MVD) +/- SD.

Since it has been shown that IL-1α induces expression of VEGF and therefore promotes vascularization, these tumours were immunohistochemically evaluated by a blinded investigator. Cross sections of tumours grown for 30 and 50 days were stained for van Willebrand factor (CD31), a marker for endothelial cells and thus for blood vessels (Fig. 2.3.4B, C). Tumours in anti-IL-1α
vaccinated mice showed significantly lower blood vessel density than control mice vaccinated with VLPs only.

The tumor growth experiment shown in Fig. 4.4 was repeated for confirmation with the following changes. Firstly, the number of injected DA/3 tumour cells was reduced from $5 \times 10^5$ to $1 \times 10^5$ with the intention to enhance the effect in IL-1α vaccinated mice. Secondly, a group treated daily with 375 mg Anakinra (IL-1R antagonist) was included to compare the efficacy of anti-IL-1α vaccination to an approved and successfully used drug (118). Again, we could observe reduced tumour growth in mice vaccinated for IL-1α compared to control animals. The efficacy of IL-1α vaccination was comparable to a daily Anakinra treatment. It should be noted, however, that Anakinra is directed against human IL-1RI. Although the doses we used in our experiments are published recommended doses in the mouse, it cannot be excluded, that Anakinra in the mouse does not work as well as in humans.

Figure 2.3.5 | Anti-IL1α vaccination reduces tumour growth. Female BALB/c mice ($n = 3 - 5$ per group) were vaccinated against IL-1α, with control VLPs or treated daily with 375 mg Anakinra, and then subcutaneously challenged with $10^5$ DA/3 mammary carcinoma cells. Tumour growth was followed for 7 weeks.

2.3.3.3 Growth of an IL-1α-negative tumour can also be inhibited with an anti-IL-1α therapeutic antibody

The experiments above showed that growth of IL-1α expressing tumours could be inhibited by anti-IL-1α treatment. In order to elucidate whether or not anti-IL-1α treatment also worked in the tumours which did themselves not express IL-1α or whether it is sufficient that infiltrating cells like
macrophages and stromal cells provide a source of IL-1α, we challenged C57BL/6 mice with 2x10^7 IL-1α negative murine EL4 tumour cells embedded in Matrigel into the flank. Tumours were allowed to grow for 8 days. Animals were treated with serum obtained from mice vaccinated against IL-1α, IL-1β or with control VLPs. An additional group was treated with Bevacizumab (Avastin®), a human VEGF inhibitor. Figure 2.3.6 demonstrates that treatment with serum containing anti-IL-1α antibody significantly reduced growth of the IL-1α negative tumour when compared to the control group (P < 0.05, Mann-Whitney-U test). At least in this model and after this rather short time, Avastin® treatment appeared to have no effect, at least not with doses we used. Again it must be noted that, although we used doses, which are published to be effective in mice, Bevacizumab targets human VEGF, and may not unleash its full efficacy in mice.

![Figure 2.3.6](image)

**Figure 2.3.6 | Maximal tumour areas of EL-4 lymphomas.** 2 X 10^7 EL4 cells in Matrigel were injected s.c. into female C57BL/6 mice and grown for 8 days. Mice were treated with serum from donors vaccinated for IL-1α (alpha), IL-1β (beta), control (ctrl), or treated with 5mg/Kg Bevacizumab (Avastin®). Tumour sections were H&E stained and tumour growth quantified through calculating the size of stained tumour area.

This result indicates that an anti-IL-1α tumour treatment could be effective independently of tumour-associated IL-1α expression probably targeting the pro-inflammatory tumour infiltrating macrophages and stromal cells.
2.3.4 Discussion

The data presented above shows that active anti-cytokine vaccination blocking IL-1α as well as anti-IL-1α antibody administration offered a promising approach to reduce tumour vascularization and growth. It would further be interesting to correlate the antibody titers achieved after vaccination, to protection against tumour growth. These experiments should give more profound insight into the therapeutic capacity of anti-IL-1α antibodies and make an impact especially in a clinical setting using high dose monoclonal antibody therapy. Furthermore, we were able show that the anti-IL-1α treatment was effective in reducing tumour growth in cells with or without expression of IL-1α. In the past few years Apte and co-workers devoted their research to the contribution of IL-1 to tumour progression and malignant inflammation. They found that NK cells isolated from IL-1α deficient mice are defective in development and function (119). NK cells have long been thought to play a minor role in targeting tumour cells, however, in recent years they gained more attention and today are considered important players that offer meaningful anti-tumour protection (120). Furthermore, Apte describes the involvement of tumour microenvironment-derived IL-1α in immunoediting during carcinogenesis (119). However, to better understand the mechanisms by which IL-1α promotes tumour progression, the role of tumour derived IL-1α and IL-1α produced by the inflammatory infiltrate must be dissected. Moreover, since IL-1α has three distinct localization sites within the cell, namely expression on the cell surface, intracellular as well as intranuclear localization, and the secreted form, the relative contribution and importance with respect to tumour progression have to be evaluated. Ron Apte and his team speculate that probably the cell-associated form of IL-1α is most effective form in tumour progression (personal communication). In addition, they also examine the role of IL-1β and found it to be more prominent during invasiveness of the tumour (40, 121, 122) and angiogenesis (123). Most of their studies are performed in knockout mice. Thus, in this study we confirm their already convincing data about the involvement of IL-1 in tumour progression using different settings including a vaccination against IL-1α or a therapeutic anti-IL-1α antibody as a new potential treatment option.
2.3.5 Materials and Methods

2.3.5.1 Mice and tumour formation

Female 6-10 week-old C57BL/6 or BALB/c mice were purchased from Harlan (Horst, The Netherlands). Mice were kept under specified pathogen-free (SPF) conditions. All animal experimentation was approved by Swiss Veterinary authorities. Tumours were injected subcutaneously in presence of matrixgel (BD Bioscience, Heidelberg, Germany) and were grown until the maximal allowed size of 1 cm$^3$.

2.3.5.2 Cell lines

The IL-1 reporter cell line EL4-6.1 was kindly provided by Prof. Robsen McDonald (Institute of Developmental Immunology, Ludwig Institute for Cancer Research, Lausanne, Switzerland) and grown in DMEM supplemented with 5% FCS. MDA-MB231 human breast cancer cell line was obtained by ATCC (Manassas, VA, USA) and grown in RPMI supplemented with 10% FCS. The DA/3 mammary carcinoma cell line was grown in RPMI supplemented with 10 % FCS and kindly provided by Timothy C. Thompson, Baylor College of Medicine, Houston, Texas, USA. EL4 thymoma cell line was purchased from ATCC (Manassas, VA, USA) and grown in DMEM supplemented with 10 % FCS.

2.3.5.2 ADCC

We prepared the Ficoll separated PBMC effector cells in AIM-V medium (Invitrogen, Carlsbad, CA, USA) at a concentration of either 7.5x10$^6$ cells/ml for the 25/1 ratio or 3.0x10$^6$ cells/ml for the 10/1 ratio. 3.0x10$^5$ MDA-MB231 cells were pre-incubated in the presence or absence of anti-human IL-1α kindly provided by John Simard (XBiotech, USA) for 20 min at room temperature. Subsequent adding of effector PBMCs and incubation for 4 hours at 37°C in a humified atmosphere containing 5% CO$_2$. 
Killing of target cells was assessed by quantifying lactate dehydrogenase (LDH) in the supernatant released from damaged cells using the Cytotoxicity Detection kit (Roche Diagnostics, Rotkreuz, Switzerland) performing according to the manufactures protocol. The assay was carried out in triplicates in a 96-well plate. Maximal release was determined from wells containing only target cells and 1% Triton X-100. Percentage of specific antibody-mediated killing was calculated as follows: 

\[ V_{\text{max}} = \frac{(x-SR)}{(MR-SR)} \times 100 \]

where \( x \) is the mean of \( V_{\text{max}} \) at a specific antibody concentration, \( SR \) is the mean of \( V_{\text{max}} \) of spontaneous release and \( MR \) is the mean of \( V_{\text{max}} \) of the maximal release.

2.3.5.3 Vaccination using IL-1\( \alpha \)- and/or IL-1\( \beta \)-VLPs.

Cytos Biotechnology AG (Schlieren) was kind enough to provide us with IL-1\( \alpha \) and IL-1\( \beta \) displayed on VLP of the bacteriophage Qb (79). Mice were immunized in a bi-weekly fashion for 3 times using 5 \( \mu \)g VLPs. Antibody titer were followed by ELISA at several time points.

2.3.5.4 ELISA for serum antibody titer determination

The antibody titer of anti-IL-1\( \alpha \) antibodies in the serum of mice was detected by an antibody capture assay. 50 \( \mu \)l per well of 0.5 \( \mu \)g/ml recombinant murine IL-1\( \alpha \) (eBioscience, San Diego, CA, USA) coated over night at 4°C. Blocking with 1% BSA/PBST for 1 hour at room temperature. Subsequently 50 \( \mu \)l murine sera diluted starting with 1:20 and titrated with 1:10 were incubated 2 hours at room temperature. Incubation withl 1:2000 diluted secondary antibody HRP-goat anti-mouse IgG (H+L) (Zymed Laboratoties invitrogen immunodetection, Basel, Switzerland) at room temperature for 30 min. Final incubation with 100 \( \mu \)l of ABTS detection reaction solution (Roche Diagnostics GmbH, Mannheim, Germany) per well. The lightning properties of this substrate are converted by the HRP-enzyme bound to the secondary antibody. The enzyme reaction was stopped with 2% oxalic acid solution per well and the OD was measured using an ELISA reader at 450 nm.
2.3.5.5 Neutralization assay

IL-1RI expressing murine reporter cell line EL4-6.1 were stimulated with different concentrations of rmIL-1α (eBioscience, San Diego, CA, USA) (25 ng/ml; 2.5 ng/ml; 0.25 ng/ml; 0.025 ng/ml; 2.5*10^{-3} ng/ml; 2.5*10^{-4} ng/ml; 2.5*10^{-5} ng/ml; 2.5*10^{-6} ng/ml) and subsequent IL-2 secretion was measured in a Sandwich-ELISA (ELISA Kit, R&D Systems, USA). For neutralization testing, serum containing anti-IL-1α antibodies was pre-incubated for 30 min with the recombinant protein. Incubation of protein or protein/serum-antibody mixture, respectively, with 5x10^5 cells/ml EL4-6.1 cells for 24 hours at 37°C in the incubator. The assay was carried out in triplicates in a 96-well plate. Finally the plate was centrifuged at 900 x g for 10 min and 100 µl of supernatant was collected to quantify IL-2 secretion by IL-2 ELSIA (R&D Systems, Switzerland).

2.3.5.6 Flow cytometry

Cells were incubated with an anti-Fc-R blocking antibody (mouse: 1:200, human: 1:20) for 5 min before staining with anti IL-1α-PE antibody (mouse, 1:100; human, 1:20) and incubated for 30 min on ice. Antibodies were purchased from BD Bioscience (Heidelberg, Germany), eBioscience (San Diego, USA) or Miltenyi Biotech (Bergisch Gladbach, Germany). Cells were fixed in 2% paraformaldehyde (PFA) and acquired using a BD FACS Canto and analysed with the FlowJo software 8.5.2 (Tree Star, Ashland, USA).

2.3.5.7 Sandwich ELISA

Sandwich ELISAs were performed according to the manufactures protocol (R&DSystems, Abingdon, UK).
2.3.5.8 Immunohistochemistry

Tumours were excised and fixed in formaldehyde and small tumour pieces were embedded in paraffin. Tumour sections were stained by haematoxylin and eosin (HE). For immune histochemistry the slides were probed with antibodies against vanWillebrand factor (DAKO, Glostrup, Denmark). Staining of this antibody was detected using an alkaline phosphatase anti-alkaline phosphatase (APAAP)-immunohistochemistry technique (reagents from DAKO, Glostrup, Denmark).

2.3.6. Acknowledgments

We thank Manfred Kopf, Martin Bachmann and members of Kündig, Oxenius and French group for discussion.

2.3.7 Contribution

For this project I designed and performed the ADCC and flow cytometry experiments. Moreover, I conducted the tumour study using EL-4 cells. I also contributed to the immunization regimen of mice and designed and performed the IL-1α-dependent IL-2 secretion assay using the IL-1 reporter cell line.
Chapter 2.4

Relief of zmp1-mediated arrest of phagosome maturation is associated with facilitated presentation and enhanced immunogenicity of mycobacterial antigens

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2.4.1 Abstract

One of the immune escape mechanisms which evolved in Mycobacteria is to interfere with the antigen presentation machinery of the host by blocking the phagosome-lysosome fusion and thus directly escaping the host's innate immunity and indirectly also affecting adaptive immunity. The current standard vaccine against *Mycobacterium tuberculosis* (Mtb) uses a live attenuated strain of *Mycobacterium bovis* BCG, but this vaccine has recently been found to have only limited efficacy. In order to improve the immunogenicity of a mycobacterial vaccine we genetically deleted a mycobacterial zink metalloprotease (zmp1), which is responsible for escaping phagosome-lysosome fusion. Immunogenicity of that mutant was enhanced showing improved antigen-presentation as assessed by delayed-type hypersensitivity (DTH), antigen-specific lymphocyte proliferation and interferon-γ (IFN-γ) production of CD4 and CD8 T-cells.

2.4.2 Introduction

Mtb manipulates the host immune response by interfering with the antigen-presentation machinery by arresting the phagosome-lysosome fusion (124). Within the phagolysosomes immunogenic Mtb peptides are loaded onto major histocompatibility class (MHC) II complexes (125). Mtb peptides presented on MHC II by infected cells can then be recognized by CD4+ T-cell, which will initiate an immune response to fight the pathogen. By inhibiting peptide-MHC II loading, Mtb avoids being recognized by the immune system and escapes a CD4+ T-cell response (126). Although CD4+ T-cells are crucial in controlling Mtb infection (127), the arrest of phagosome maturation can also have an effect on CD8+ T-cells, activated by peptides presented on MHC I either by cross-presentation (128) or by fusion of phagosomes with endoplasmatic reticulum containing MHC I molecules (129, 130). Additionally, Mtb is able to escape actively other immune surveillance mechanisms of the host (126, 131, 132). The poor immunogenicity of Mtb during an infection can be noticed by the fact that re-infected individuals are not necessarily protected from previous exposure thus having failed to
generate a memory response against Mtb (133).

The same is true for the *mycobacterium bovis* BCG strain, which is the only available Mtb vaccine. However, the efficacy in terms of protection against Mtb is limited (42). One protein expressed by both Mtb and BCG, a zink metalloprotease (zmp1), is known to have the capacity of interfering with activation of inflammasomes and phagosome maturation in host cells (43). Thus, zmp1 might be important for the immune evasion of mycobacteria. In order to improve the current vaccine against Mtb in terms of immunogenicity, we evaluated the BCG wild-type (wt) in comparison to the BCG zmp1 mutant for the ability of antigen-presentation *in-vitro* and *in-vivo*.

2.4.3 Results

2.4.3.1 Zmp-1 dependent phagosome maturation arrest

Mtb infection of macrophages is characterized by an arrest of phagosomal maturation. Thus, the bacterium is able to replicate in a vacuole that offers a normal high pH with limited hydrolytic activity and therefore low antigen presentation activity (124). BCG*wt* arrests phagosome maturation at the stage of an early endosome. However, BCG*zmp1* mutant was no longer able to perform that arrest. BCG*zmp1* infected bone marrow-derived macrophages colocalized with an early endosomal marker LAMP-1, whereas BCG*wt* did not (Fig. 2.4.1).

