universität wien

DIPLOMARBEIT

Titel der Diplomarbeit:

Evaluating IC31®-induced cellular and mucosal humoral immune protective mechanisms against intracellular bacterial infections using a well established *Chlamydia pneumoniae* model

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angestrebter akademischer Grad:

Magister rerum naturalium

Wien, 2010

Studienkennzahl lt. Studienblatt: a0106023
Studienrichtung lt. Studienblatt: Mikrobiologie/Genetik (A441)
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Abbreviations

aa	Amino acid
ab	Antibody
ag	Antigen
AF	Alexa Fluor
AIP	Antigen identification program
APC	Antigen presenting cell, Allophycocyanin
Arg	Arginine
ATP	Adenosine triphosphate
BALT	bronchus-associated lymphoid tissue
BSA	Bovine serum albumin
CCD	Charge-coupled device
CD	Cluster of differntiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
ConA	Concavanalin A
CopN	*Chlamydia* outer protein N
Cpn	*Chlamydia pneumoniae*
CT	Cholera toxin
Ct	*Chlamydia trachomatis*
CTL	Cytotoxic T lymphocyte
CV	Column volume
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMSO	Dimethyl sulfoxid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbent assays
ELIspot	Enzyme-linked immunospot assay
FACS	Fluorescence activated cell sorting
FCM	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GTP	Guanidine triphosphate
GuaHCl	Guanidine hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
i.d.	Intra dermal
IFA	Incomplete Freud’s adjuvant
IFN	Interferon
IFU	Inclusion forming unit
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
i.n.	Intra nasal
i.p.	Intra peritoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K6	Kajaani 6
LB	Luria broth/Luria-Bertani broth
LPS	Lipopolysaccharide
MAb/mab	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MOMP	Major outer membrane protein
NALT	Nasopharynx-associated lymphoid tissue
o/n	Over night
ORF	Open reading frame
pAPC	Professional antigen presenting cell
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
PE	Phycoerythrin
PRR	Pattern recognition receptor

RB	Reticulate body
RCAS	Rat ConA supernatant
RPHPLC	Reversed phase HPLC
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
s.c.	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEB	*Staphylococcus aureus* enterotoxin B
SEC	Size exclusion chromatography
SFC	Spot forming cell
SPG	Sucrose-phosphate-glutamic acid
SSC	Side scatter
TARP	Translocated actin-recruting phosphoprotein
TCM	T cell medium
TGF	Transforming growth factor
T_H/Th	T helper
TLR	Toll-like receptor
TOF	Time-of-flight mass spectrometer
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TTSS	Type III secretion system

1 Abstract

Chlamydia pneumoniae (*Cpn*) is a generally widely distributed respiratory human pathogen causing infections in the great majority of individuals once in a lifetime. Intensive and long lasting use of antibiotics is efficient in treating *Cpn* acute and persistent infections. Though, the majority of infections are asymptomatic or subclinical and especially persistent infection has been associated with a variety of chronic respiratory tract inflammatory diseases and cardiovascular diseases. Furthermore the development of antibiotic resistances has been observed *in vitro* which underlines the importance and advantages of a future prophylactic and therapeutic vaccine against acute and chronic *Cpn* infections.

Given the essential role of T_H1-type immune responses, primarily $CD8^+$ T cells and $CD4^+$ T cells but also humoral responses, in immunity to *Cpn*, potent induction of respective immune effector mechanisms is a requirement for successful vaccine design. The two-component adjuvant IC31® has been shown to efficiently induce $CD8^+$ cytotoxic T lymphocyte (CTL) and $CD4^+$ T helper (Th) responses to T cell antigens following their systemic administration. Additionally the efficacy of mucosal adjuvanticity following intra nasal immunization was demonstrated for IC31® using the protective *Cpn* antigen CopN.

Further promising *Cpn* candidate antigens have been identified through the Antigen Identification Program (AIP®) by antibodies from infected/ exposed humans. During this study IC31®-induced cellular and humoral immune responses to 6 AIP®-discovered conserved recombinant *Cpn* antigens were evaluated in immunization and protection studies. Furthermore, the relevance of these antigens in *Cpn* infection in terms of cellular and humoral immunogenicity was assessed in infection primed immune response studies using BALB/c and C57BL/6 mice. Moreover, by applying immuno-fluorescence microscopy the 6 candidate antigens could be located using sera from immunized animals.

This study demonstrated the efficacy of IC31® in the induction of cellular and humoral responses in mucosal as well as systemic applications, supporting previous findings of a dominant T_H1-type in a mixed T_H1/T_H2-type induction of immune responses. In addition two out of the 6 antigen candidates, namely CP0177-1 and CP0020-1, were found to substantially reduce the bacterial burden in *Cpn* challenged mice following intra nasal immunization in combination with IC31®. Moreover immunogenic epitopes of these two

antigens were identified by IFN-γ ELIspot and CFSE-$CD3^+$-$CD4^+$ flow cytometry in protein immunized and infection primed BALB/c and C57BL/6 mice using peptide libraries.

All together the results demonstrate the high potential of IC31® as a mucosal adjuvant and its application in nasal vaccines, as well as the efficiency of the AIP® technology for the identification of novel subunit vaccine candidates.

2 Zusammenfassung

Chlamydia pneumoniae ist ein generell weit verbreitetes, bakterielles, humanes Pathogen der Atemwege und verursacht ein- oder mehrmals pro Lebenszeit Infektionen bei der Mehrheit aller Menschen. Behandlungen von akuten und persistierenden *Cpn* Infektionen durch intensive und lang anhaltende Verabreichung von Antibiotika haben sich als effektiv erwiesen. Die meisten Infektionen verlaufen jedoch asymtomatisch oder in Form subklinischer Erkrankungen. Besonders persistierende *Cpn* Infektionen wurden in Zusammenhang mit chronischen inflammatorischen Erkrankungen der Atemwege sowie kardiovaskulären Erkrankrungen gebracht. Zusätzlich haben *in vitro* Studien gezeigt, dass Chlamydien Antibiotikaresistenzen entwickeln können. Gemeinsam unterstützen diese Tatsachen die Wichtigkeit und Vorteilhaftigkeit eines prophylaktischen und therapeutischen Impfstoffes, welcher sowohl vor akuten als auch vor chronischen *Cpn* Erkrankungen schützt.