![Figure 2.4.1 | Zmp1 colocalization with LAMP-1.](image)

Figure 2.4.1 | Zmp1 colocalization with LAMP-1. Bone-marrow-derived macrophages infected with BCG*wt* (upper panel) and BCG*zmp1* (lower panel). FITC-labeled bacteria in green (left), LAMP-1-labeled late endosomes in red (middle), merge and colocalization in yellow (18). Stainings were performed 1-60 min after...
infection. BCGzmp-1 but not BCGwt colocalizes with LAMP-1. One representative out of 3 independent experiments shown.

2.4.3.2 In vitro antigen presentation of BCG

As described earlier BCGzmp1 interferes with innate immune responses (43) and therefore might have an effect on the capacity to present mycobacterial antigens to T-cells. To address this issue, we infected bone marrow-derived dendritic cells (bmDCs), i.e. professional antigen-presenting cells (APCs) at different MOIs with BCGwt, BCGzmp1, or BCGcompl. The infected bmDCs were incubated with the MHC class II-restricted T-cell hybridoma cells DE10 and 2E5, specific for the mycobacterial epitopes Ag85A(241-260) (I-A^b) and Ag85A(101-120) (I-E^d), respectively. IL-2 secretion was quantified as a marker of antigen-stimulated T-cell activation. Increased amounts of IL-2 secretion was observed in infections with BCGzmp1 compared to BCGwt, suggesting enhanced antigen presentation for the mutant strain due to the inability to arrest the phagosome-lysosome maturation (Fig 2.4.2). The improved antigen presentation of BCGzmp1 infected APCs was independent of MHC haplotype. It was observed in both H-2^b C57BL/6 (Fig. 5.2A) (P=0.04) and H-2^d (BALB/c) (Fig. 5.2B) (P < 0.0001) mice.

**Figure 2.4.2 | In-vitro presentation of mycobacterial Ag85A.** BmDCs were prepared from H-2^b C57BL/6 (A) or H-2^d BALB/c (B) mice and infected with various MOIs with BCGwt (open bars), BCGzmp1 mutant (closed bars) or the zmp1-complemented strain BCGcompl (hatched bars). The infected DCs were cocultured for 20 hours with the I-A^b-restricted Ag85A(241-260)-specific T-cell hybridoma DE10 (A) or with the I-E^d-restricted Ag85A(101-120)-specific T-cell hybridoma (B). IL-2 secretion by the T-cell hybridoma was quantified by ELISA. One representative of three independent experiments shown with means plus standard deviation of 3 (DE10) and 6 (2E5) replicates. Statistical differences between BCGwt and BCGzmp1 were analysed by two-way ANOVA, the variable being MOI and BCG genotype.
2.4.3.3 Immunogenicity of the BCG wild type and Zmp1 mutants

In order to test the contribution of Zmp1 to in vitro immunogenicity, we immunized mice with titrated doses of BCG wt, or BCG zmp, and challenged these mice with a footpad injection of PPD, a major antigen of mycobacteria. Such injection elicits delayed type hypersensitivity (DTH), caused by mycobacteria-specific T cell responses, which manifest as a measurable swelling. Immunization of mice with an inoculum of $10^3$ CFU BCG zmp1 was sufficient to induce a DTH response. In contrast, when immunizing BCG wt strain inoculation with $10^4$ CFU was necessary (Fig. 2.4.3A). Also, BCG zmp1 strain induced a significantly stronger response than BCG wt at $10^3$ CFU ($P = 0.0004$) with only borderline significance at $10^4$ CFU ($P = 0.08$) (Fig. 2.4.3B). If this data was combined with the data of an independently performed second experiment the significance ($n = 78$) could be enhanced at $10^3$ CFU ($P = 0.0001$) and $10^4$ CFU ($P = 0.04$). Also, when analysing the kinetics of the DTH response for $10^4$ CFU, we observed a faster and stronger response for BCG zmp1 than for BCG wt.

![Figure 2.4.3](image)

**Figure 2.4.3 | DTH reactions in BCG-immunized mice.** C57BL/6 mice were immunized with $10^2$, $10^3$, or $10^4$ CFU BCG wt or BCG zmp1 mutant and challenged with PPD in the footpad on day 21. (A) Footpad swelling measured 48 hours post challenge ($n=66$). The box plots and whiskers show the minimum, maximum, 25th and 75th percentiles, and median. (B) In a different experiment, footpad DTH was measured 24, 48, and 96 hours post challenge of mice immunized with $10^4$ CFU BCG wt (open symbols) or BCG zmp1 (closed symbols) ($n=12$). The data are means ± standard errors and $P$ values were calculated by the Kruskal-Wallis test (A) and ANOVA (B).

To further investigate antigen specific immunogenicity, mice were immunized with $10^6$ CFU BCG wt, BCG zmp, and BCG compl. Splenocytes of immunized and untreated control mice were harvested and restimulated with PPD (Fig 2.4.4) or Ag85A (data not shown). BCG zmp1 immunized mice showed increase proliferation rate (Fig. 5.4A), increased INFγ secretion in ELISA (Fig. 2.4.4B) and ELISPOT assay (Fig. 2.4.4C) as well as an increased frequency of IFNγ-producing CD44-positive CD4 and CD8...
T-cells (Fig. 2.4.4D). In summary, the Zmp-1-deletion enhanced immunogenicity, thus confirming our hypothesis that Zmp-1 was crucial in interfering with phagolysosome fusion. Such enhancement of phagosome maturation resulted in increased antigen presentation to T-cells.

Figure 2.4.4 | Analysis of antigen-specific splenocytes. Groups of six C57BL/6 mice were immunized with $10^6$ CFU BCGwt, BCGzmp1, or BCGcompl on days 0 and 21; littermate controls were left untreated. On day 28, splenocytes were harvested and restimulated in vitro with 5 or 1 µg/ml PPD or medium. (A) Lymphocyte proliferation by $[^3H]$thymidine incorporation was measured after 4 days, and the stimulation index (SI) was determined against medium stimulated cultures. (B, C) IFNγ secretion was measured in supernatants in 3-day cultures by ELISA (B) and ELISPOT showing the number of IFNγ-secreting cells per 200,000 splenocytes (C). (D) Flow cytometry analysis showing the percentage of IFNγ-producing CD44-positive CD4+ or CD8+ lymphocytes. The histograms show mean and standard errors, while box blots show minimum, maximum, 25th and 75th percentiles, and median. P values were obtained from two-way ANOVA comparing BCGzmp1 with BCGwt (A-C) or by the Mann-Whitney test (D). One out of 2 or 3 representative experiments is shown.

2.4.3.4 BCG persistence and growth in wild type and SCID mice.

To address the persistence of the BCGwt and BCGzmp1 mutant strain, we intravenously infected immunocompetent C67BL/6 mice with $10^6$ CFU of either wt or mutant strain. General signs of disease and bacterial load within various organs were monitored over a time period of 85 days. No signs of clinical disease symptoms were recorded and comparable bacterial burden were found in liver and spleen (Fig. 2.4.5).
Figure 2.4.5 | BCG persistence in wild-type mice. C57BL/6 mice received $10^6$ CFU BCG<sub>wt</sub> or BCG<sub>zmp1</sub> by intravenous injections ($n = 40$). At different time periods post injection, mice were euthanized and the spleens and livers were harvested and homogenized. Titrated amounts of the tissue homogenates were plated on 7H10 agar plates for determination of the bacterial load. The data are means ± standard errors. No statistical difference was observed between BCG<sub>wt</sub> and BCG<sub>zmp1</sub>.

For safety evaluations we also intravenously infected immunocompromized SCID mice with $10^6$, $10^7$, $10^8$ titrated CFU of BCG<sub>wt</sub> and BCG<sub>zmp</sub> and recorded weight loss and survival. A 20% weight loss was previously determined for a termination. Both groups of mice started to loose weight independently of BCG associated Zmp1 expression (Fig. 2.4.6A). In line with this the Kaplan-Meier curves did not manifest differences in survival of mice infected with BCG<sub>wt</sub> or BCG<sub>zmp</sub> altogether suggesting a comparable persistence and safety (Fig. 2.4.6B,C).
Figure 2.4.6 | BCG safety testing in SCID mice. SCID mice received BCGwt or BCGzmp1 by intravenous injections (n = 36). The body weights were measured before injection and individually monitored thereafter. Upon a weight loss of 20%, the animals were euthanized. (A) Weight monitoring of groups of six SCID mice that received 10⁶ CFU of either BCG strain. (B) Survival of SCID mice in groups of six receiving 10⁶, 10⁷, or 10⁸ CFU of either BCG strain illustrated by Kaplan-Meier curves. (C) The hazard ratio (HR) and log rank analysis of relative risks demonstrated no statistical difference between BCGwt and BCGzmp1.

2.4.4 Discussion

The only existing vaccination against tuberculosis is a vaccination using the live attenuated strain *M. bovis* BCG which confers only very limited protection. Almost all cases of disease had been previously BCG vaccinated (134). Thus, enormous effort was made trying to improve efficacy of a vaccination. Most attempts focussed on overexpression of *Mtbc* antigens (135, 136) or reintroduction of genes, which were deleted during attenuation process (136). The most promising vaccine candidate to date is probably a BCG strain expressing lieteriolysin, which facilitates endosomal escape and cytosolic antigen delivery inducing MHC class I-restricted antigen presentation (137, 138). This strategy, similar to our ZMP1 deletion strategy, also involves inhibition of phagosome-lysosome fusion accompanied with an accumulation of bacteria within the early endosome, which are then not processed and therefore do not induce protective immunity. However, the exact mechanism how
fusion with the lysosome is prevented is still unclear. Phagosome maturation is a complex series of reactions involving Rab proteins (139-142).

In terms of antigen presentation the phagosome-lysosome fusion is required to induce an acidic environment for degrading and unfolding of proteins and for substitution of the invariant chain that subsequently allows binding of peptides to MHC class-II molecules. Therefore, triggering of phagosome maturation is expected to improve immunogenicity of antigens and thus enhance effectiveness of a vaccine.

Our approach to enhance BCG vaccination efficacy with the deletion of a gene encoding for a zinc-metalloprotease (*zmp1*) compared the classical used BCGwt vaccination strain with the BCG*zmp1* mutant strain. Zmp1 is known to interfere with the phagosome maturation probably by preventing inflammasome activation and caspase-1-dependent IL-1β secretion (43). In experiments comparing both strains for their capacity of antigen-presentation and antigen-specific T-cell induction we noticed improved T-cell responses in situations with Zmp1 deletion. All assays performed in this study, DTH responses and restimulation assays with splenocytes of immunized mice approaching antigen-specific proliferation, IFNγ secretion and levels of intracellular IFNγ of antigen experienced T-cell, indicated the higher and more efficient T-cell responses with the BCG*zmp1* mutant. Our data suggests a correlation of phagosome maturation and antigen presentation efficacy, opening a new alley towards development of future vaccines. Our data also provide for a better understanding of the immune evasion of Mtb. However, the protection efficacy of an BCG*zmp1* vaccination towards a Mtb infections remains to be elucidated.

2.4.5 Materials and Methods

2.4.5.1 Mice

Female BALB/c (*H-2d*) or C57BL/6 (*H-2b*) mice were obtained from Harlan (Horst, Netherlands). Female SCID (CB-17/Scr-Prkdc<sup>SCID</sup>) mice were obtained from the Jackson Laboratory (Bar Harbor,
ME). All mice were obtained and kept under specific-pathogen-free (SPF) conditions in facilities at the University Hospital Zurich and were used at 6 to 10 weeks of age. The experiments were reviewed by the local ethical committee and were performed according to Swiss experimental and ethical guidelines.

2.4.5.2 Preparation and cultivation of bacteria

Mycobacterial strains were grown and propagated according to standard microbiological techniques. The construction of the BCGzmp1 knockout mutant has been described previously (43). BCGzmp1 was complemented by transformation with the zmp1-containing plasmid, pMV361-hyg-zmp1, and the complemented BCG strain is henceforth referred to as BCGcompl. Zmp1 expression in the complemented mutant is under the control of the zmp1 promoter.

Western blot analyses using rabbit anti-Ag85 antiserum (gift from Colorado State University) showed comparable expression levels for Ag85 in all three strains: BCG, BCGzmp1, BCGcompl (data not shown).

2.4.5.3 Confocal imaging

Bone marrow stem cells were isolated from mice femurs and differentiated for 7 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 10% L cell conditioned medium, and penicillin/streptomycin on petri dishes. The cells were then mounted on 0.7-mm glass coverslips in 24-well plates at 1.5 x 10^5 to 2 x 10^5 cells per well and infected with BCG as described previously (143). Colocalization studies were done as described previously (143), and the coverslip contents were left unidentified before analysis.
2.4.5.6 Antigen presentation studies in dendritic cells

Dendritic cells (DCs) were prepared from C57BL/6 and BALB/c mice as described previously (144). Briefly, femurs were aseptically harvested and bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FCS, glutamine, sodium pyruvate, penicillin, and streptomycin in the presence of 10% supernatant from granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting X-63 cells. The X-63 cell line was transfected and kindly provided by A. Rolink (University of Basel). After 6 to 7 days, 1 x 10^5 DCs per well were plated in 96-well flat-bottom culture plates and infected with BCGwt, BCGzmp1, or BCGcompl at different multiplicities of infection (MOIs) (10, 1, 0.1, 0.01, or 0). Fluorescence-activated cell sorter (FACS) analyses of DCs infected with Alexa Fluor 488-labeled mycobacteria demonstrated comparable mean fluorescence levels for BCGwt, BCGzmp1, and BCGcompl, and fluorescence microscopy of macrophages showed similar infection rates for all three strains (data not shown). Within 1 h, 1 x 10^5 DE10 (H-2b) or 2E5 (H-2d) T-cell hybridoma cells specific for the M. tuberculosis Ag85A peptides comprising amino acids 241 to 260 [Ag85A(241-260)] and amino acids 101 to 120 [Ag85A(101-120)], respectively, were added to each well. The antigens were purchased from EMC (Tübingen, Germany), and the hybridomas were kindly provided by C. Leclerc (Institut Pasteur, Paris). After incubation at 37°C for 20 h, supernatants were collected and frozen for later analysis of interleukin-2 (IL-2) by standard enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, United Kingdom).

2.4.5.7 Analysis of delayed-type hypersensitivity (DTH)

C57BL/6 mice were immunized with different doses of BCGwt or BCGzmp1 in 100 µl phosphate-buffered saline (PBS) by subcutaneous injections in the neck region. After 3 weeks, the mice were challenged by injection of 50 µl of a 5-µg/ml solution of tuberculin purified protein derivative (PPD) (SSI, Copenhagen, Denmark) in saline, into the plantar side of the hind right footpad. Injection of saline alone was used as a negative control. One to 4 days later, a delayed-type hypersensitivity (DTH) reaction was analysed by measuring the swelling of the footpad in comparison to the pre-challenge
thickness of the footpad. The measurements were done using a spring-loaded digital micrometer from Mitutoyo (Kawasaki, Japan).