Die bewiesene zentrale Rolle von $CD8^+$ T Zellen, aber auch $CD4^+$ T Zellen und der humoralen Immunantwort vom Typ T_H1, verlangt eine leistungsfähige Stimulation der entsprechenden Effektormechanismen um bei der Entwicklung eines Impfstoffes gegen *Cpn* erfolgreich zu sein. Für das aus zwei Komponenten bestehende Adjuvans IC31® wurde bereits gezeigt, dass dieses ein wirkungsvoller Stimulator von $CD8^+$ cytotoxischen T Lymphocyten (CTL) und $CD4^+$ T Helfer (Th) Immunantworten in Folge systemischer Verabreichung von T-Zell Antigenen ist. Des Weiteren wurde für IC31® die Effizienz des adjuvantiven Effekts in der mukosalen Anwendung, unter Verwendung des protektiven *Cpn* Antigens CopN, bewiesen.

Weitere vielversprechende Protein Antigene wurden im Verlauf des AIP® (Antigen-Identifizierungs-Programm) unter Verwendung von humanen Seren, welche von infizierten oder von Krankheit regenerierten Patienten stammen, identifiziert. Während dieser Studie wurden die durch IC31® induzierten zellulären sowie humoralen Immunantworten gegen sechs im

Prozess des AIP® identifizierten, konservierten, rekombinanten *Cpn* Antigenen in Immunisierungsstudien und Protektionsstudien evaluiert. Zusätzlich wurde die Relevanz dieser Antigene im Verlauf einer *Cpn* Infektion, hinsichtlich zellulärer und humoraler Immunogenität, in durch Infektion angeregten Immunantworten von BALB/c und C57BL/6 Mäusen, untersucht. Außerdem konnte durch die Anwendung von Immunofluoreszenzmikroskopie und der Verwendung von aus immunisierten Mäusen stammender Seren die Lokalisation der besagten Antigene/Proteine bestimmt werden.

Diese Studie hat gezeigt, dass das Adjuvans IC31® eine überaus wirkvolle Induktion von antigenspezifischen zellulären und humoralen Immunantworten bewirkt und dies sowohl bei mukosaler als auch systemischer Verabreichung. Des Weiteren unterstützen die Ergebnisse die dominante Induktion einer Immunantwort vom Typ T_H1 in einer gemischten T_H1/T_H2 Immunantwort durch IC31®. Zusätzlich konnten aus den 6 Kandidaten 2 identifiziert werden welche einen substanziellen, reduzierenden Effekt auf die Anzahl an Bakterien in den Lungen von infizierten Mäusen aufwiesen, wenn diese zuvor in Kombination mit dem Adjuvant IC31® intra nasal verabreicht wurden. Diese Kandidaten sind CP0177-1 und CP0020-1.

Des Weitern konnten immunogene Epitope dieser beiden Proteine in immunisierten und infizierten BALB/c und C57BL/6 Mäusen durch IFN-γ ELIspot und CFSE-$CD3^+$-$CD4^+$ durchflusscytometrische Analysen identifiziert werden.

Zusammengefasst zeigen die Ergebnisse einerseits das große Potential des Adjuvanten IC31® in mukosalen Anwendungen oder als Bestandteil nasaler Impfstoffe und andererseits die hohe Effizienz des AIP® beim Auffinden neuartiger protektiver Impfstoffkandidaten.

3 Introduction

3.1 *Chlamydia pneumoniae*

Chlamydia pneumoniae (*Cpn*) is an obligate intracellular eubacterium that belongs to the Gram-negative group of bacteria. *Cpn* is one of the two species (spp.) in the family of the *Chlamydiaceae* that are common pathogens in humans and was first isolated in 1965 from the conjunctiva of a Taiwanese child participating in a trachoma vaccine trial [10]. The other species is *Chlamydia trachomatis*. The species *Cpn* consists of one human biovar (TWAR) and two animal biovars of which one infects horses (biovar equine) and the other infects frogs [1] and koalas (biovar koala [2]). The genome size is rather small with approximately 1.2 Mbp [3].

C. pneumoniae can be morphologically distinguished from other *Chlamydia* by its pear-shaped structure of elementary bodies (EB) and lose outer membrane [4]. Interestingly not all *Cpn* isolates possess pear-shaped elementary bodies as a characteristic feature [5-8]. Accordingly the presence of a large periplasmic space between the cytoplasm and the outer membranes, which is not seen in other chlamydial species, was demonstrated by scanning electron microscopy in *Cpn* elementary bodies [9, 11]. *Chlamydia pneumonia*, as other Gram-negative bacteria, has LPS in its cell membrane. The LPS is present in both elementary bodies and reticulate bodies (RB). Chlamydial LPS is a genus-specific group of antigens and therefore suitable as a marker for chlamydial infection [20].

In distinction from other Gram-negative bacteria *Chlamydia* do not have peptidoglycan in the periplasmic space [22, 23], although the genes for the synthesis are present [3, 21] and chlamydial growth has shown to be sensitive to penicillin [24].

All *Chlamydia* replicate in a biphasic developmental cycle inside eukaryotic host cells. The smaller extracellular elementary body represents the metabolically inactive but infective stage of the developmental cycle. The primary step in chlamydial entry, which occurs minutes after infection, is the attachment of the EB to the host cell. This process of entry has been described to be mediated by a complex network where the proteins chemokine C-X-C motif receptor 7 (CXCR7), integrin beta 2 (ITGB2), platelet-derived growth factor beta polypeptide (PDGFB), vascular endothelial growth factor (VEGF), vascular cell adhesion molecule 1 (VCAM1), and GTP binding protein play a key role [12]. Uptake of the EB by endocytosis is highly dependent on the

reorganization of the actin cytoskeleton. Various different signaling pathways involved in actin dynamics and reorganization have been shown to be targeted by *Chlamydia* by inducing tyrosine phosphorylation of regulatory proteins. Examples are the PI3-kinase (PI3K) and MEK-ERK kinases and the Rho family of GTPases [13-16]. The differentiation of *chlamydial* EBs to reticulate bodies (RB) occurs at 8-12 hours post infection inside a protected vacuole, which is called an inclusion [16].