2.4.5.8 Immunogenicity testing

C57BL/6 mice were immunized by subcutaneous (s.c.) injection of $10^6$ CFU BCG on days 0 and 21. On day 28, the mice were euthanized and spleens harvested. The spleens were homogenized, the erythrocytes were lysed in a hypotonic buffer, and triplicates of $2 \times 10^5$ splenocytes were restimulated in vitro with 5 or 1 µg/ml PPD for determination of proliferation and cytokine secretion. After 3 days of cultivation, supernatants were collected and frozen for later analysis of IFN- secretion by ELISA (R&D Systems). Parallel cultures were pulsed with $^3$H-labeled thymidine at 1 µCi per well for another 16 h for analysis of proliferation by â-scintillation. IFNγ-producing cells were analysed by enzyme-linked immunospot (ELISPOT) assay (Diaclone, Besançon, France) by restimulating $2 \times 10^5$ splenocytes from immunized mice with or without antigen for 16 h in multiscreen 96-well Millipore polyvinylidene difluoride (PVDF) plates (Fisher Scientific AG, Wohlen, Switzerland) precoated with 1 µg/ml anti-IFN-. The ELISPOT plates were then washed and developed according to the manufacturer's protocol. The spots were analysed on an AID EliSpot reader system from Autoimmun Diagnostika (Strassberg, Germany), and the results were expressed as spots per $2 \times 10^5$ splenocytes.

IFNγ-producing splenocytes were analysed by intracellular staining and flow cytometry. Triplicates of $2 \times 10^6$ splenocytes were restimulated in 24-well plates at 37°C for 16 h with 5 µg/ml PPD, the last 4 h also with 2.5 µg/ml brefeldin A (Sigma-Aldrich, Buchs, Switzerland). The cells were washed, fixed in protein-free PBS/paraformaldehyde (1%) on ice for 10 min, washed, and then permeabilized in PBS/NP-40 (0.1%) on ice for 3 min. After washing, the cells were resuspended in PBS/FCS (2%), incubated on ice for 5 min with anti-CD16/CD32 for Fc-receptor blocking, and then stained with anti-CD4 (fluorescein isothiocyanate [FITC]), anti-CD8 (PerCP-Cy5), anti-CD44 (phycoerythrin [PE]), and anti-IFN- (allophycocyanin [APC]) antibodies for 40 min. After washing, the cells were acquired using a FACSCanto (BD Biosciences, San Jose, CA). All antibodies were purchased from BD
Pharmingen (Basel, Switzerland) or from eBioscience (Bender MedSystems, Vienna, Austria). The analysis was done using the FlowJo 8.5.2 software from Tree Star, Inc. (Ashland, OR), and the frequency of IFN--producing cells was defined by gating on CD44-positive CD4 or CD8 lymphocytes.

2.4.5.9 Persistence of BCG in immunocompetent mice

The effect of Zmp1 on the growth of BCG in immunocompetent mice was tested after tail vein injection of $10^6$ CFU of BCG<sub>wt</sub> or BCG<sub>zmp1</sub> in C57BL/6 mice ($n = 40$). At different time points thereafter, 5 to 7 animals from each group were euthanized, and the lungs, spleen, and frontal liver lobe of each animal were harvested aseptically in 5 ml PBS. The tissues were homogenized using a Polytron PT 3000 from Kinematica (Littau, Switzerland), incubated for 1 h in 0.5% quillaja bark saponin (Sigma-Aldrich), serially diluted, and plated on Middlebrook 7H10 plates for the determination of the numbers of BCG bacteria. The colonies were enumerated after 3 weeks of incubation at 37°C.

2.4.5.10 Pathology of BCG in SCID mice

To test whether the deletion of Zmp1 in BCG affects its pathogenicity in immunodeficient mice, the survival of SCID mice upon inoculation with BCG<sub>wt</sub> and BCG<sub>zmp1</sub> was assessed. The two BCG strains were administered by tail vein injections at $10^6$, $10^7$, and $10^8$ CFU in groups of six SCID mice. Body weight and survival were assessed over a period of 6 months. The animals were euthanized when they met the preestablished endpoint of 20% weight loss compared to the weight at the day of inoculation.

2.4.5.11 Statistical analysis

For the analysis of dependent observations, a random-effects analysis of variance (ANOVA) was performed, with the BCG strain, immunization dose, and type of antigen used for restimulation as
fixed factors and the individual mouse as a random effect. Prior to ANOVA, the data were square root transformed to meet the equal variance assumption. Nonparametric and independent data were analysed by the two-sided Mann-Whitney U test for two groups or by the Kruskal-Wallis test for three groups or more. For analysis of the survival data, mean survival time and hazard ratios (with 95% confidence intervals) were computed. Kaplan-Meier survival curves were compared with the use of the Cox proportional-hazards models, and statistical significance was tested by log rank test. Analyses were done using the statistical software R HERE (124) or GraphPad Prism. The significance level was set at 5%.

2.4.6. Acknowledgements

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2.4.7 Contribution

For this project I performed and designed the in vitro antigen-presentation assays illustrated. Moreover, I conducted one set of in vivo DTH response experiments. I also contributed to the immunization and in-vitro restimulation experiments for the analysis of antigen-specific splenocytes including antigen-specific proliferation by thymidine incorporation, IFNγ secretion measured by
ELISA and the quantification of intracellular levels of IFN\(\gamma\) in CD44-positive CD4 and CD8 T-cells by flow cytometry. Finally, I monitored the safety testing in the SCID mice for weight loss and survival summarized.
Chapter 2.5

A novel vaccine stimulates T-cell independent IgG responses enabled through TLR4- and TRIF-dependent stimulation of B cells

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2.5.1 Abstract

In general, B-cell class-switching from IgM to IgG, IgA, or IgE is T-cell dependent. Antigen-presenting cells (APCs) and T cells stimulate the formation of plasma- and memory B cells in order to establish a long-lived immune response. Certain antigens stimulate B cells independent of T help (TI). Repetitive antigens such as present on bacteria or viruses may strongly crosslink B-cell receptors, sufficient to stimulate antibody production without T cell help. However, such stimulation typically induces only short-lived IgM responses. Our studies shows that immunization of mice using a liposome displaying a peptide and a TLR4 ligand (MPLA) can induce long-lasting and high titered IgG responses, independent of T-cell help. This was evidenced in athymic, CD4-T-cell depleted, and mice deficient for major histocompatibility complex (MHC) class II, T-cell receptor (TCR), CD40L or CD28, which were all still able to develop high and long-lived IgG titers comparable to those in wild-type (wt) mice. The IgG response was however abrogated in TLR-4 deficient mice. These TLR4-induced TI IgG responses were found to be TRIF- but not MyD88-dependent.

2.5.2 Introduction

There are two different ways to activate B cells. The classical T-cell dependent (TD) pathway is specified by the requirement of T-cell help and is used by most antigens (44). The TI pathway is used only by few antigens, mostly polysaccharides (PS) and lipopolysaccharides (145) (Figure 6.1). An important characteristic of TD B-cell activation is costimulation via CD40/CD40 Ligand (CD40L) interaction. APCs process antigens and present the resulting peptides on MHC class II, which is then recognized by the TCR of a T cell. Moreover, the APC provides CD40 upregulation and cytokine release to activate specific T-cells via CD40L and cytokine receptors (146). Conversely, the activated T cells then interact with the specific CD40-expressing B cells. These B cells have on one hand native antigen bound to their B-cell receptor (BCR) and on the other hand also internalized, processed and expressed the same analogous peptide on MHC class II acting as a professional APC. The B cell
undergoes a class switch and secretes high affinity and long-living IgG antibodies. The class switch of B-cells occurs in the germinal centers of secondary lymphoid organs and involves follicular dendritic cells (FDCs). Apart of the isotype class switching, hallmarks of the follicular pathways are B-cell proliferation, polyclonal activation, establishment of immunologic memory, and affinity maturation including somatic hypermutation to increase the affinity of the antibodies to their antigens. Finally long-living plasma cells that secrete the IgG and memory B-cells are generated. In contrast, antigens that activate B cells independent of T cells are also able to crosslink the BCR so efficiently that they become activated without T-help and costimulation. Such TI B-cell activation is characterized by inducing the transient secretion of an IgM response. Usually, no IgG, IgA or IgE is generated in the absence of T cells (147). TI antigens can be of two types. TI type 1 (TI-1) antigens are polyclonal B-cell activators such as bacterial cell wall components such as lipopolysaccharides (LPS), which are also known as mitogens, activating B cells regardless of their antigen specificity. TI type 2 (TI-2) antigens typically consist of repetitive biochemical structures, such as polymeric protein antigens, bacterial capsular polysaccharides or repetitive viral glycoproteins (46).

In our study, we designed liposomes consistent of phospholipids that formed a lipid bilayer with the potential to integrate antigens. We densely packed our antigen on the surface of these liposomes in order to generate TI-2 antigens. A 15-mer peptide with no known T-cell epitopes was palmytoylated and mixed with phospholipids and monophosphoryl A (MPLA) to allow spontaneous formation of liposomes with densely arranged peptides on the outer surface. The palmitoylated model peptide is derived from human beta-amyloid (Aβ, aa1-15) and adopts an aggregated β-sheet conformation on liposomes (47).
We found that this liposomal vaccine was able to generate long-lasting peptide specific IgG responses in mice, even in the absence of T cell help. TI isotype switching has so far not been previously reported occur in the context of a non-replicating protein vaccine (148).

2.5.3 Results

2.5.3.1 Rapid Aβ-specific IgG antibody response with no evidence of T-cell activation by Aβ-peptide liposomes

Our liposomes contained the palmitoylated Aβ1-15 peptide (PalmAβ1-15) anchored in the liposomal unilaminar bilayer through four palmitoyl chains, and with the adjuvant MPLA, a TLR4 agonist, integrated into the lipid bilayer through its six fatty acid chains (Fig. 2.5.2A and 2.5.2B). Using these liposomes for vaccination of C57BL/6 mice, we induced high titers of anti-Aβ IgG antibodies when compared to a PBS control. These titers reached a maximum already seven days post immunization and could not be further increased by additional boosts at day 14 and 28 (Fig. 2.5.2C). Further analysis of the IgG subclasses revealed the presence of IgG2a/c, IgG2b, and IgG3 antibodies in the serum of immunized mice and identified IgG2b as the dominant subclass (Fig. 2.5.2D). Previous studies suggest that exactly the rapid appearance of specific IgG antibodies including an IgG3 production is in favour of a TI antibody production (149).

In contrast to the full-length Aβ peptide (Aβ1-42), the peptide Aβ1-15 we were using in our study did not contain any T-cell epitopes shown by the lack of antigen-specific IFNγ (Fig. 2.5.2E) or IL-4 (Fig. 2.5.2G) secretion of splenocytes restimulated in vitro with Aβ. The TD antigen ovalbumin (OVA), mixed with the adjuvants CpG and aluminium hydroxide, could induced significant IFNγ (Fig. 2.5.2F) and IL-4 (Fig. 2.5.2H) secretion.
2.5.2 Rapid Ab-specific IgG antibody response with no evidence of T-cell activation by Aβ-peptide liposomes. (A) Liposomes coated with palmitoylated Aβ1-15 peptide (PalmAβ1-15) anchored in the liposomal unilaminar bilayer through four palmitoyl chains, and with the adjuvant MPLA integrated into the lipid bilayer through its six fatty acid chains. (B) Electronmicroscopy of these liposomes. (C) Pronounced anti-Aβ IgG antibody response of wt mice already seven days after one subcutaneous immunization (indicated by arrow) using Aβ-liposomes (closed circles) compared to PBS control (open circles). Repeated injections on day 14 and 28 (97) did not boost the titers. Data shows mean and standard deviation with one representative of two independent experiments with n=6 mice per group (D) Analysis of anti-Aβ IgG subclasses showed presence of IgG2a/c, IgG2b and IgG3 with a dominant attendance of IgG2b. (E-F) Peptide-specific secretion of IFNγ upon splenocytes restimulation using either Aβ1-15 (E) or OVA/CpG/Alum (F) measured by ELISPOT. Data shows mean and standard deviation with one representative of two independent experiments with 10 mice per group. (G-H) Peptide-specific secretion of IL-4 upon splenocytes restimulation using either Aβ1-15 (G) or OVA/CpG/Alum (H) measured by ELIPOT. Data shows mean and standard deviation with one representative of two independent experiments with 10 mice per group.

2.5.3.2 Peptide-specific IgG responses induced by Aβ-peptide are independent of T cells

In order to further rule out the CD4+ T-helper cell involvement in the generation of Aβ-specific IgG antibody responses, wt and CD4-depleted mice were immunized with Aβ-peptide liposomes. Confirming the previously assumed CD4 T-cell independency, both groups showed high and comparable anti-Aβ IgG antibodies (Fig. 2.5.3A). In contrast to that, the OVA-specific IgG antibody response in CD4 T-cell depleted mice was strongly reduced due to the known T-cell dependency (Fig. 2.5.3B). CD4 T-cell depletion was confirmed by flow cytometry (data not shown). Similar results were obtained when using MHC II-deficient mice that lack mature CD4+ T-cells (150) (Fig. 2.5.3C).

To further confirm the CD4 T-cell independency, anti-Aβ IgG titers were compared in immunized wt, athymic nude mice, and T-cell receptor (TCR)-deficient mice, lacking both αβ and γδ T-cells. These
TCR-deficient mice were used as an additional control for nude mice, which have been shown to develop and accumulated with age low numbers of extra-thymic T cells (151). All anti-Aβ IgG titers were found to be high and of similar kinetics upon immunization with the liposomes (Fig. 2.5.3D). The generation of anti-Aβ IgM titers were comparable in wt, nude and TCR-deficient mice (Fig. 2.5.3E). Further subclass analysis of the anti-Aβ IgG antibodies in wt, nude and TCR-deficient mice confirmed the previously seen pattern of a predominant IgG2b expression in combination with the TI-associated IgG3 (Fig. 2.5.3F-H).

**Figure 2.5.3 | Peptide-specific IgG responses induced by Aβ-peptide liposomes are independent of T-cells.**

(A, B) Control (closed circles) and CD4+ T-cell depleted mice (open circles) were immunized (indicated by arrows) with Aβ liposomes (A) or OVA/alum (B) three days post depletion and anti-Aβ, or anti-OVA titer respectively were followed. CD4+ T-cell depletion did affect anti-OVA-titer, but not anti-Aβ titer. (C) CD4+ T-cell depleted wt (grey bars) and MHCII-deficient mice (open bars) were immunized with Aβ-liposomes resulting in comparable anti-Aβ IgG responses. One representative of two independent experiments showing mean and standard deviation of n=6 mice per group. (D-H) Comparable anti-Aβ IgG (D) and IgM (E) after Aβ liposomes immunization (indicated by arrows) in wt (closed circles), nude (open circles) and TCR-deficient mice (grey circles). Dominant and similar levels of IgG2b and IgG3 subclasses in wt (F), nude (G), and TCR-deficient mice (H).
2.5.3.3 Peptide-specific IgG responses induced by Aβ-peptide liposomes do not require APC-mediated co-stimulation

The presented data above implies that our model liposomes stimulate a T cell independent IgG production and therefore a CD4 T-cell independent isotype class switch in B cells. However, previous publications show the importance of costimulatory interaction via CD80/CD28, CD86/CD28, and CD40/CD40L (146, 152-154). These interactions could be provided by APCs. In order to evaluate the contribution of such costimulation, wt, CD40L- and CD28-deficient mice were immunized with Aβ IgG antibody titers were found to be similar in the different mice strains, indicating a minor role for costimulation within IgG generation (Fig. 2.5.4A). Likewise, analysis of IgM titers (Fig. 2.5.4B) and IgG subclasses showed comparable results and a predominant IgG2b and IgG3 response (Fig.2.5.4C-E).

![Figure 2.5.4](image)

**Figure 2.5.4 | Peptide-specific IgG responses induced by Aβ-peptide liposomes do not require APC-mediated co-stimulation.** (A-E) Comparable anti-Aβ IgG (A) and IgM (B) after Aβ liposomes immunization (indicated by arrows) of wt (closed circles), CD40L- (open circles) and CD28-deficient mice (grey circles). Dominant and similar levels of IgG2b and IgG3 subclasses in wt (C), nude (D), and TCR-deficient mice (E). One representative of two indepedent experiments showing mean and standard deviation of n=6 mice per group.

2.5.3.4 TLR4 activation is required for TI anti-Aβ responses stimulated by Aβ-peptide liposomes

To address the role of MPLA on the surface of the liposome, wt mice were immunized using the above described normal liposomes in comparison with liposomes lacking MPLA. In the absence of
MPLA, the liposomes did not induce specific IgG (Fig. 2.5.5A) or IgM (Fig. 2.5.5B) responses in wt mice. Hence, MPLA as a second signal is required to activate the B-cell for antibody production. MPLA is the active component of LPS and thus an agonist for either to TLR4/MD2 or CD14. Aβ liposome immunized wt, CD14−, and TLR-deficient mice highlight TLR4 to be the relevant receptor for MPLA mediated anti-Aβ IgG (Fig. 2.5.5C) and IgM (Fig. 2.5.5D) response, since, an antibody response was observed only in wt and CD14-deficient mice but not in TLR4-deficient mice. The subclass distribution in wt and CD14-deficient mice was similar to previous experiments with predominant IgG2b and IgG3 (Fig. 2.5.5E).

**Figure 2.5.5 | TLR4 activation is required for TI anti-Aβ IgG responses stimulated by Aβ-peptide liposomes.** (A, B) Liposomes lacking MPLA (open circles) did not induce a anti-Aβ IgG (A) or IgM (B) response following several immunizations (indicated by arrows) in contrast to normal MPLA containing liposomes (closed circles). (C, D) Aβ liposomes immunized (indicated by arrows) wt (closed circles) and CD14-deficient mice (open circles) did induce an anti-Aβ IgG (C) and IgM (D) response in contrast to TLR4-deficient mice (grey circles). (E) Comparable IgG subclass distribution in wt (closed bars) and CD14-deficient (open bars), but not detectable in TLR4-deficient mice (grey bars). One representative of two independent experiments showing mean and standard deviation of n=6 mice per group.
2.5.3.5 TLR4 and TRIF activation in B lymphocytes is needed to induce a TI IgG response by peptide liposomes

After having identified the importance of TLR4 activation for TI specific IgG class-switching, the question raised whether it is necessary to present the TLR4 agonist and the peptide on the surface of the same liposome; B cells are known to express TLRs (155-157), thus being able to be activated both through BCR and TLR stimulation. To address this question, we prepared liposomes that only contained MPLA and liposomes only containing the PalmAβ1-15-peptide. Immunizing mice with a mixture of both liposome formulation produced a much lower anti-Aβ IgG response than compared to normal MPLA-PalmAβ1-15-containing liposomes (Fig. 2.5.6A) suggesting a required close proximity of PalmAβ1-15 antigen and MPLA adjuvant for efficient induction of a TI IgG antibody response in B cells. Thus, a TI IgG response is more efficient when both signals are provided together on one liposome. However, to test whether or not the TLR signal has to directly been given to the B cell itself, B-cell containing splenocytes derived from either wt or TLR4-deficient mice were adoptively transferred into B-cell deficient µMT mice in order to generate a system were the TLR4 is specifically lacking on B-cells (Fig. 2.5.6B). In contrast to “wt-B-cell mice” “TLR4-deficient-B-cell mice” were not able to produce specific anti-Aβ IgG responses when immunized with Aβ liposomes. This suggests that liposomes induced TI IgG responses directly activate TLR4 on B cells (Fig. 2.5.6C).

Within a cell, TLR4 can trigger two distinct pathways involving two separate adapter molecules, MyD88 and TRIF. To further evaluate which adapter molecule is used for intracellular B cell signalling after TLR4 stimulation, immune responses in wt, MyD88-, and TRIF-deficient mice were compared after Aβ liposome immunization. The titers of anti-Aβ IgG were found to be significantly reduced (p<0.001) in TRIF-deficient mice indicating the involvement of the TRIF adapter molecule (Fig. 2.5.6D). In parallel to the transfer experiment above, we generated mice with B cells that specifically lacked the TRIF adapter molecule by transferring TRIF-deficient splenocytes into µMT mice and as control mice wt splenocytes into µMT mice. We found reduced anti-Aβ IgG responses in mice lacking the TRIF molecule specifically on B cells, thus suggesting a required TRIF-dependent TLR4 activation on B cells for liposome-induced TI IgG responses (Fig. 2.5.6E).
Figure 6.6 | TLR4 and TRIF activation in B-lymphocytes is needed to induce a TI IgG response by peptide liposomes. (A) Aβ liposomes with MPLA and Aβ-peptide present on the same liposome (closed circle) induced higher anti-Aβ IgG responses after liposome immunization (indicated by arrows) than when MPLA and Aβ-peptide are present on different liposomes (open quadrates). (B) Aβ liposome immunization of B-cell deficient μMT mice that were adoptively transferred with splenocytes derived from either wt or TLR4-deficient μMT mice in order to generate mice that specifically lack TLR4 on B-cells and their control. (C) No anti-Aβ IgG antibody response in TLR4-deficient B-cell mice (open bars) when compared to wt B-cell mice (grey bars). (D) Anti-Aβ IgG titers following an Aβ liposome immunization (indicated by arrows) in TRIF-deficient (open circles) were significantly reduced compared to wt (closed circles), MyD88-deficient (grey circles) mice. (E) Aβ liposome immunization of μMT mice that were adoptively transferred with splenocytes derived from either wt or TRIF-deficient donors in order to generate mice that specifically lack TRIF on B-cells and their control. Almost no detectable anti-Aβ IgG response following an Aβ liposome immunization (indicated by arrows) in TRIF-deficient B-cell mice (open bars) compared to wt B-cell mice (grey bars). One representative of two independent experiments showing mean and standard deviation of n=4 mice per group.

2.5.3.6 Aβ-induced germinal center (GC) formation is T-cell independent

Antigen stimulated B-cell proliferation, somatic hypermutation, antibody class switch, and differentiation to memory B cells or antibody-producing plasma cells is performed together with supporting FDCs in germinal centers of secondary lymphoid organs (158, 159). Germinal centers are mainly formed during TD antigen responses, however, such formation has also been observed in some cases of TI responses (160-162). Germinal center B cells are characterized by the expression of GL-7 and by the ability to bind high levels of peanut agglutinin (PNA). Moreover, IgD negativity is another indicator of GCs as IgD-negative B cells were found to participate far more efficiently in GC formation than IgD-positive B cells (163). To test whether or not the Aβ liposomes were able to induce GC B cells, spleens were analysed for GL-7 expression by flow cytometry, spleens and lymph nodes and PNA or IgD expression by immunohistochemical methods. Immunization of WT mice with
Aβ liposome significantly ($p=0.0294$) increased GL-7 expression on B cells (Fig. 2.5.7A) and strongly increased PNA staining with inversely correlating IgD staining on spleens (Fig. 2.5.7B) and lymph node (data not shown) when compared to untreated control mice on day eight. Conclusively, our TI response mediating liposomes were able to induce GC formation upon immunization. When comparing GC formation in immunized wt and nude mice on day 14 after two immunizations, we observed comparable GC formation levels of GL-7 expression (Fig. 2.5.7C). In athymic mice, lymph nodes were expectedly smaller than in WT mice, and formation of germinal centers was less prominent (Fig. 2.5.7D upper panel). In spleen, germinal center were formed to a comparable extent in athymic and WT mice (Fig. 2.5.7D lower panels). Similar observed isotype switching kinetics of IgG titers in athymic and WT mice (Fig. 2.5.7E).

**Figure 2.5.7 | Aβ-induced germinal center (GC) formation is T-cell independent.** (A, B) Splenocytes of untreated and twice Aβ liposomes vaccinated mice were analysed for GC formation. Significantly higher GC formation in vaccinated mice quantified by GL-7 expression of B220+/CD19+ B-cells by flow cytometry (A). Data shows mean and standard deviation. Immunohistochemistry of splenic sections for PNA, staining GC, and IgD, staining naïve B cell, confirmed the results generated by flow cytometry. (B). (C, D) Splenocytes of Aβ liposomes vaccinated wt and nude mice were analysed by flow cytometry for GC formation and were found to have comparable levels of GL-7+/B220+/CD19+ expressing B-cells(C). Data shows mean and standard deviation. Immunohistochemistry of splenic sections for PNA and IgD confirmed flow cytometry results (D). Data shows one representative of two independent experiments.
2.5.3.7 *In vitro* Aβ-liposomes loaded DCs did not mediate anti-Aβ IgG responses

To further evaluate the possibility that DCs may play a role during induction of immune responses, we prepared bmDCs from wt, TLR-4, and MyD88-deficient mice, loaded the cells with 5 µg/1x10⁶ cells Aβ liposomes *in vitro* and intravenously injected these into wt, nude and MHCII-deficient mice. Anti-Aβ IgG could be induced in all mice, irrespective of TLR4 and MyD88 (Fig. 2.5.8). The establishment of antibody responses in nude mice indicates the T-cell independency of the response (Fig. 2.5.8A). In all liposomes loaded DC transfer experiments, we extensively washed the liposomes loaded DCs with PBS in order to remove free or only DC associated liposomes. However, to test whether free or DC surface-associated liposomes are present in our system, we transferred TLR4- or MyD88-deficient DCs into MHCII-deficient mice. This excludes presentation of potentially free liposomes on host MCH class II and additionally excludes liposomes mediated activation of DCs due to TLR4- or MyD88-deficiency (Fig. 2.5.8B). Although, irrespective of extensive DC washing before injecting, it seemed that we had enough free or DC-associated liposomes in the system to induce anti Aβ IgG responses. Further evaluation in mice, which directly were intravenously injected with either 10 µg or 1µg of liposomes, showed high titers of anti Aβ IgG (Fig. 2.5.8C). This confirms that intravenous injection of liposomes was able to induce isotype switching and leading to the conclusion that IgG antibody responses in our DC system were induced by enough free liposomes associated to DCs that directly activate B cells in the host. Therefore, liposomes processed and presented on DCs cannot induce isotype switching in B cells, thus demonstrating a DC independent B cell activation.

Figure 2.5.8 | Adoptive transfer of *in-vitro* Aβ-liposomes loaded DCs did not induce anti-Aβ antibodies. *In-vitro* generated bmDCs were over night loaded using 5µg/ml Aβ-liposomes, thoughtfully washed twice and 1x10⁶ cells were intravenously injected into recipient mice. Anti-Aβ IgG antibodies were quantified in the serum 7 and 14 days post transfer. (A) wt DCs into wt and nude mice. (B) wt, TLR4, MyD88 DCs into MHC II-deficient mice. (C) Intravenous injection of 10 or 1 µg of Aβ liposomes into WT mice. Anti-Aβ IgG antibodies were quantified in the serum 7 and 14 days post transfer.
2.5.4 Discussion

T-helper cell independent antibody production can be achieved by displaying multiple copies of molecules on the surface of cells and requires the simultaneous stimulation of 10-20 BCRs (164, 165). Such semi-crystalline arrays of antigens displayed on virus-like particles have been exploited to increase immunogenicity of the antigen and to enable TI stimulation of B cells (148). However, in the absence of T help, antibody responses stimulated by non-replicating vaccines are characterized by short-lived IgM without immunoglobulin class-switch to IgG, IgA or IgE. This is for example seen after immunization with virus-like particles produced from hepatitis B core antigens or Qβ bacteriophage capsid proteins (148). Similarly, antigen-specific TI IgG responses are detected in mice infected with live polyoma-virus but not in mice immunized with synthetic polyoma-like particles (166). This suggests that live and replicating organisms provide additional and co-stimulatory signals that are crucial for the isotype switching. To our knowledge, the present study is the first to show that IgG responses to a protein antigen may not be impaired by the lack of T-cell-mediated help and bystander effects. Liposomes displaying an array of densely packed peptide and TLR4 ligand on their surface induced long-lived IgG antibody responses in several T-cell deficient mouse strains including athymic nude and TCR-deficient mice.

The repetitive display of peptide alone was not sufficient to induce antibody responses, but required the presence of a TLR ligand, here, MPLA. MPLA is a synthetic and detoxified derivative of lipid A, derived from LPS of Gram-negative bacteria and has recently been approved for human use in cervical cancer, hepatitis and influenza vaccines (167). MPLA mediates immune responses through activation of the TLR4/CD14/MD-2 co-receptor (168). In our study, stimulation of TRL4 with MPLA was required for the induction of both IgM and IgG responses, demonstrating that the TLR4 signalling is crucial for initial B-cell activation and not only for class-switch recombination, as recently suggested (169). Whether the stimulation of antibody responses requires the involvement of TLRs in general is currently a matter of debate (157, 170, 171), but it has become clear over the last years that TLR signalling can influence B-cell responses directly (172). In vitro, the direct stimulation of TLR4 (169) or TLR9 (172) on B cells has been shown to strengthen B-cell receptor signalling and immunoglobulin
class-switch. The requirement for direct triggering of TLR on B lymphocytes was recently demonstrated for TD antigens displayed on synthetic nanoparticles, where TLR4 and TLR7 ligands synergistically increased antigen-specific antibody responses (156). We observed that when B cells are directly triggered by an array of peptide antigens and a TLR4 ligand, germinal center formation and isotype class switch is allowed and IgG antibodies can be produced in total absence of T cells. In addition, TLR4-facilitated B-cell activation was mediated by TRIF (also known as TICAM-1) and not by MyD88. In summary, this study presents the first evidence that peptide antigens can directly stimulate production of TI IgG by B cells providing parallel signalling through TLR, and that the signal is transmitted by TRIF in B cells.

TLR4 is the only TLR identified until now that activates two signalling pathways, the MyD88-dependent and the TRIF-dependent pathways (173). MyD88 transmits signals culminating in NF-κB activation and production of inflammatory cytokines whereas TRIF activation leads to Type I interferon production. The low toxicity of MPLA compared to LPS or Lipid A has been associated with the preferential activation of TRIF and reduced MyD88 activation (174). A change from diphosphoryl to monophosphoryl lipid A enabled TRIF-biased and MyD88-independent TLR4 signalling (175). More recently, the reduced MyD88 activation of MPLA was shown to be associated with impaired inflammasome priming and reduced Caspase-1 activation and IL-1β production (176, 177). Consistent with these studies, we found that the IgG secretion after immunization with MPLA-containing liposomes was impaired in TRIF-deficient, but not in MyD88-deficient B cells. Thus, the low toxicity of MPLA, associated with TRIF-biased signalling and reduced production of inflammatory cytokines, still allows for TI activation of B lymphocytes and robust IgG responses.

MyD88-independent LPS and Lipid A signalling in macrophages have previously been shown to require CD14 expression (178). Surprisingly though, the TI IgG production after immunization with Aβ-peptide and MPLA-containing liposomes did not require TLR4 signal transduction through CD14 or MyD88. As mentioned above, the TRIF-bias and MyD88-independency of MPLA compared to Lipid A suggest that minor structural changes can have major impact on downstream signalling pathways. It is possible that these structural changes can also explain the combined CD14- and MyD88-independency of MPLA as opposed to Lipid A. The fact that CD14 is not expressed on B
lymphocytes but most only on myelomonocytic cells (179), further suggest that antigen presentation does not take place by APCs such as DCs, macrophages and monocytes, but that the peptide-MPLA assembly directly stimulate B-cell responses through ligation of B-cell receptors and TLR4. Since little is known about TLR4 signalling in B lymphocytes, it remains to be determined whether TLR4 signalling pathways differ between B lymphocytes, and macrophages, particularly with regards to the requirement for CD14, MyD88 and TRIF and how such differences may affect humoral responses.