Cpn actively inhibits phagosome-lysosome fusion [40] and promotes the association with exocytic vesicles avoiding intracellular killing in this way [39]. Additional beneficial modulations for chlamydial survival inside the host are inhibition of apoptosis in macrophages [41] and epithelial cells [42] and down regulation of the expression of the major histocompatibility complex class I and class II molecules [43].

The intracellular form, the reticulate body, is metabolically active, replicates, but is not infectious. Between 12-48 hours post infection the RBs replicate inside the inclusion. RBs are highly dependent on the supplementation of iron, amino acids and energy in form of ATP by the host cell [17, 18]. After several rounds of multiplication, the RBs differentiate back into infectious EBs. An intermediate state which is called the intermediate body (IB) is present during the transition from the chlamydial RB to the EB.

At 72- 92 hours post infection, when the replication of *Chlamydia* is complete, infectious EBs are released. Two different mechanisms by which EBs leave the host cell have been described for *Chlamydia trachomatis*. The first one is lysis of the host cell by rupture of the inclusion at the cell membrane in a sequential process [19]. The second way of exiting is called extrusion. Chlamydial extrusion leaves the host cell intact. Only a portion of the *Chlamydia*-containing vacuole is pinched-off and infectious EBs are released during this process. An intact part of the inclusion remains inside the host cell. Extrusion has been suggested to also play a role in chlamydial persistence [19]. The persistent state of chlamydial infection is thought to be an incomplete developmental cycle whereby *Chlamydia* are capable of causing long-term infection ranging for month or years in the absence of treatment. Low iron and tryptophan concentrations as well as antibiotic treatment are known inducers of persistence [18, 25]. *In vitro* IFN-γ treatment has become a common method to induce persistence through its activation of the host tryptophan-degrading enzyme indoleamine 2, 3-dioxygenase (IDO) which leads to the inability of *Cpn* RBs to differentiate into EBs [18].

A variety of antibiotics, such as tetracyclines, macrolides, rifamycines and erythromycin have been shown to be effective in treating *Cpn* infection [11, 26]. Though, antibiotic treatment is recommended to be intensive and long

lasting (3-4 weeks) in order to avoid reinfection by persistent bacteria which is even more difficult to treat effectively [26]. It has been seen that *Chlamydia* can develop antibiotic resistance *in vitro.* Therefore antibiotic resistance might become a problem in future treatment [33-36].

Chlamydia pneumoniae as a disease is generally widely distributed. Seroepidemiologic studies showed an antibody prevalence of 50% at an age of 20 years and 70-80% at an age of 60-70 years [26]. The human pathogen *Cpn* is transmitted via aerosol droplets and respiratory secretions from person to person. The majorities of *Cpn* infections are asymptomatic or subclinical mild upper respiratory tract infections. *Cpn* is the causative agent of diseases such as pneumonia, acute bronchitis, common cold, persistent cough, pharyngitis, sinusitis and otitis media [27]. In addition, *Cpn* infection has been associated with a variety of chronic respiratory tract inflammatory diseases. Examples are chronic bronchitis and chronic obstructive pulmonary disease (COPD) as well as sarcoidosis [27]. Several studies indicate an association between *Cpn* and asthma [28]. Finally, *C. pneumoniae* has been associated with cardiovascular diseases, especially with atherosclerosis which is based on data from seroprevalence studies and by the direct detection of the organism within artheromatous plaques [29, 30-32].

Studying the mechanisms underlying protection against *Cpn* infection revealed that protective immunity strongly depends on the viability of the T cell compartment. $CD4^+$ T cells and $CD8^+$ T cells have been found at the site of infection in humans and in mice. Both $CD4^+$ T cells and $CD8^+$ T cells contribute to protection mainly through the secretion of the type 1 cytokine IFN-γ. Several studies showed that IFN-γ secreting $CD4^+$ T cells of the T_H1 subset can inhibit *Chlamydia* replication [46]. Furthermore, IFN-γ levels produced by macrophages during *Cpn* infection were efficient in reducing bacterial loads which underlines the importance of this cytokine [48]. Additionally the absence of IFN-γ dramatically increased the bacterial burden in murine challenge studies [47].

Murine depletion models of $CD4^+$ T cells and $CD8^+$ T cells showed a deteriorated course of infection in both cases, but especially $CD8^+$ T cells have been shown to play a critical role in protective immunity to *Cpn* infection [38]. Pathogen specific $CD8^+$ T cells which exert an *ex vivo* cytolytic effector function have been isolated from *Chlamydia trachomatis* (*Ct*) and *Cpn* infection models. Furthermore adoptive transfer experiments of *Ct* specific type 1 cytokine-producing $CD8^+$ cytotoxic T cells were partially protective [38]. Acquired immunity to *Cpn* infection was found to be dominantly dependent on $CD8^+$ T cells in a murine infection model [44, 45]. This findings stand in contrast to data from a *Ct* infection model, where the effect of CD4+ T

cell depletion on the course of disease was more pronounced compared to $CD8^+$ T cell depletion. However, as $CD4^+$ T cells are often required for the induction and maintenance of a functional $CD8^+$ T cell compartment the role of $CD8^+$ T cells should not be underestimated [38]. The importance of the $CD8^+$ T cell population in protection against *Cpn* infection is not surprising given the intracellular nature of this pathogen and the MHC class I presentation of peptides commonly derived from the cytosol to $CD8^+$ T cells. However, MHC class I molecules can also present peptides originating from exogenous soluble or cell-associated proteins, including antigens which are not secreted from intravacuolar and cytosolic pathogens by cross-presentation. MHC class I presentation of antigens to $CD8^+$ T cells can be enhanced by the interaction with B cells across professional antigen-presenting cells (pAPC) such as dendritic cells (DC), by their Fc receptor mediated uptake of antigen bound by IgG2a or IgA. In fact, mice lacking both $CD8^+$ T cells and B cells show a dramatically increased chlamydial burden compared to $CD8^+$ T cell depletion alone. Furthermore the depletion of IgA alone and in combination with the depletion of $CD8^+$ T cells leads to an increased bacterial burden in murine challenge models which underlines the importance of this immunoglobulin in protection against infection by the respiratory pathogen *Chlamydia pneumoniae* [49, 50].

3.2 Antigen Identification Program (AIP®)

The identification of few protective components from the entirety of molecules present in a pathogen as well as the validation of the immune response against any candidate antigen are generally complex and time consuming tasks in the design of a subunit vaccine. The approach combining the advantages of the post-genomic era and serological antigen identification is an effective way to meet these challenges. This process has been first applied to *Staphylococcus aureus* by the use of a small-fragment genomic-surface display library and antibodies from human serum [51].

Intercell's AIP® technology uses human sera from previously exposed and diseased individuals as primary screening selection criteria on a genome-wide basis. The antigen screening is performed against a bacterial surface display library of *E. coli* outer membrane proteins LamB and FhuA [52-54] thereby providing a subset of all genome-encoded proteins of a pathogen that are expressed in vivo and induce antibodies in humans. The validation procedure in general includes PCR studies to confirm the conservation of the encoding gene in various clinical isolates, peptide ELISA assays to prove the

immunogenicity in humans and FCM analyses to show that the antigen is localized on the surface of the pathogen. Bactericidal assays are performed to test the functionality of the antibodies directed against a particular antigen in animal models. This technology has been successfully applied to various human pathogens such as *S. aureus, S. epidermidis, S. pneumoniae, S. agalactiae, S. pyogenes* [52], *Enterococcus faecalis, K. pneumoniae, Borrelia spp., ETEC, Shigella, Campylobacter jejuni, non-typable Haemophilus influenzae, Moraxella catarhalis* and *Chlamydia pneumoniae.*

In the case of *Cpn* the AIP® technology has led to the identification of 84 antigenic proteins out of 1052 open reading frames. From those 34 high priority antigens were selected on the basis of conservation in different clinical isolates. 6 out of those high priority antigens were selected for the evaluation of IC31®-induced immune mechanisms during this study. The criteria by which these antigens were chosen included conservation, quality of recombinant protein expression, solubility and the fact of being uncharacterized hypothetical proteins.

3.3 *Chlamydia* outer protein N (CopN)

CopN is a chlamydial type III secreted protein that is also present in the inclusion membrane and inclusion body as demonstrated by immuno-fluorescence staining using sera obtained from mice immunized with the recombinant protein [55]. As an equivalent to Yersinia YopN, CopN is described to be the lid of the type III secretion system (TTSS) apparatus regulating the gating function of type III secretion [56].

Regarding the function of CopN, heterologous expression of the protein in mammalian and yeast cells induced a cell cycle arrest at the G2/M phase, possibly due to reorganization of the cytoskeleton [57]. Additionally, the essential role of CopN in chlamydial growth was demonstrated by functional knock-out of the protein leading to the inhibition of *Cpn* growth in infected cells. Immunization studies with CopN in mice and hamster showed a protective effect against *Cpn* challenge when administered via the intra nasal or intra peritoneal route [58, 59]. The protective effect was even more pronounced when CopN was delivered in combination with *E.coli* heat-labile toxin [59]. Previous in house experiments further showed a protective effect of CopN when BALB/c mice where immunized intra nasally with recombinant CopN in combination with IC31® as an adjuvant. Resembling a potent *Chlamydia* vaccine candidate recombinant CopN was used as a control in immunological assays and *Cpn* challenge experiments throughout this study.

3.4 *Chlamydia* Major Outer Membrane Protein (MOMP)

The major outer membrane protein of *Chlamydia* is a 40 kDa immunodominant protein located on the surface of chlamydial EBs and RBs. MOMP has been well characterized as a porin and an adhesin. Further, MOMP is used to determine chlamydial genus and species specificity. Sequence analysis of MOMP in different chlamydial species has revealed a structurally similar gene organization containing conserved regions [11]. As MOMP induces neutralizing antibodies and T cell responses [60, 61], it is regarded as one of the most promising vaccine candidates for an anti-chlamydial subunit vaccine. The protective effect after immunization with MOMP was demonstrated in various studies in mice using the *Ct* [62-64] and the *Cpn* [49, 65] model. This results where further confirmed by in house *Cpn* intra nasal challenge studies where recombinant MOMP was intra nasally administered either alone or in combination with cholera toxin (CT) as well as IC31® as an adjuvant. Besides recombinant CopN, MOMP was used as an additional control antigen during this study.

3.5 Adjuvants

The two-component adjuvant IC31® is composed of a synthetic immuno-stimulatory oligodeoxynukleotide containing deoxy-inosine/deoxy-cytosin repeats (ODN1a; 5'ICICICICICICICICICICICIC-3') and the cationic peptide $KLKL_5KLK$ (KLK). The immunostimulatory effects of IC31® originate from the immunopotentiating effects of KLK, stimulating protein-specific type-2 adaptive immune response after prime-booster immunizations [67] and enhancing antigen uptake through dendritic cells by binding and integrating into the membrane, specifically targeting lipid rafts [66]. The specificity of activation is supported by the upregulation of CD40, CD80 (B7.1), CD86 (B7.2) and IL12p40 only in dendritic cells associated with IC31® [68]. Additionally, KLK mediates depot formation of antigen injected in combination with IC31® at the injection site which is of great advantage for sustained antigen and adjuvant release [67].

ODN1a, as second part of IC31® has its adjuvantive effect by signaling via TLR9 and MyD88, thereby activating NF-κB and initiating immune responses. These responses include a specific type 1 dominated mixed type 1 and type 2 and cytotoxic T cell adaptive immune response as well as IgG1 and IgG2 B cell responses. In addition to the enhancement of antigen-specific T

cell responses in mice this adjuvantive effect on T cell responses was also observed in monkeys and humans. Furthermore, a variety of toxicological studies did not show any toxic effect of IC31® [69]. Recent studies using CopN as a model antigen and IC31® as an adjuvant in a murine mucosal model showed a strong antigen and epitope specific local and systemic T cell response in terms of IFN-γ secretion, and enhanced antigen specific IgG titers (Zimmel. D, 2009; unpublished data).