While previous reports have suggested that non-replicating protein vaccines require T cells for antibody isotype switching (148, 166), the present study demonstrates that a switch is feasible with a correct assembly of antigen and adjuvant. Independent of direct signalling or secondary bystander effects from T-helper cells, high titers of long-lasting IgG antibodies could be produced upon immunization of mice with a densely packed array of peptide and MPLA on particles made up of phospholipids. One practical implication of this finding is the immunotherapeutic utilization in situations where T cells are less abundant due to disease, e.g. AIDS, drug-mediated immune suppression, e.g. organ transplant recipients and cancer patients, or where T cell activation may cause pathology, e.g. induction of encephalitis following vaccination against Alzheimer’s disease (180). The Aβ-peptide used in our Aβ liposomes is a peptide that is produced in the brain and is known to be involved in the development and progression of Alzheimer’s disease. Aβ1-42-peptide is derived upon cleavage of the amyloid precursor protein (APP). The healthy conformation of Aβ is a random coil, however, in case of disease, the proteins misfold into a β-sheet that accumulates and forms toxic oligomers leading to the observed plaque formation in Alzheimer’s patients (181). For potential vaccines against Alzheimer’s disease, it is important to develop a T-cell independent vaccine as activated T-cells could lead to severe tissue damage of the brain. Our previous experiments suggested that Aβ liposomes is such a T-cell independent vaccine, however, a potential DC involvement has to be further investigated.
2.5.5 Materials and Methods

2.5.5.1 Mice

Mice deficient for CD14 (B6.129S-Cd14tm1Frm/J), CD28 (B6.129S2-Cd28tm1Mak/J), CD40L (B6.129S2-Cd40ltm1Imx/J), TCR αβ and γδ (B6.129P2-Tcrbtm1Mom Tcrdtm1Mom/J), MHC class II (B6.129S2-Citiatm1Ccum/J), TLR4 (B6.B10ScN-Tlr4lps-del/JthJ), MyD88 (B6.129P2(SJL)-Myd88tm1Defr/J), TRIF (C57BL/6J-Ticam1Lps2/J), µMT (B6.129S2-Ighmtm1Cgn/J) as well as nude mice (B6.Cg-Foxn1nu/J) were purchased from Jackson Laboratories (ME, USA). All mice were on a C57BL/6 background. Wt C57BL/6 mice were purchased from Charles River (France) or Harlan (Netherlands). All treatments were approved by the Local Committee for Animal Use and were carried out in accordance to state and federal regulations.

2.5.5.2 Preparation of liposomes with peptides and MPLA

Aβ-peptide liposomes (ACI-24, (Hickman et al., 2011; Muhs et al., 2007) were prepared by solubilizing dimyristoyl phosphatidyl choline (Lipoid, Switzerland), dimyristoyl phosphatidyl glycerol (Lipoid), cholesterol (Solvay, Netherlands) and MPLA (Avanti Polar Lipids, Inc. AL, USA) at molar ratios 9:1:7:0.06 in EtOH at 40-60°C. Palmitoylated human Aβ antigen aa1-15 (H-K(Palm)-K-(Palm)-DAEFRHDSGYEVHHQ-K(Palm)-K(Palm)-OH, PalmAβ1-15, Bachem, Switzerland) was dissolved in 1% octyl-β-D-glucopyranoside (Pentapharm, Switzerland) in PBS. Both solutions were sterile filtered and the phospho-lipid solution injected into the octyl-β-D-glucopyranoside/PBS solution followed by further dilution in PBS. The generated liposomes were concentrated by ultra-diafiltration and then sterile filtered. The Aβ-peptide liposomes were sterile and endotoxin free. Each dose (200 µl) contained 78 µg PalmAβ1-15 and 12 µg MPLA.

For the experiments mentioned below, liposomes were prepared as previously described (Muhs et al., 2007). For experiments with Aβ-peptide liposomes +/- MPLA, each immunization dose (200 µl) contained 26 µg PalmAβ1-15 and 30 µg MPLA. For the experiment with mixed liposomes, MPLA-
containing liposomes (19 µg/dose) were mixed with liposomes containing PalmAβ1-15 (94 µg/dose) at a 1:1 ratio prior to injection. For preparation of Tau-peptide liposomes, the tetrapalmitoylated phosphopeptide (H-K(Palm)K(Palm)-RQEFVMEDHAGTY[PO3H2]GL-K(1)K(1)-NH2, human Tau 5-20 with a phospho group on Y18) was used and each immunization dose contained 13 µg Tau5-20 [pY18] and 16 µg MPLA.

2.5.5.3 Preparation of OVA vaccines

10 mg of ovalbumin (OVA; Sigma-Aldrich) was dissolved in 1 ml of sterile water and diluted in PBS to obtain a solution of 1 mg/ml. Aluminum hydroxide (Alum; Alhydrogel, Brenntag Biosector, Denmark) was added to obtain a final concentration of 5 mg/ml. The OVA/Alum solution was mixed with CpG (CpG ODN 1668, phosphorothioate-modified as indicated by asterisks in the 5'-3' sequence: T*C C ATG ACG TTC CTG A*C*G *T*T, Microsynth, Zurich, Switzerland). Each vaccine dose (200 µl) contained 100 µg OVA, 1000 µg Alum and 60 µg CpG.

2.5.5.4 Immunizations

Mice were immunized subcutaneously (s.c.) with 200 µl of liposomes and the administration was typically repeated after 2 and 4 weeks or as indicated in the figure legends. Blood serum samples were prepared before and at various time points after immunization and frozen at -20 °C for later antibody analysis by ELISA. For analysis of T-cell responses, mice were immunized s.c. with Aβ-peptide liposomes or with OVA, aluminum hydroxide and CpG on days 0 and 10. On day 15, spleens were harvested for cell-proliferation assay and ELISPOT analysis.

2.5.5.5 Quantification of OVA- and Aβ-specific cytokine producing T cells by ELISPOT

Cytokine production of OVA- or Aβ-specific T cells was assessed by ELISPOT. Precoated multiscreen 96-well nitrocellulose plates were used according to the manufacturers’ instructions
(Mabtech, Sweden). Single-cell suspensions were prepared from spleens of immunized mice and incubated with OVA (2 µM), Aβ1-42 (2 µM) or Concanavalin A (5 µg/ml, GE Healthcare, Sweden) at 37°C at 5% CO₂ for 48 hours. The plates were then washed and incubated for 2 hours at room temperature with biotinylated anti-mouse IFN-γ or IL-4 monoclonal antibodies. After washing, the plates were incubated for 1 hour at RT with streptavidin-alkaline phosphatase (AP), and after another washing, spots were developed by adding a substrate (BCIP-NBT) for 20 minutes. The number of spots per well was then counted automatically, and the number of spots per 10⁶ cells for each individual mouse was calculated.

### 2.5.5.6 Measurement of Aβ-specific and OVA-specific antibody responses

Aβ1-42-specific and OVA-specific IgG and IgM antibodies were determined by ELISA. Plates were coated with 10 µg/ml of Aβ1-42 (Bachem) or 30 µg/ml of OVA overnight at 4°C. After washing with 0.05% Tween 20 in PBS and blocking with 1% BSA in PBS, serial 1:2 dilutions of serum (1/100 to 1/12600) were added to the plates and incubated at 37°C for 2 hours. After washing, plates were incubated with AP conjugated anti-mouse IgG total antibody (Jackson ImmunoResearch, PA, USA), HRP-conjugated IgM antibody (Pharmingen BD, CA, USA) or subclass specific antibodies (IgG1-AP, IgG2a/c-biotin, IgG3-biotin from Pharmingen BD, CA, USA or IgG2b-AP from Zymed Laboratories, CA, USA) at 37°C for 2 hours. For IgG2a/c (Morgado et al., 1989) and IgG3 antibodies, an additional incubation of 45 minutes at RT with Streptavidin-horse radish peroxidase was carried out. After final washing, plates were incubated with AP (pNPP) or horseradish peroxidase (ABTS) substrate and measured at 405 nm using an ELISA plate reader. The results for IgG are expressed with reference to a commercial available antibody (6E10, Covance, CA, USA) and as O.D. at a non-saturated plasma dilution for IgM, IgG1, IgG2a/c, IgG2b and IgG3.
2.5.5.7 Cryo-TEM measurements

An EM-grid with a holey carbon film was held by tweezers and 4-5 µl of the sample solution were applied on the grid. The tweezers were mounted in a plunge freezing apparatus (guillotine) and after blotting, the grid was immediately immersed in liquid ethane present in a small metal container that was cooled from outside by liquid nitrogen. The image acquisition was made at -170°C in a Philips CM12 EM (The Netherlands) operating at 100 kV and equipped with a cryo-specimen holder Gatan 626 (PA, USA). Digital images were recorded with a Gatan MultiScan charge coupled device (CCD) camera. The image processing software was a Gatan Digital Micrograph.

2.5.5.8 T-cell proliferation assay

Single-cell suspensions were prepared from spleens of immunized mice and restimulated as for ELISPOT. The lymphocyte proliferation was measured using a MTT kit (Promega, Switzerland) according to the manufacturer’s instructions. Briefly, 15 µl of dye solution was added to each well and plates were incubated at 37°C for 4 hours. Next, 100 µl per well of solubilization/stop solution was added per well and the plates were incubated at 37°C for one 1 hour. The optical density (O.D.) was measured at 570 nm (measurement) and 690 nm (background) wavelengths and the absorbance at 690 nm subtracted from the 570 nm absorbance.

2.5.5.9 CD4 depletion and flow cytometry

Depletion of CD4⁺ T cells was achieved by weekly i.p. injections (200 µl) of 100 µg (optimized in house, data not shown) anti-CD4 antibody (Clone YTS191.1; AbD Serotec, UK) starting 3 days before immunization. The animals were bled on days 7, 14 and 21 for analysis of CD4 depletion using fluorescence-activated cell sorter (FACS). Red blood cell lysis was performed by incubating blood preparations with ACK Lysing Buffer (Invitrogen, UK) for 10 minutes at 4°C. Remaining cells were washed in cold PBS and incubated with APC-labeled anti-CD3 (clone KT3; AbD Serotec) and FITC-
labelled anti-CD4 (clone RM4-5; AbD Serotec) at 4°C for 30 minutes. The cells were then washed in cold PBS, resuspended in 1% paraformaldehyde in PBS, and fluorescent events acquired with a CyAn ADP flow cytometer analyser (Beckman Coulter GmbH, Switzerland). Data was analysed using the Summit V4.3.01 software (Beckman Coulter GmbH).

2.5.5.10 Transfer of splenocytes to μMT mice

Single cell suspensions of splenocytes were prepared from TLR4-deficient, TRIF-deficient or wt mice after red blood cell lysis. The cells were washed and resuspended in PBS, and 100 millions cells were injected intravenously into each μMT mouse. Mice were immunized as above with Aβ-peptide liposomes 18 hours after transfer.

2.5.5.11 Bone-marrow derived dendritic cells (bmDCs) and peptide loading

Murine bmDCs were prepared as previously described (John E. Coligan, 2005 #4235). Briefly, bone marrow cells were cultured in RPMI supplemented with 10% FCS, Glutamine, Sodium pyruvate, Penicillin and Streptomycin for 6 to 8 days in the presence of GM-CSF containing X-63 cell supernatant. BmDCs were loaded over night using 5 µg/ml Aβ liposomes at a density of 2x10⁶ cells in 6-well plates.

2.5.5.12 Adoptive transfer of peptide loaded bmDCs

Following peptide loading (5 µg / 1x10⁶ bmDCs), the cells were washed three times in PBS to remove excessive liposomes that have not been taken up by the bmDCs. BmDCs were prepared and intravenously infected using 1x10⁶ cells in 200 µl PBS at day 0. Bleeding of mice at days -1, 7, 14 and 28 for analysis of anti- Aβ-peptide antibodies.
2.5.4.15 Analysis of Germinal Center formation

Athymic and C57BL/6 mice were immunized once (day 0) or twice (day 0 and 7) subcutaneously with Aβ-peptide- and MPLA-containing liposomes as described above. On day 8 or 14 mice were euthanized. Spleens and axillary and mesenteric lymph nodes were harvested for the analysis of germinal center formation. Lymph nodes and half of the spleen were snap frozen for immunohistology, while half the spleen was freshly homogenized for analysis by flow cytometry. The frozen sections were stained with PNA for germinal centers and anti-IgD for immature follicular B cells. Single cell suspension of splenocytes were analysed for CD19-, CD220-, CD23-, and GL7 expression (eBioscience, San Diego, CA, USA) after red blood cell lysis.

2.5.5.18 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, CA, USA). Data were analysed by one-way or two-way ANOVA followed by Tukey post-hoc analysis for multiple comparisons or by Kruskal-Wallis non-parametric test (when n<10). The significance level was set at 0.05.

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2.5.7 Contribution

For this project I performed the GC formation flow cytometry. Moreover, I conducted the adoptive transfer of \textit{in vitro} peptide loaded DCs experiments. I also contributed in writing the manuscript.
Chapter 2.6

Clemastine causes immune suppression through inhibition of extracellular signal regulated kinase ERK dependent pro-inflammatory cytokines

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2.6.1 Abstract

**Background:** Antihistamines are considered safe and used worldwide against allergy, pruritus, nausea, cough, and as sleeping aids. Nonetheless, a growing number of reports suggest that antihistamines also have immunoregulatory functions. **Objective:** We examined the extent and by what potential mechanisms histamine 1-receptor (H1R) antagonists exert immune suppressive effects.

**Methods:** Immune suppression of antihistamines and immunosuppressants were tested in mice infected with *Listeria monocytogenes*. Potential modes of action were studied in vitro using murine and human cells. We also tested if injection of clemastine in healthy volunteers affected the activation of peripheral macrophages and monocytes. Finally, therapeutic application of clemastine-mediated immune suppression was tested in a murine model of sepsis.

**Results:** Clemastine and desloratadine strongly reduced innate responses to Listeria in mice similar to that of dexamethasone. The immune suppression was MyD88-independent and characterised by inhibition of the MAPK-ERK signalling pathway, leading to overall impaired innate immunity with reduced TNF-α and IL-6 production. Surprisingly, the observed effects were H1R independent as demonstrated in H1R-deficient mice. Moreover, in a double-blind placebo-controlled clinical trial, one intravenous administration of clemastine caused reduced TNF-α secretion potential of peripheral blood macrophages and monocytes. This inhibition could be exploited to treat sepsis in mice.

**Conclusions:** The safety profile of antihistamines may need to be revisited. However, antihistamine-mediated immune suppression may also be exploited and find applications in the treatment of inflammatory diseases.

2.6.2 Introduction

Histamine is an important mediator of the allergic response. It is stored in the granules of mast cells and basophils, and is released when an allergen cross-links surface-bound IgE, but also by complement and other chemical or physical stimuli. Histamine leads to hives, itching, pain, smooth
muscle contraction and increased vascular permeability – the typical mucosal symptoms of hay fever or asthma. Histamine-1-receptor (H1R) antagonists are widely used to treat such symptoms and considered very safe, illustrated by the more than hundred million prescriptions and sales of over $4.3 billion in the United States (182, 183) annually. Despite the fact that antihistamines are well tolerated, it is suggested that signalling through the H1R may influence facets of adaptive and innate immunity (48-52). With this in mind, we tested if antihistamine treatment affected innate immune reactions. The results surprisingly revealed that severe biological consequences of H1R interference. Clemastine suppressed inflammation by interfering with the pERK-mediated production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6. This enhanced the susceptibility of mice to bacterial infection, and the effect was surprisingly not dependent on the H1 receptor. However, the results may pave the way for new therapeutic applications of antihistamines, as shown by the inhibition of sepsis in mice.

**2.6.3 Results**

**2.6.3.1 Antihistamines enhanced susceptibility to *Listeria* infection**

C57BL/6 mice have a high resistance to *L. monocytogenes* and show no or little clinical signs of infection with $10^4$ CFU of the bacteria (Fig. 2.6.1a). Clemastine treatment caused five out of seven mice to die within three days of inoculation (HR 0.120, 95% CI 0.016-0.877; p=0.037). Clemastine facilitated bacterial growth (Fig. 2.6.1b, p=0.026) in a dose-dependent manner over a broad range of inoculation doses (not shown). The strength of clemastine-mediated immune suppression was comparable to that of the classical immunosuppressant dexamethasone and superior to that of azathioprine (Fig. 2.6.1c). Suppression of anti-listerial resistance was also observed for the antihistamine desloratadine, while treatment with cetirizine and dimetindene had no significant effect on general health (Fig. 2.6.1d) and listerial growth (Fig. 2.6.1e).
2.6.3.2 Listeriosis in antihistamine-treated mice cause liver abscess and disruption of splenic architecture

In untreated mice, listerial infection caused few, small and evenly distributed infectious foci in livers, while parallel clemastine treatment was associated with formation of numerous abscesses (not shown). In spleen, the lymphoid structures were maintained in infected but otherwise untreated mice, and no prominent staining of *L. monocytogenes* (179) could be observed. While the number and distribution of splenic (F4/80) as well as metallophilic (MOMA-1) and marginal-zone (ERTR-9) macrophages did not notably change in infected mice as compared to non-infected mice, neutrophils strongly infiltrated the red bulb of the spleen upon infection (Fig. 2.6.1f). In contrast, clemastine treatment was associated with massive destruction of lymphoid structures with lack of MOMA-1 and ERTR-9 macrophages framing the follicles and lack of lymphocyte (CD3 and B220) compartmentalisation (Fig. 2.6.1f). Sections from infected mice treated with clemastine also stained positive for *Listeria* and showed dense stains of neutrophils, suggesting necrotic lesions. The stainings also suggested that numbers of B220 B cells and CD3 T cells were reduced in infected mice treated with clemastine. However, due to the lack of clear lymphoid structures, it is difficult to quantify these changes, and whether the differences are due to infection and necrosis of lymphocytes or due to distribution differences because of the break down of follicle structures. Similar splenic features were observed after infection and treatment with desloratadine (not shown).

![Figure 2.6.1](image-url) | Fatal growth of *L. monocytogenes* in antihistamine-treated mice. (a) Survival of C57BL/6
mice infected with 1 104 CFU *L. monocytogenes* and treated daily with 100 µg clemastine (n=7) or not (n=4) starting one day pre-infection. (b) Bacterial counts in spleens harvested three days post-infection with 1x10⁵ CFU *L. monocytogenes*. Data are representative of three experiments (n=4). (c) Bacterial growth in spleen of mice (n=4) treated daily with 100 µg of clemastine (184), azathioprine (24), or dexamethasone (Dex) starting one day pre-infection (1x10⁷ CFU). The experiment was repeated once (n=4). (d-f) Infected mice were treated daily with 100 µg clemastine (184), desloratadine (185), cetirizine (Cet) or dimetindene (Dim). (d) Changes in body-weight were monitored, and (e) bacterial growth were determined (n=6). (f) Histological analysis of spleens from infected mice. The arrowheads indicate *Listeria* (179).

### 2.6.3.3 Clemastine suppressed secretion of TNF-α and IL-6

Mortality within three days suggested that antihistamines affected the innate immune system. Consistent with this, the production of IL-6 (p=0.006) and TNF-α (p<0.019) by in-vitro-infected peritoneal macrophages was reduced by clemastine in a dose-dependent manner (Fig. 2.6.2a). Clemastine also reduced IL-6 and TNF-α production in LPS-stimulated macrophages (Fig. 2.6.2b; p=0.003 and 0.016, resp.). Gene transcripts of TNF-α, IL-6, IL-12 and RANTES were downregulated, whereas those of MIP-1b, MCP-1 and LIX were up-regulated after clemastine treatment (Fig. 2.6.2c). Clemastine at 10 µg/ml did not affect the viability of the macrophages within six hours of incubation, but after 24 hours, a slight difference in viability of clemastine-treated (80-85%) and untreated (95%) was observed (not shown). To analyse the effect of antihistamine on the cytokine secretion in vivo, mice were treated with clemastine and infected with *L. monocytogenes*. After four hours, peritoneal cells and cardiac blood was collected. Analysis of IL-6 and TNF-α in serum and in overnight cultures of peritoneal cells revealed suppression of both cytokines in clemastine-treated mice as compared to infected but otherwise untreated mice (Fig. 2.6.2d). No effect of clemastine on serum IFN-γ was observed 1 and 3 days post-infection (not shown). The role of TNF-α was also tested upon infection of TNF-α deficient mice. While no immunosuppressive effects of clemastine were observed for an infection inoculum of 100 CFU (not shown), antihistamine treatment enhanced susceptibility to listeriosis in mice infected with 5x10⁴ CFU. The effect of clemastine did not reach statistical significance in terms of loss of mouse body weight (Fig. 2.6.2e, p=0.063), but the listerial counts in both liver (p=0.019) and spleen (p=0.029) were significantly higher in clemastine-treated than in untreated mice (Fig. 2.6.2f). Whereas partial disruption of the follicle structures was observed
in infected TNF-α-deficient mice, splenic disruption was more severe in clemastine-treated mice (not shown). Liver histology revealed abscess formation in both untreated and clemastine-treated infected mice (Fig. 2.6.2g). While infection caused strong migration of neutrophils into these lesions, infection with clemastine treatment was associated with severe necrosis of neutrophils in the periphery of the lesions.

Figure 2.6.2 | Immunosuppression by clemastine is TNF-α and IL-6 dependent. (a) Cytokine secretion from peritoneal macrophages cultured in triplicates with or without clemastine (184) before being infected with *Listeria* (179). The experiment was repeated once. (b) Cytokine secretion from macrophages cultured in triplicates with clemastine or histamine (His) and with LPS. The experiment was repeated twice. The p-values were calculated without Dunn’s corrections. (c) Real-time PCR analysis of cytokine and chemokine expression in macrophages MACS-isolated from mice treated with clemastine and infected. The experiment was repeated once for analysis of secreted cytokines and chemokines on a protein array. (d) Mice were pre-treated with clemastine or not before infection. Four hours post-infection, blood (left panel) and peritoneal cells (right panel) were harvested and analysed for secretion of cytokines (n=5). (185) TNF-α-deficient mice were treated daily with clemastine or not and infected with 5x10⁷ CFU *Listeria*. Body-weight loss (e), bacterial growth in livers and spleens (f), and liver histology (g) were analysed three days post-infection.

2.6.3.4 Immunosuppression by clemastine was not mediated through H1R antagonism

Because clemastine is a specific H1-receptor antagonist, the immune suppressive effect of the drug was expected to be mediated by interaction with this receptor. To verify this, H1-receptor deficient mice were infected with *L. monocytogenes*. Infected but otherwise untreated mice showed no clinical signs of disease. In contrast, infected mice that were treated daily with clemastine succumbed to
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severe listeriosis as measured by weight loss (Fig. 2.6.3a), bacterial growth (Fig. 2.6.3b), and based on histological features of spleen and liver (Fig. 2.6.3c). Moreover, the in-vitro secretion of TNF-α by macrophages in response to LPS was reduced by clemastine in cells from H1R-deficient and wild-type mice to the same extent.

Figure 2.6.3 | Clemastine suppresses antibacterial immunity independent of histamine 1-receptor expression. (a-c) H1R deficient mice were infected with *L. monocytogenes* and treated (184) or not (Untr) with clemastine (n=4-5). Listeriosis was measured by monitoring (a) body weight, (b) bacterial growth, and (c) and histology in spleens and livers. The histochemical analysis shows sections stained for *L. monocytogenes* (179). Equivalent experiments were performed thrice. (d) TNF-α secretion from peritoneal macrophages isolated from wild type or H1R deficient mice and stimulated with LPS in the presence or absence of clemastine. The experiment was performed twice.

2.6.3.5 Clemastine delayed sepsis development in mice

While the in vivo and in vitro experiments revealed adverse immune suppression by clemastine, this same effect could be applied in the treatment of diseases caused by excess inflammation, such as sepsis, a life threatening and complex disease caused by an overly-active host response to infection. Using a murine model where sepsis is induced by LPS injection, we found that seven out of eight mice succumbed to sepsis within 16 hours of LPS injection (Fig. 2.6.4). In contrast, daily administration of clemastine significantly delayed the fatal outcome of LPS injection (mean survival time was 20 hours for untreated mice, 43.5 hours for clemastine-treated mice, p=0.015). By way of comparison, one group of mice received daily injections with the immunosuppressant dexamethasone, which showed comparable protection.
Figure 2.6.4 | Clemastine suppresses septic shock in mice. Survival of BALB/c mice (n=8) after LPS-induced sepsis. Mice received immunotherapy with clemastine (184), dexamethasone (Dex), or not (Untr). The p-value describes the significance of untreated versus clemastine-treated mice as calculated by Gehan-Breslow-Wilcoxon test (p=0.015). The experiment was repeated twice.

2.6.3.6 Clemastine exerted immunosuppressive effects also in humans

A key question was whether the above observations were mouse-specific or also relevant for humans. Therefore, monocytes were isolated from human PBMCs and stimulated with LPS. Clemastine treatment was associated with reduced production of TNF-α and IL-6 after one hour stimulation with LPS (Fig. 2.6.5a-b), and the inhibited response was maintained after four hours of LSP stimulation (not shown). Similar results were obtained for human monocytes treated with desloratadine, but cetirizine and dimetindene did not affect the potential of human monocytes to produce TNF-α and IL-6 (not shown). The effect of clemastine was confirmed in a small double-blind placebo-controlled clinical trial. A single intravenous administration of 2 mg clemastine reduced the mRNA expression of TNF-α (p=0.026) and IL-6 (p=0.075) from PBMC-derived cells that were stimulated with LPS in vitro (Fig. 2.6.5c-d). Hence, therapeutic doses of a widely-used antihistamine could impair the innate immune responses in man.
Figure 2.6.5 | Clemastine hampers both TNFα and IL-6 secretion from and ERK signalling in human monocytes. (a+b) Human monocytes were isolated from PBMC and pre-cultured in quadruples with or without clemastine and then stimulated with LPS. The supernatants (n=4) were analysed for (a) TNF-α and (b) IL-6. P-values are calculated by Kruskal-Wallis without Dunn’s corrections. (c+d) Healthy volunteers were infused with 2 mg clemastine (n=5) or placebo (n=5) in a clinical trial. Cells from venous blood collected before and after the treatment, stimulated with LPS, and analysed for (c) TNF-α and (d) IL-6. (e) Monocytes isolated from human PBMCs were stimulated as described above. The cell lysates were analysed by immunoblot as indicated. The experiment was repeated twice.

2.6.3.7 Clemastine inhibited cytokines signalling through the MAPK-ERK pathway independent of MyD88

The cytokine pattern of down-regulated TNF-α, IL-6, IL-12 and RANTES suggested that treatment with clemastine interfered with the NF-κB or the MAPK/ERK signal transduction pathway. NF-κB is a universal trigger of pro-inflammatory cytokines, while extracellular signal-regulated kinase ERK is associated with pro-inflammatory signals produced by MyD88-dependent toll-like receptors. ERK regulates AP-1 binding activity and the stability of transcripts encoding TNF-α, IL-6 and other cytokines associated with the innate immune response (186). We stimulated human monocytes with LPS and analysed the effect of clemastine on various signal transductions molecules. No abrogation of stimulation of the p38 signalling pathways was observed, but clemastine reduced phosphorylation of extracellular signal-regulated ERK (Fig. 2.6.5e). The expression of the NF-κB-associated Inhibitor IκBα was slightly reduced in clemastine-treated monocytes, while phosphorylation of IκBα was not notably changed. Surprisingly, the effect of clemastine on the resistance against listeriosis in mice was not exclusively MyD88 dependent. Treatment of MyD88-deficient mice with clemastine caused
increased bacterial growth (Fig. 2.6.6) similar to that seen in wild-type mice.

![Graph showing bacterial growth comparison]

**Figure 2.6.6** | Clemastine suppresses antibacterial immunity independent of the Toll-like receptor associated anchor protein MyD88. MyD88-deficient mice were infected with 1x10⁵ CFU *L. monocytogenes* and treated daily or not (Untr) with 100 µg clemastine (184) (n=5). The development of listeriosis was measured by monitoring (a) body weight and (b) bacterial growth in spleens (left panel) and livers (right panel) three days post-infection.

### 2.6.4. Discussion

Antihistamines are over-the-counter drugs widely used in acute and chronic situations against a variety of indications. Their usage is considered very safe. Generally, antihistamines act by the blocking of the effect of histamine released from mast cells and basophils by IgE or other triggers. This study revealed that short-term administration of the antihistamines clemastine, which has selective affinity to the H1-receptor but not to other H-receptors, fatally hampered listerial resistance in mice. Also the antihistamine desloratadine caused increased susceptibility to infections with *Listeria* in mice. Immune suppression was mediated through an inhibition of innate immune responses, in particular secretion of TNF-α and IL-6, apparently by inhibition of MAPK-ERK and IκB signal transductions. Surprisingly, the immune suppression was also observed in H1-receptor deficient mice. Hence, at least the two antihistamines clemastine and desloratadine appear to have additional targets different from H1 receptor. Interference at these targets may be exploited in the treatment of inflammatory diseases. Although antihistamines are not expected to have immunosuppressive effects, such properties would likely be associated with the fact that the antihistamine blocks signalling via H1R. This interpretation can be derived from previous reports suggesting that stimulation of H1R signalling may lead to activation of the transcription factor NF-kB (187), which is ubiquitous in regulating the production of pro-inflammatory cytokines, hematopoietic growth factors, adhesion proteins, and acute-phase proteins (188). Consequently, antihistamines could be expected to suppress immune reactions...
mediated by NF-κB. Also, reduced adaptive immune responses in H1R deficient mice have been demonstrated (48), and we have recently shown that clemastine suppressed antibody response to proteins (52). With regard to the effect of H1-receptor signalling on innate immunity, the intracellular survival of *Mycobacterium bovis* BCG was improved in macrophages isolated from histidine-decarboxylase knock-out mice, which are unable to produce histamine, and the bacterial growth in wild type macrophages was enhanced by treating the cells with the H1-antihistamine pyrilamine (189).