In some experiments chlora toxin (CT) was used as an adjuvant in combination with MOMP. Cholera toxin is a common adjuvant for studying mucosal immunity in murine models due to its strong immunostimulatory effects. However, CT is not applicable to humans because of its toxic properties [70].

3.6 Mucosal Immunity in the Lung and Nasal Vaccination

The mucosal immune system includes the gastrointestinal tract, the upper and lower respiratory tracts, the urogenital tract as well as exocrine glands associated with these organs such as the pancreas, the conjunctivae and lachrymal glands of the eye, the salivary glands and the lactating breast. In total it covers an area approximately 200 times larger than the skin of an adult. The functions of the mucosal immune system involve the protection of the mucous membranes against colonization and invasion by potentially harmful microbes, the prevention of the uptake of unprocessed antigens originating from ingested food, inhaled particles or commensal microorganisms and the prevention of harmful immune reactions to those antigens [71].

Mucosal surfaces are protected from the environment by a thin layer of epithelial cells where most pathogens actually invade. Protection is maintained by the epithelium itself resembling a physical barrier, by secretion of mucins and antimicrobial proteins and a highly specialized innate and adaptive immune system. Besides acting as a physical line of defense, epithelial cells act as sensors recognizing common components of dangerous microbes by pattern-recognition-receptors (PRR) such as Toll-like receptors (TLR). Through the secretion of cytokines and chemokines epithelial cells respond and signal to underlying mucosal cells such as DCs and macrophages in order to initialize nonspecific innate immune defenses and specific adaptive immune responses [71, 72].

Antigen-specific immune responses are initiated at so called microfold cells (M cells) located in the epithelium and above follicles of the mucosa-associated lymphoid tissue (MALT). At this site all cells which are required for

the generation and regulation of an immune response, T cells, B cells and professional antigen-presenting cells (pAPC), can be found. Furthermore, DCs can actively capture antigen and as well as other pAPCs directly present those to T cells present at the inductive site.

IgA is the prevalent type of antibody in the mucosal immune system, and is produced in a ratio of IgA1 to IgA2 of 3:2 almost exclusively as a dimmer by plasma cells expressing the mucosal homing receptor α_4:β_7 and/or α_4:β_1 as well as the chemokine receptors CCR9 and/or CCR10. However, homing of plasmablasts in the airways depends on the expression of CD62L, α_4:β_1, CCR7 and CCR10. Subsequent production and secretion by B cells, IgA is transported from the inner side of the epithelial wall into the lumen by transcytosis through epithelial cells expressing the polymeric immunoglobulin receptor (poly-Ig receptor). In humans, isotype switch of B cells to IgA production and differentiation to IgA secreting plasma cells was demonstrated to be dependent on transforming growth factor (TGF)-β, IL-10 and IL-4 [78].

The functions of secretory IgA at mucosal surfaces include neutralization of pathogens and toxins either on the mucosal surface or inside endosomes and the binding of antigens in the lamina propria enabling export into the lumen [71, 73]. Opsonization and antibody-dependent cell-mediated cytotoxicity (ADCC) by IgA are supposed to be of minor importance compared to the effector mechanisms described above.

In the respiratory tract nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are important inductive sites for the generation of mucosal immunity. The common mucosal immune system (CMIS) connects these inductive sites with mucosal tissues and allows communication between organized mucosa-associated lymphoid tissues, thereby enabling the generation of antigen-specific T_H2-cell-dependent IgA responses, T_H1-cell and cytotoxic T lymphocyte (CTL)-dependent immune responses that also act at distant sites of the induction as well as the induction of a systemic immune response [72]. Regarding the T cell environment present in NALT, mRNA characterization of T_H1 and T_H2 cytokines in murine NALT $CD4^+$ T cells revealed a prevalent T_H0 profile, indicating the capability of these cells for becoming T_H1 and T_H2 cells by antigen exposure of the nasal tract. The commitment for a specific type of T cell response was shown to be dependent on the type of nasally administered antigen and adjuvant [74-77].

In general, nasal vaccination has shown to be an efficient way for the stimulation of the respiratory immune system eliciting both, humoral and cell mediated responses in mucosal and systemic immune compartments [74-77]. Further advantages of nasal immunization include the generation of cross-protective immunity in the reproductive tract as well as in the upper respiratory

tract, the need of a smaller dose of antigen in comparison to oral vaccination, and the redundancy of injection and trained medical personnel.

4 Results

4.1 Antigen purification

Based on results obtained by the Antigen Identification Program® 6 conserved uncharacterized hypothetical proteins were selected for recombinant expression in *E. coli*, and were subsequently isolated by gravity column affinity purification. The selected candidate proteins were: CP0529, CP0177-1, CP0020-1, CP0504, CP0409 and CP0879. For the evaluation of IC31®-induced immunity and immunogenicity these proteins were chosen to build the basis for intra nasal and intra peritoneal immunization studies, intra nasal immunization-challenge studies as well as infection primed immune response studies. In order to ascertain an optimal quality of soluble recombinant protein for *in vivo* and *in vitro* application following parameters were implicated respecting the final solution of recombinant *C. pneumoniae* proteins: LPS ≤ 200 EU/ml, Tween20 ≤ 0.1%, protein concentration ≥ 0.55 mg/ml, L-Arg concentration < 100 mM. The applied procedures for antigen purification are described in section 6.1, materials and methods.