However, to our knowledge, it has never been shown that antihistamine treatment can increase susceptibility to bacterial infection. Protection against *L. monocytogenes* is biphasic (190). In the first few days, the cells of the innate immune system play a central role, the cells representing both a major habitat for the bacteria as well as being major effectors in defence. In the following days and weeks, the adaptive immune system gains importance. C57BL/6 mice have a high resistance to *L. monocytogenes*, which is cleared by innate immune responses within a week. The fact that a single injection of clemastine abrogated bacterial resistance so that the mice died within two to three days, therefore suggested that the drug affected the innate immune system, especially macrophages, monocytes and neutrophils, which typically eliminate the bacteria by direct phagocytosis and by production of TNF-α and IL-6 (190-193). We observed that both murine and human macrophages lost their cytokine-secreting potential when treated with clemastine. TNF-α is a strong activator of NF-κB and thereby a key mediator of inflammation and antibacterial immunity (194). Together with IFN-γ, TNF-α triggers the activation of phagocytes, which contain the infection and prevent the pathogen from spreading to other organs. We showed that clemastine, but also desloratadine, prevented efficient TNF-α production and facilitated bacterial spread and growth. Likewise, the reduced IL-6 production observed is of special importance for liver pathology. IL-6 is typically produced by liver macrophages, i.e., Kupffer cells. IL-6 recruits neutrophils to the site of infection, activates hepatocytes to synthesize acute-phase proteins, and influences the oxidative burst and degranulation capacity of neutrophils (195). It has been shown that antihistamines can reduce the release of enzymes, peroxidases and oxides from neutrophils (196, 197). The infiltration of neutrophils to the liver was not notably impaired by clemastine in the present study, but they turned necrotic and caused more severe liver damage than did neutrophils from infected but otherwise untreated mice. Since *L. monocytogenes*...
multiplies rapidly in hepatocytes, it is crucial for neutrophils to gain access to the organ in order to mediate antibacterial effects. For host survival, the mobilisation of neutrophils or their precursors should occur within 24 hours of infection (193). In agreement with the result that clemastine impaired the production of pro-inflammatory cytokines such as TNF-α in response to Listeria or LPS, we found that the resistance to infections with 100 CFU *L. monocytogenes* was not impaired by clemastine in TNF-α-deficient mice, suggesting that the majority of the clemastine effect was attributed to the suppression of TNF-α. However, at higher doses (5×10⁴ CFU), clemastine weakened the resistance to *Listeria*, implying that TNF-α is not the only cytokine with anti-listerial functions.

Under normal conditions, multiple and, sometimes, redundant pathways cooperate to induce a rapid antimicrobial defence. When one signalling pathway is inhibited or removed from the system, the other pathways are still capable of mounting a sufficient response to ensure survival of the host (198). Hence, during weak infections, TNF-α-independent innate immune responses, such as those mediated by IL-6, IL-1, and IL-12, may be sufficient for the control of bacterial growth and spreading (190, 193). Moreover, high inoculates of *L. monocytogenes* is also known to directly affect the output of neutrophils from the bone marrow (199), and may explain the aggravated infection also in TNF-α-deficient mice when 5×10⁴ CFU was inoculated. The fact that clemastine suppressed innate immune responses in murine and human macrophage cultures that were free of mast cells or basophils, the major sources of histamine, was very surprising. However, monocytes and macrophages have been described to express histidine decarboxylase (200), to release histamine (201, 202), and to have G-proteincoupled histamine receptors with spontaneous activity independent of receptor occupation by an agonist (203) or antagonist (187, 204, 205). Hence, even in the absence of extrinsic histamine, certain degree of macrophage activation can be expected hampered by antihistamine treatment. However, the fact that the suppressive effects were also observed in H1R deficient mice, demonstrated that the H1R was not the target of clemastine.

At present, it is unknown which target clemastine and desloratadine use to exert this unexpected immunosuppressive effect, but we were able to show that clemastine inhibits MAPK-ERK signalling in macrophages; this is consistent with the observed down-regulation of TNF-α, IL-6, IL-12 and RANTES. ERK is associated with pro-inflammatory signals produced by MyD88-dependent toll-like
receptors and regulates AP-1 binding activity and the stability of transcripts encoding TNF-α, IL-6 and other innate cytokines (186). However, in the present study, the anti-inflammatory effect of clemastine was MyD88 independent, since clemastine increased the susceptibility of listeriosis also in mice deficient for MyD88. Nonetheless, the impaired phosphorylation of ERK aligns with earlier observations that histamine activates the phosphorylation of protein kinase C (PKC), which again plays a crucial role in the activation of Raf/MEK/ERK and IKK/IκB/NF-κB as well as the expression of cytokine and adhesion molecules (185, 206). Other reports have suggested that H1R stimulation can activate transcription factor NF-κB (187). We therefore analysed the NF-κB inhibitor κB, hypothesising that clemastine might have direct affinity to IκB, thereby preventing its phosphorylation and degradation, and by consequence, the activation of NF-κB. In contrast, we rather observed a degradation of IκB suggesting nuclear translocation and potential activation of inflammatory reactions. At present, we can only speculate on this, but if clemastine is affecting the NF-κB signalling, it rather looks as though it might having a decoy effect by which it either prevents translocation of the NF-κB complex or at least the translation of NF-κB transcription signals. However, since no previous (185, 187, 206) studies included H1R-deficient models, so it would be interesting to repeat those experiments in light of our results. An H1R-independent mechanism of immune suppression might account for the activation of kinases in all cases. Although our observations reveal so far unknown immunosuppressive properties of antihistamines, they also open new therapeutic alleys in inflammatory diseases. For example, sepsis is a serious and complex disease caused by an overly active host response to infection. Sepsis is the leading cause of death in critically ill patients, the mortality rates are as high as 60%, with an estimated 210,000 deaths annually in the United States (207). Based on the assumption that sepsis results from an uncontrolled pro-inflammatory response, a considerable amount of effort has been devoted to the development of anti-inflammatory therapies. Despite research effort, there has been limited success in clinical trials of anti-inflammatory therapies with corticosteroids, or TNF-α and IL-1α blocking agents (208). The fact that we were able to inhibit septicaemia in mice by treatment with clemastine may open up new therapeutic perspectives for this and similar drugs, perhaps as a complement to other anti-inflammatory treatment options. Collectively, our observations rise concerns regarding the safety of some antihistamines as
these increased the susceptibility of mice to *Listeria* infections inhibited the potential of human monocytes to produced antibacterial cytokines in response to *Listeria* or LPS. However, the data also suggest that some antihistamines may modulate innate immunity resulting in down-regulation of pro-inflammatory immune responses. It is therefore intriguing to consider the use of clemastine or desloratadine to modulate inflammatory diseases, such as sepsis, but also rheumatoid arthritis, artherosclerosis, and inflammatory bowel diseases.

### 2.6.5. Materials and Methods

#### 2.6.5.1 Mice and reagents

Female C57BL/6 and BALB/c mice were purchased from Harlan (Horst, the Netherlands) and used age-matched at 6-12 weeks of age. MyD88-deficient mice (B6.129P2[SJL]-Myd88tm1Defr/J) were purchased from Jackson Laboratories (ME, USA). TNF-α-deficient mice were kindly provided by Adriano Aguzzi and Tobias Suter (University of Zurich), while H1R-deficient mice were kindly provided by Felix Tanner (University of Zurich). All transgenic mice were on a C57BL/6 background. The antihistamines clemastine (Tavegyl®, Novartis), desloratadine (Aerius®, Essex Chemie), dimetindene (Feniallerg®, Novartis), and cetirizine (Cetirizin-Teva®, Teva) and the immunosuppressants Azathioprine (Imurek®, GlaxoSmithKline) and dexamethasone (Dexamethason-Helvapharm®, Helvapharm) were purchased from the hospital pharmacy of Zurich (Kantonsapotheke). Aqueous histamine was in the form of a skin-prick test solution from ALK-Abelló (Trimedal Ltd., Switzerland). Lipopolysaccharide (LPS) from *E. coli* clones 026:B6 and 0111:B4 was from Sigma Aldrich (Switzerland). Antibodies for immunoblotting were purchased from Cell Signaling Technology (Germany). All procedures on animals were approved by the local review board and the veterinary authorities in the canton of Zurich. The experiments were done and the animals kept according to guidelines formulated by the Swiss Federal Veterinary Office.
2.6.5.2 *Listeria* infection and determination of colony forming units

*Listeria monocytogenes* was grown in tryptic soy broth and a log–phase culture was used for intraperitoneal injections in C57BL/6 mice. If not otherwise specified, the infection dose was $10^5$ colony-forming units (CFU). Typically, liver and spleen were collected after three days for bacterial counts. For this, organs were homogenised, cells lysed in 0.5% saponin, and serial lysate dilutions in PBS plated on supplemented tryptic soy agar for 24 hours. The CFU results are illustrated as geometric means (± s.e.m.). Alternatively, infected mice were euthanized after 4 hours, peritoneal cells lavaged in 4 ml cold PBS/FCS, and cardiac blood collected. Cytokines were assessed directly in serum or from an overnight culture of $2 \times 10^6$ peritoneal cells. Survival was measured by monitoring body weight once per day. The endpoint was defined as a 15% reduction in body weight as compared to pre-infections weights and the mice were then euthanized. Changes in body-weight was also illustrated as relative change comparing the pre-infection body weights with those measured three days post-infection.

2.6.5.3 Immunohistology

Freshly removed organs of *Listeria*-infected mice were immersed in O.C.T.TM (Tissue-Tek, Japan) and snap-frozen in liquid nitrogen. Tissue cryosections of 5 µm were stained with hematoxylin and eosin (H/E) or with antibodies against *L. monocytogenes* (179), neutrophils (Gr-1), macrophages and monocytes (F4/80 or CD68), metallophilic macrophages (MOMO-1), marginal zone macrophages (ERTR-9), dendritic cells (CD11c), follicular dendritic cells (FDC), T lymphocytes (CD3), B lymphocytes (B220). Antibody-stained sections were visualised using alkaline-phosphatase.

2.6.5.4 Analysis of in vitro cytokine secretion and expression by ELISA

C57BL/6 mice were euthanized and peritoneal cells collected by lavage. The cells were washed, re-suspended in supplemented IMDM, and plated in 48-well plates with various concentrations of
clemastine or histamine (0.1, 1 and 10 µg/ml). After 90 minutes incubation, the wells were gently washed to remove non-adherent cells. The adherent cells were added fresh medium with 1.0 µg/ml LPS clone 026:B6 ± clemastine or histamine. After 60 min, the supernatants were collected and frozen for later analysis of cytokines using Quantikine® ELISA kits from R&D Systems (Abingdon, United Kingdom). Alternatively, macrophages were pre-treated with clemastine as described above and then infected with 10³ CFU *L. monocytogenes* for two hours in the presence of clemastine. The supernatants were collected and frozen for later analysis of cytokine, and results expressed as means ± s.e.m.

### 2.6.5.5 Determination of cytokine secretion in human monocytes

Human PBMCs were prepared from buffy coat by ficoll centrifugation. Quadruples of 500’000 cells were cultured in supplemented RPMI 1640 medium in 24-well plates for 90 min in the presence of clemastine or not. Non-adherent cells were removed, and adherent cells were incubated with clemastine as well as 1 µg/ml LPS clone 026:B6 for another 60 minutes or 4 hours. The supernatants were collected and analysed for cytokines.

### 2.6.5.6 Analysis of cytokine expression by Real-Time PCR

C57BL/6 mice were pre-treated with PBS or 100 g clemastine one hour prior to infection with 10⁵ CFU *L. monocytogenes*. After 3 h, mice were euthanized and peritoneal cells were collected by lavage. Cells were washed and macrophages were isolated using CD11b MACS sorting. mRNA was extracted by the Trizol® method (Invitrogen) and transcribed to cDNA using the Promega Reverse Transcription System. The cDNA was quantified for cytokine expression by real-time PCR analysis using designed primers (Primer 3) from Microsynth (Switzerland) and a LightCycler from Roche Diagnostics (Switzerland). S18 was analysed in parallel in every PCR and used as the standard. Expression levels were calculated and displayed as fold change.
2.6.5.7 Immunoblots

Human PBMCs were prepared from buffy coat by ficoll centrifugation and cultured in supplemented RPMI 1640 medium in 6-well plates for 90 min. Non-adherent cells were removed and adherent cells were pre-treated with 10 µg/ml clemastine for 1 h, followed by incubation with 1 µg/ml LPS for 10 or 30 min. Cells were lysed in NETN buffer (20mM Tris–HCl pH 7.5, 150mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40 40mM NaF, 1mM Na3VO4, Complete Mini (Roche) protease inhibitor). Equal amounts of protein from lysates were immunoblotted (209).

2.6.5.8 LPS-induced sepsis

BALB/c mice received 300 µg LPS clone 0111:B4 by i.p. injection. Selected groups of mice also received 100 µg clemastine or 150 µg dexamethasone by i.p. injection one hour before LPS injection and again once daily. Survival was monitored every 6 to 12 h.

2.6.5.8 Clinical trial

Healthy volunteers (n=10) were treated intravenously with 2 mg clemastine or placebo (118) in a double-blind placebo controlled trial; 2 mg clemastine is the recommended human dose by anaphylactic reactions, angiooedema, or prophylactically against allergic or pseudo-allergic reactions, e.g. to contrast agents and blood transfusions. Venous blood was collected before and 60 min after treatment. PBMCs were purified after centrifugation on ficoll, and 5×10^5 cells cultured for 90 minutes in 24-well plates. Non-adherent cells were removed and adherent monocytes and macrophages were cultured for another 60 minutes with 1 µg/ml LPS. The supernatants were analysed for TNF-α and IL-6 by ELISA. The results are shown as post-treatment cytokine secretion relative to pre-treatment values for each individual study subject, and differences are analysed by the Mann-Whitney U test. The study was conducted with approval of the ethical review board at the University Hospital Zurich and by the federal Swissmedic. All patients gave written consent.
2.6.5.9 Statistics

Independent and non-parametric distributed data were analysed using two- sided Mann Whitney U tests or two-sided Kruskal-Wallis H tests with Dunn’s post test. P-values indicated in histograms and box plots illustrate the difference between antihistamine-treated groups and the untreated control group. Kaplan Meyer survival curves were compared with the use of Cox proportional-hazards models and statistical significance tested by Mantel Cox log rank test and Gehan-Breslow-Wilcoxon test. The significance level was set at 5%.

2.6.6 Acknowledgements

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2.6.7 Contribution

For this project I isolated monocytes from buffy coats and further titrated Clemastine on LPS treated human monocytes for the analysis of pro-inflammatory TNF-α and IL-6.
Chapter 3

Discussion
Our innate immune system has evolved to sense foreign and danger. Within this innate immune system DCs play a central role because they provide the major link between innate and adaptive immunity. The immune response must also be tightly regulated, since pathogens call for a rapid and strong immune response, but tissue damage mediated by inflammatory cells and T-cells, on the other hand must be avoided. The immune system plays an important role in many, if not nearly all, diseases that today represent the main challenge to medicine and humanity. Today it has become clear that disease, which were previously known as “metabolic” or “degenerative”, such as diabetes or atherosclerosis, actually are caused by chronic inflammatory processes in the insulin producing islets of the pancreas or within the vessel walls, respectively. Also, cancer progression, characterized by neoangiogenesis and breakdown of surrounding tissue matrix, is mediated by the same immune mechanisms that normally, for example repair wounds (210). Therapeutic manipulation of the immune system to treat the above named diseases and many others, therefore, is a difficult balancing act. The more we understand about the pathogenic and healthy immune response, the better we get a chance to treat immune mediated disease, without compromising the protective function of the immune system. Understanding these mechanisms might provide the scientific basis for the development of new treatment therapies and vaccines.