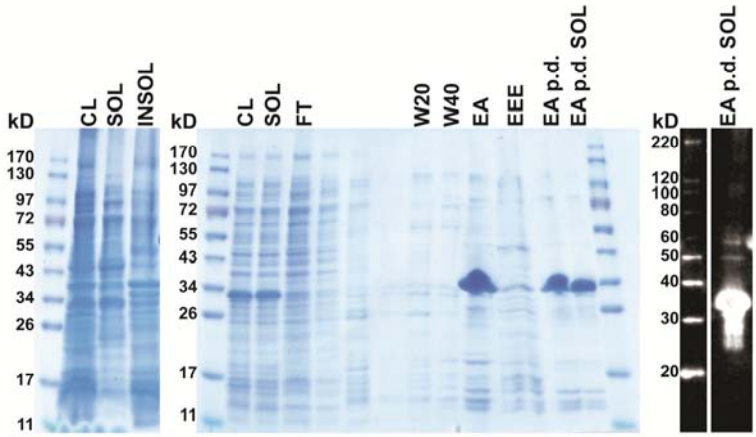

Figure 1: SDS-PAGE, Western Blot; Purification of CP0529: CL (crude lysate), SOL (soluble fraction), INSOL (insoluble fraction), EA (eluate under native conditions), EEE (eluate under denaturating conditions), p.d. (post dialysis). Procedure of protein purification is shown chronologically from left to right.

The procedure for the isolation of CP0529 is shown in figure 1. CP0529 has the size of 231 amino acids (aa) containing 1 cysteine (cys) and a molecular weight of 25.0 kDa. As found by using the protein basic local alignment search tool (BLAST) provided by the National Center of Biotechnology Information (NCBI) the ortholog in *Ct* is the uncharacterized hypothetical protein CT181. CP0529 was purified from the soluble fraction of recombinant *E. coli* lysate. Dialysis of the fraction eluted under native conditions was performed against 50 mM TrisHCl, pH 8.0, 150 mM NaCl. The final preparation of recombinant CP0529 contained 1.51 mg/ml protein and 23.78 EU/mg LPS. Size exclusion chromatography (SEC) revealed that 95.5% of the protein was present as a monomer in the final buffer.

As CP0529, the recombinant protein CP0177-1, consisting of aa 1-380 of CP0177, containing 2 cys and with a molecular weight of 40.8 kDa, also showed to be expressed in the soluble fraction. The solution used for immunizations and *in vitro* stimulation was prepared in 50 mM TrisHCl, pH 8.0, 150 mM NaCl and contained 1.68 mg/ml protein and 53.98 EU/mg LPS. SEC analysis showed 83.8% of protein to be present as a monomer in solution. Figure 2 shows the purification procedure of CP0177-1 chronologically. Interestingly CP0177-1 aberrantly migrated at approximately 60 kDa

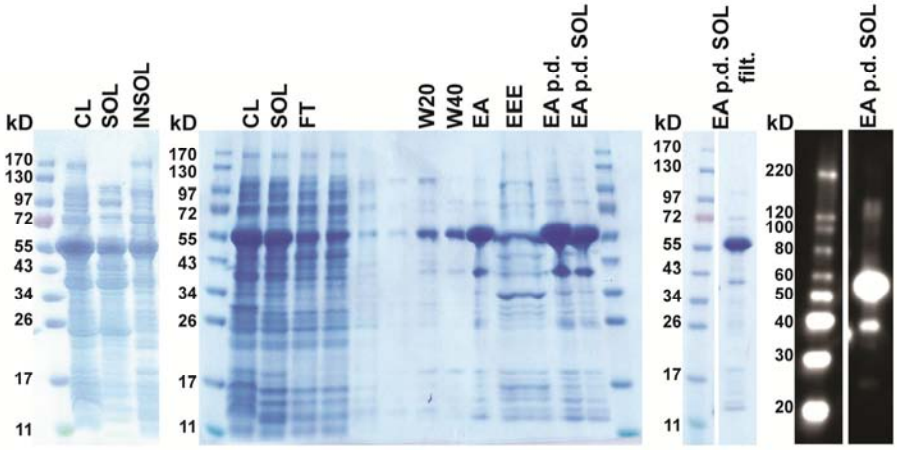

Figure 2: SDS-PAGE, Western Blot; Purification of CP0177-1: CL (crude lysate), SOL (soluble fraction), INSOL (insoluble fraction), FT (flow through), EA (eluate under native conditions), EEE (eluate under denaturating conditions), p.d. (post dialysis), filt. (0.2 µm filtered). Procedure of protein purification is shown chronologically from left to right.

in SDS-PAGE and western blot while it has a molecular weight of 40.8 kDa, a property of this protein that was also seen in orthologs [81]. However, for validation of the protein identity N-terminal sequencing was performed (Alta

Bioscience, University of Birmingham) which confirmed the purification of CP0177-1.

The ortholog of *Cpn* CP0177 in *C. trachomatis* is CT456. CT456 protein is also called translocated actin-recruting phosphoprotein (TARP) and was found to be present in *C. pneumoniae, C. caviae, C. muridarum and C. trachomatis* EBs [79]. Furthermore, *Ct* TARP was demonstrated to be a type III-secreted protein that is involved in the recruitment of actin by targeting host small GTPases and inducing F-actin reorganization at the entry site of the chlamydial EB [80]. In contrast to its orthologs *Ct* TARP possesses several tyrosine-rich repeats in its N-terminal region which become tyrosine-phosphorylated inside the target cell. However, tyrosine phosphorylation was shown not to be involved in the process of actin recruitment nor was it essential for infection of target cells by *Chlamydia* [79].

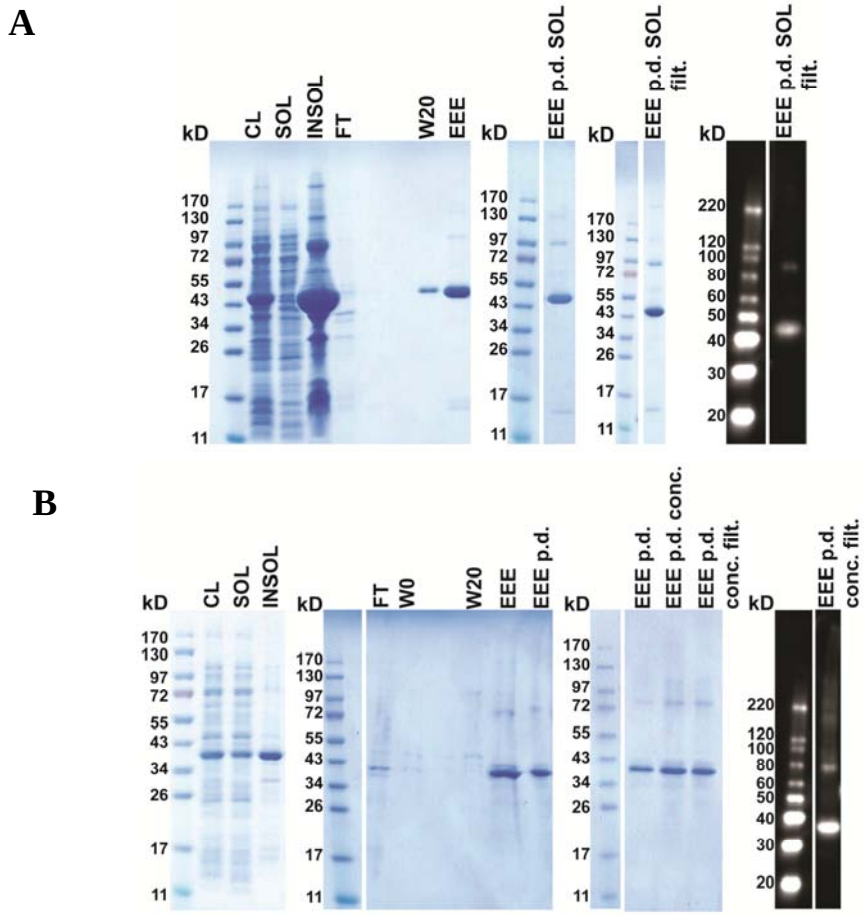

Figure 3: SDS-PAGE, Western Blot; **A**: Purification of CP0020-1; **B**: Purification of CP0879: CL (crude lysate), SOL (soluble fraction), INSOL (insoluble fraction), FT (flow through), EEE (eluate under denaturating conditions), p.d. (post dialysis), filt. (0.2 µm filtered), conc. (post centrifugal concentration). Procedure of protein purification is shown chronologically from left to right.

The C-terminal domain of TARP contains the greatest sequence similarity between orthologs. Interestingly all TARP orthologs contain an actin-binding helix and proline-rich oligomerization domain in the conserved C-terminal region [79]. In 2009 J. Wang *et al* demonstrated in a murine *C. muridarum* vaginal infection model the induction of TARP specific cellular and humoral immune responses.

Furthermore, intra muscular immunization of BALB/c mice with *Ct* TARP adjuvanted with CpG-incomplete Freud's adjuvant (IFA) led to the reduction of bacterial loads in the lower genital tract of challenged mice and attenuated inflammatory pathologies in the fallopian tube tissue [81].

CP0020-1 as well as candidate protein CP0879 were isolated under denaturating conditions from purified inclusion bodies after recombinant expression in *E. coli*. CP0020-1 consists of amino acid 2-418 of *Cpn* CP0020, and has a molecular weight of 46.9 kDa. The sequence contains 3 cys. The ortholog in *Ct* is CT620.

Recently genetic vaccination using the vector pCMVi-UB carrying the CP0020 gene was shown to protect mice from death in a high dose *Cpn* challenge study, but did not reduce disease or bacterial lung loads [82].

The purification procedure of CP0020-1 and CP0879 are shown in figure 3A and 3B respectively. CP0020-1 was stepwise dialyzed against 500 > 250 > 100 mM L-Arg, 50 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.1% Tween20. The final protein solution contained 1.26 mg/ml protein and 119.18 EU/mg LPS. SEC analysis of the final CP0020-1 protein solution showed strong oligomerization with no monomers of CP0020-1 present.

CP0879 protein has a molecular weight of 40.6 kDa and contains amino acids 2-343 of 343, 5 of these being cys. The protein solution which was eluted under denaturating conditions was stepwise dialyzed against 500 > 250 > 100 mM L-Arg, 50 mM TrisHCl, pH 8.5, 150 mM NaCl, 0.1% Tween20.

The CP0879 solution used for immunizations and in vitro stimulation contained 0.93 mg/ml protein and 182.58 EU/mg LPS. 100% of CP0879 were present as oligomers in the final solution, as assessed by SEC. Protein BLAST with the aa sequence of CP0879 did not reveal an ortholog in *Ct*.

The sequential purification procedures for candidate proteins CP0504 and CP0409 are shown chronologically in figure 4A and 4B respectively. Both proteins were purified under denaturating conditions from purified inclusion bodies.

As proteins CP0504 and CP0409 showed to precipitate during dialysis using a variety of different buffer systems, these two candidates were subjected to refolding experiments. The procedure of refolding and subsequent isolation is described in section 6.1.6, materials and methods.

CP0504 protein has the size of 287 aa (5 cys) and a molecular weight of 33.1 kDa. The ortholog of CP0504 in *Ct* is CT143, an uncharacterized hypothetical protein. The final protein solution of CP0504 contained 2.01 mg/ml protein and 71.06 EU/mg LPS. The final buffer consisted of 50 mM TrisHCl, pH 8.5, 150 mM NaCl, 100 mM L-Arg and 0.1% Tween20. 5% of CP0504 in the final buffer were assessed to be present in monomers by SEC. 95% were present as strongly interacting oligomers which were not resolved during SDS-PAGE as can be seen in figure 4A.

The ortholog of CP0409 in *Ct* is the uncharacterized hypothetical protein CT066. CP0409 consists of 171 aa (2 cys) and has a molecular weight of 20.6 kDa. The final preparation of CP0409 contained 0.58 mg/ml protein, 5.38 EU/mg LPS and showed 100% oligomerization in SEC analysis.