3.1. Regulation of IL-1α secretion vs. surface expression

There are eleven pro- and anti-inflammatory members form the IL-1 family of cytokines, which are important regulators of immunity (13) and are often involved in inflammatory diseases (15). Most members have been discovered only recently, whereas IL-1α and IL-1β were the first two cytokines described. Both are highly pro-inflammatory and are produced as intracellular precursors (36). After appropriate stimulation only small amounts of these intracellular precursors are getting actively secreted in mature forms, but most stays within the cell (211). The secretion mechanisms underlay a tight and complex regulation. In case of IL-1β the regulation of secretion was unravelled soon after its discovery. Intracellular pro-IL-1β is expressed after activation of the transcription factor NFκB that
lies downstream of the TLR pathway. This TLR-dependent NFκB activation is also called signal 1. To achieve biological activity, pro-IL-1β requires further maturation. The proteolytical cleavage depends on a second type of stimulation, named signal 2, that activates a second type of PRR, the inflammasomes. There are different types of inflammasomes sensing different PAMPs and DAMPs all leading to activation of caspase-1 (10). Caspase-1 then cleaves pro-IL-1β into its active mature form and ensures the secretion of mature IL-1β by providing unconventional secretion (33, 36). For IL-1α the situation is a bit more complex as it exists not only as a secreted form, but also as a membrane-associated form on the cell surface. The signal 1 leading to intracellular pro-IL-1α expression is shared with the IL-1β pathway. The cleavage, however, is not mediated by caspase-1, but by calpains, a large group of calcium-dependent neutral proteases, which are located closely to the inner cell membrane (39, 74). However, IL-1α secretion is not caspase-1 independent. Already in 1995 Kuida et al. described a decrease of IL-1α secretion in caspase-1-deficient mice (34). Later in 2008 this was confirmed and explained by Keller et al. who suggested that caspase-1 was a regulator of unconventional protein secretion facilitating protein secretion independent of the ER/Golgi pathway and therefore regulating secretion of proteins that lack the classical secretion sequence (31). Caspase-1-dependent secretion implies that inflammasome activation is necessary to activate the caspase-1. Hence, IL-1α secretion additionally requires the same second signal 2 as described above for IL-1β.

We were able to show that not only inflammasome-dependent activation of caspase-1 is required for an efficient secretion of IL-1α, but also the presence of IL-1β. More specifically, IL-1β binds IL-1α and thus seems to providing a shuttle through the cell (Chapter 2.1). Our model for IL-1α secretion suggests that pro-IL-1β binds to pro-IL-1α. We speculate that IL-1β as a substrate for caspase-1 will then target the IL-1α to caspase-1. Caspase-1 cleaves pro-IL-1β into mature IL-1β and the whole complex consisting of pro-IL-1β, mature IL-1β, active caspase-1 and pro-IL-1α translocates through the cell. Shortly before passing the membrane, calpains can cleave the pro-IL-1α into the mature form so that all three mature IL-1α, -β, and caspase-1 are secreted by the cell (Fig. 3.1). It is likely that between both pathways, the IL-1α secretory and IL-1α surface expression pathway, there is crosstalk. If one of the pathways is blocked IL-1α is more likely to take the other pathway, and vice versa.
Indeed, in cells stimulated with both signals, we found that additional treatment with a calpain inhibitor decreased IL-1\(\alpha\) secretion but also increased IL-1\(\alpha\) surface expression. To our surprise, IL-1\(\beta\) secretion was also strongly decreased. So far nothing is known about the relationship of calpains and IL-1\(\beta\), which therefore requires further investigation.

**Figure 3.1 | Our IL-1\(\alpha\) surface expression and secretion model.** Pro-IL-1\(\alpha\) and pro-IL-1\(\beta\) are intracellularly expressed upon TLR/NF\(\kappa\)B stimulation. For IL-1\(\alpha\) there are two distinct pathways within the cell, either it is translocated to the cell surface or it will enter the secretion pathway via IL-1\(\beta\) and caspase-1. In order to activate the secretion pathway cells have to receive a second stimulus activating the inflammasome and subsequently caspase-1. Pro-IL-1\(\beta\) acts as a shuttle for pro-IL-1\(\alpha\) to be directed to caspase-1, where IL-1\(\beta\) will be cleaved into its mature form. Pro-IL-1\(\alpha\), IL-1\(\beta\) and active caspase-1 will translocate to the cell membrane where calpains will cleave pro-IL-1\(\alpha\) into mature IL-1\(\alpha\) and finally mature IL-1\(\alpha\), -\(\beta\), and active caspase-1 will be secreted by the cell.

In summary, both IL-1\(\alpha\) and IL-1\(\beta\) require signal 1 for their secretion resulting in NF\(\kappa\)B-dependent production of pro-forms. Also, both IL-1\(\alpha\) and IL-1\(\beta\) require signal 2, which activates caspase-1 via the inflammasomes. The difference between IL-1\(\alpha\) and IL-1\(\beta\) is that IL-1\(\beta\) is directly cleaved by caspase-1, whereas IL-1\(\alpha\) requires an additional mechanism for cleavage, most likely the calpains. Furthermore, without IL-1\(\beta\) providing a shuttle for IL-1\(\alpha\) to target caspase-1, which mediates unconventional protein secretion, there is no secretion of IL-1\(\alpha\). In fact, bmDCs derived from IL-1\(\beta\) deficient mice could not secrete any detectable mature IL-1\(\alpha\) after combined stimulation of TLR4 and
NALP3, while production of the pro-forms of IL-1α was similar in IL-1β−/− compared to +/- cells (Chapter 2.1).

However, with time IL-1β−/− cells with time stimulation with signal 2 leads to the release of pro-IL-1α and pro-IL-1β in the supernatant. We interpret this such that prolonged inflammasome-dependent activation of caspase-1 by ATP can be toxic for cells. Caspase-1 is a so-called inflammatory caspase and not an apoptotic caspase, although it is believed that there is a strong correlation between inflammation and death. Too much inflammation causes cell death (212) and maybe this is not an on-off state but rather a flowing transition.

As mentioned above the IL-1β deficient bmDCs did not secrete mature IL-1α, but released significant amounts of pro-IL-1α into the supernatant (Chapter 2.1). Thus, it is important to distinguish “true” secretion describing an active and regulated process of shuttling intracellularly matured cytokine towards the extracellular space from “release” of the cytokine, an unspecific process occurring due to cell rupture or pyroptosis-associated cell death (212). Also, in the case of “release” of the cytokine pro-forms can be extracellularly matured by extracellular proteases, i.e. cathepsin G, elastase, proteinase-3 and chymase (82). The cleavage sites are not exactly involving the same amino acids, though very similar proteins activities are achieved compared to caspase-1-, or calpain-cleavage, respectively.

Interestingly, we found that the cell surface-associated form of IL-1α was independent of inflammasome-mediated caspase-1 activation (Chapter 2.1). Only signal 1 provided by NFκB activation is required. Anchoring to the cell membrane occurs most probably via glycosylated sites on IL-1α which binds to cell surface lectins. The biological role of the membrane form of IL-1α is most likely different from the role of the secreted IL-1α. In certain infections such as Legionella pneumophila the secreted IL-1α could mainly contribute the immune response (Chapter 2.1), whereas the membrane-bound form might be more prominent in diseases like atherosclerosis with IL-1α expression on the endothelial cell walls. Indeed, a differential role of surface-bound and secreted IL-1α in different inflammatory situations has been recently suggested. Surface-bound but not
secreted IL-1α triggers senescence-associated secretion of IL-6/IL-8 (71). Furthermore, in arthritis, only surface-bound IL-1α caused inflammatory destruction of cartilages (72, 73).

In summary, we found two distinct pathways for surface expression and secretion, concluding that secreted IL-1α is not generated by cleaving-off the surface form. Thus, the presented data on the regulation of cell surface and secreted IL-1α may offer the possibility to selectively modulate the expression of the two forms of IL-1α. IL-1 is deregulated in various important diseases, i.e. atherosclerosis, rheumatoid arthritis, diabetes or Alzheimer’s disease (210). Thus, our data might have a future impact on treatment modalities of these diseases. In order to develop new effective therapies that are highly specific in order to create less side-effects it is important to better understand the mechanisms by which the cytokines are expressed, surface-associated, secreted, or released. Furthermore specific blocking possibilities of certain expression forms might contribute to the better understanding of some diseases. Further research must therefore characterize the relative importance of these two IL-1α forms in antimicrobial protection and in disease. Interesting parallels may be drawn to TNFα. Also TNFα exists in a cell surface associated and in a secreted form that are differentially regulated (213). Therapeutic monoclonal antibodies directed shown to form stable complexes with the transmembrane form (214), such as infliximab (Remicade©), show superior clinical potency than other TNFα inhibitors, such as etanercept (Enbrel®). Crohn’s disease, for example, responds only to infliximab treatment, but not to etanercept (214). By analogy, also in IL-1α, it may be important to consider which of the IL-1α forms, surface of secreted should be blocked, and this may depend on the disease. Thus, future research should further investigate the relative importance of surface IL-1α vs. its secreted form in various disease models.

Most studies on IL-1 focus on myeloid cells such as monocytes, MΦ, and bmDCs, because these cells are the main source of IL-1. However, there are other cell types that produce IL-1α or IL-1β, i.e. B-cells, epithelial cells, endothelial cells, platelets and keratinocytes. Keratinocytes or the skin and platelets are especially interesting cell types with respect to IL-1. Keratinocytes constitutively produce IL-1α (31), which likely represents a survival factor for them. The IL-1α in keratinocytes, the outermost cells of the body, is thought to initiate an immune response in case of the skin injury. Then
the already pre-produced IL-1α can be released very fast by the cells, functioning as an alarmin to initiate a rapid pro-inflammatory response. Platelets are also cells with constitutive IL-1α production (211) and, most of the IL-1α is surface-bound (17). These cells might be interesting to study effects of surface-bound IL-1α.

3.2 Summaries of side projects

3.2.1 Different vaccination strategies for different needs and therapies

Therapeutic vaccinations are under investigation for patients who suffer from allergy, atopic-asthma, cancer, patients with chronic infections and neurological disorders such as Alzheimer’s disease. These strategies require that the antigen is well characterized and that vaccination against this antigen will not damage healthy tissues. When vaccinating against self antigens, self-tolerance is a major obstacle that must be overcome by using an appropriate vaccination platform. VLPs or the liposomes described in this thesis represent such platforms which can induce B cell responses in the absence of T cell help, i.e. also in cases of central or peripheral T cell tolerance. In this thesis we analysed and evaluated an active anti-cytokine vaccination strategy targeting IL-1α by using VLPs for treating tumours, as well as a modified BCG strain for vaccination against tuberculosis, and also a T-cell independent vaccine against amyloid-β for treating Alzheimer’s disease using a liposomal vaccine platform containing MPLA.

Depletion of IL-1α to arrest tumour progression

Tumour vaccinations can either target tumour-specific antigens exclusively expressed by the tumour or tumour-associated antigens, which are also expressed by healthy cells but rather pre-dominantly expressed at tumour sites (215). Tumour vaccination targeting a self-protein like IL-1α requires a strong adjuvant like VLPs in order to break tolerance towards self. However, vaccinating mice using
IL-1α coated VLPs were able to induce high and strongly neutralizing anti-IL-1α IgG titers. In vaccinated mice tumour growth was reduced significantly when compared to control mice. This effect was independent on whether or not the tumour cells expressed IL-1α themselves. This observation may suggest a broad applicability of the anti-IL-1α depletion strategy in cancer. This suggests an important role for infiltrating immune cells during cancer progression.

**Vaccination against a bacterial pathogen: modified Bacillus Calmette-Guérin (BCG) with enhanced antigen presentation capacity**

*Mycobacterium tuberculosis* is one of the pathogens with the highest death toll, killing millions of individuals worldwide every year. The current vaccine strain BCG has limited efficacy and does not protect from infection (42). In order to enhance immunogenicity of BCG we used a modified BCG strain. This strain carried a deletion of zmp, a protein inhibiting phagosome - lysosome fusion. Phagosomes are equipped with the machinery to generate peptide - MHC II complexes in order to present *Mycobacterium* antigens to CD4+ T-cells. We were able to show an increased immunogenicity of our modified BCG strain by enhanced CD4 T-cell responses. However, it yet has to be determined whether or not this BCG zmp mutant strain can mediate protection against infection with Mtb.

**T-cell independent vaccination using a liposomal vaccine targeting Alzheimer’s disease**

For some diseases like Alzheimer’s it is important not to induce T-cell responses during vaccination because activated T-cells may cause immunopathology in the brain. Therefore vaccines targeting amyloid-β must be TI vaccines. The problem is, however, that TI vaccines usually generate merely short-lived IgM responses with low affinity and without memory induction (148). Interestingly, our analysis of a liposome based Alzheimer’s vaccine revealed that this vaccine induced not only IgM, but also long lived IgG response independent of T cell help. The liposome contained the TLR4 agonist MPLA and a densely packed palmytoylated antigen. Our analysis revealed that TLR4 stimulation was key for the TI of the vaccine, activating B cells and signalling via TLR4 adapter protein TRIF. This is the first demonstration of a non-replicating TI vaccine which can induce IgG in compete absence of T
cell help. Therefore, this vaccine platform might not only be a promising candidate to test Alzheimer’s
disease, but also to vaccinate immunocompromised patients in general.

3.2.2 Side effects of a common allergy and asthma medication

Clemastine causes immune suppression through inhibition of ERK

Antihistamines widely used drugs against allergy. Although these drugs are considered safe and are
available over the counter, there exist a small number of reports on side effects impairing innate and
adaptive immunity (48-52). In our study we found an immunosuppressive side effect of clemastine a
H1R antagonist. Clemastine enhanced susceptibility to Listeria monocytogenes with similar strength
as dexamethasome. We found that clemastine inhibited the MAPK-ERK signalling pathway leading to
reduced levels of TNFα and IL-6. Hence, the wide spread use of anti-histamines should be revisited
from a safety perspective. On the other hand antihistamines such as clemastine may also be
therapeutically exploited to treat TNFα and IL-6 mediated diseases.
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2002-2003 work and travel (New Zealand)

List of publications

A novel vaccine stimulates T-cell independent IgG responses enabled through TLR4- and TRIF-dependent stimulation of B cells
Pål Johansen, Maria Pihlgren, Alberto B. Silva, Rime Madani, Valérie Giriens, Ying Waeckerle-Men, Antonia Fettelschoss, David T. Hickman, Maria Pilar López-Deber, Dorin Mlaki Ndao, Valérie Gafner, Nathalie Chuard, Pedro Reis, Andrea Pfeifer, Thomas M. Kündig & Andreas Muhs
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Scientific presentations

2011  Oral presentation at the 1\textsuperscript{st} The IL-1 Family of Cytokines Conference, 8-11.5.2011, Clearwater Beach, Florida, USA  
Oral presentation at the 23\textsuperscript{rd} Wolfsberg Immunology Ph.D Student Retreat, 30.3.- 1.4.2011, Ernatingen, Switzerland

2010  Poster presentation at the World Immune Regulation Meeting IV 29.3.-1.4.3.2010, Davos, Switzerland

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Poster presentation at the 2\textsuperscript{nd} MIM Students Retreat, Ph.D programm Zürich, 6.-8.9.2009, Parpan, Switzerland  
Poster presentation at the SGAI, 19.-20.2009, Geneva, Switzerland  
Poster presentation at the 21\textsuperscript{st} Wolfsberg Immunology Ph.D Student Retreat, 30.3.- 1.4.2011, Ernatingen, Switzerland  
Poster presentation at the 41\textsuperscript{st} USGEB, 29.-30.1.2009, Interlaken, Switzerland

2008  Poster presentation at SGAI, 17.-18.4.2008 Fribourg, Switzerland