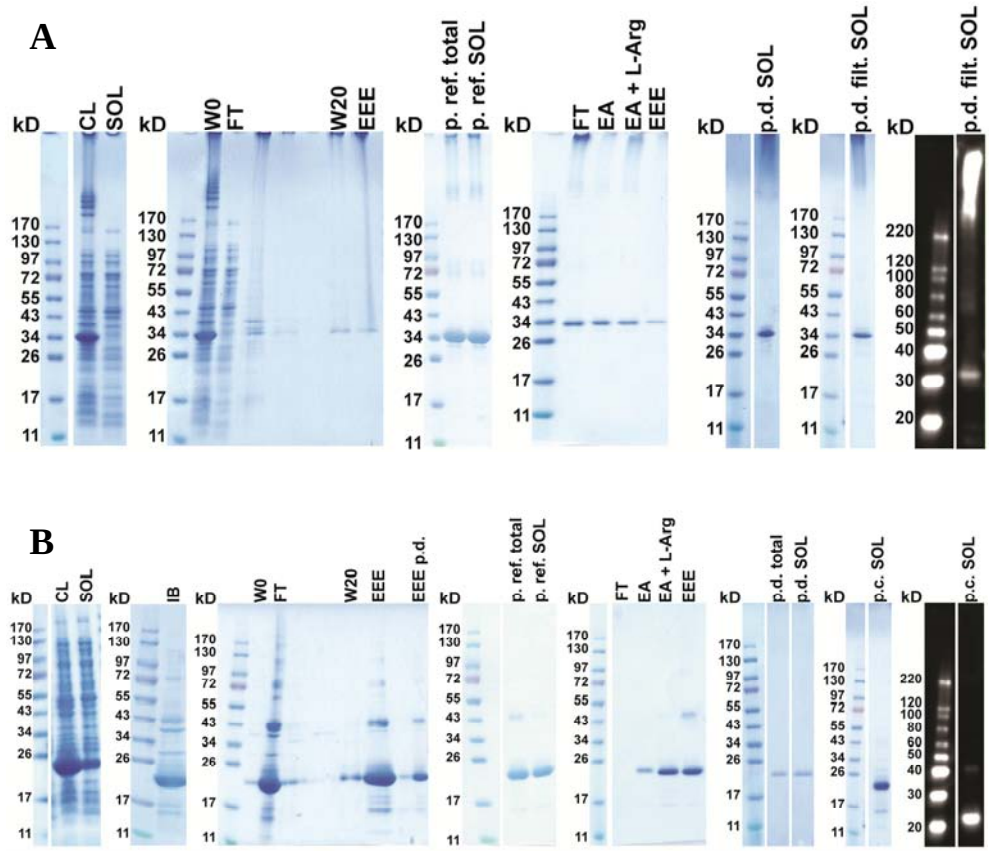

Figure 4: SDS-PAGE, Western Blot; **A**: Purification of CP0504; **B**: Purification of CP0409: CL (crude lysate), SOL (soluble fraction), IB (inclusion bodies), FT (flow through), EA (eluate under native conditions), EEE (eluate under denaturating conditions), p.d. (post dialysis), p. ref. (post refolding), p.c. (post centrifugal concentration), filt. (0.2 µm filtered), Procedure of protein purification is shown chronologically from left to right.

Table 1 summarizes the properties of the 6 purified *Cpn* candidate antigens. In general, purification of recombinant His-tag proteins from the soluble fraction, by gravity column affinity purification, showed to be more efficient but led to a higher degree of contamination with co-isolated *E.* coli proteins. In contrast all candidate antigens purified from inclusion bodies showed a higher degree of purity but a stronger tendency for oligomerization.

LPS contamination heavily varied regarding each different candidate protein, indicating the dependency of LPS binding to protein on the antigen itself and not the purification procedure. However, CP0529, CP0177-1, CP0020-1, CP0504, CP0409 and CP0879 could be successfully purified with keeping the limitations indicated in the beginning in mind.

	CP0529	**CP0177-1**	**CP0020-1**	**CP0504**	**CP0409**	**CP0879**
Annotation	Conserved hypothetical protein	Conserved hypothetical protein	Conserved hypothetical protein	Conserved hypothetical protein	Conserved hypothetical protein	Conserved hypothetical protein
Homology/ Function	Uncharacterized (CT181)	TARP (CT456)	Uncharacterized (CT620)	Uncharacterized (CT143)	Uncharacterized (CT066)	Uncharacterized (no ortholog in *Ct*)
Solubility	Soluble	Soluble	Insoluble	Insoluble	Insoluble	Insoluble
LPS	23.78 EU/mg	53.98 EU/mg	119.18 EU/mg	71.06 EU/mg	5.38 EU/mg	182.58 EU/mg
Final buffer	50 mM TrisHCl pH 8.0 150 mM NaCl	50 mM TrisHCl pH 8.0 150 mM NaCl	50 mM TrisHCl pH 8.0 150 mM NaCl 100 mM L-Arg 0.1% Tween20	50 mM TrisHCl pH 8.5 150 mM NaCl 100 mM L-Arg 0.1% Tween20	50 mM TrisHCl pH 7.0 150 mM NaCl 100 mM L-Arg 0.1% Tween20	50 mM TrisHCl pH 8.5 150 mM NaCl 100 mM L-Arg 0.1% Tween20
Estimated yield	12.5 g biomass: ~40 mg (1.51 mg/ml)	34.2 g biomass: ~150 mg (1.68 mg/ml)	1g IBs ~20 mg (1.26 mg/ml)	2 g IBs: ~18 mg (2.01 mg/ml)	2 g IBs: ~ 5 mg (0.58 mg/ml)	200 mg IBs: ~ 10 mg (0.93 mg/ml)
% oligomers	4.5%	16.2 %	100%	95%	100%	100%

Table 1: Characteristics and purification profile for selected *Chlamydia pneumoniae* candidate antigens

4.2 Immunogenicity of selected *Chlamydia pneumoniae* antigens

For the evaluation of *Cpn* candidate antigens, respecting cellular and humoral immune responses induced by immunization of BALB/c mice either with the *Chlamydia* antigen alone or in combination with the adjuvant IC31®, IFN-γ ELIspot, IL-4 ELIspot, CD154 FCM and ELISA assays where the chosen readout. The basis for this selection built the documented importance of type I interferon, especially IFN-γ production, primarily by $CD4^+$ and $CD8^+$ T cells in protective immune responses directed against *Chlmaydia pneumoniae* [46, 47]. Additionally, secretion of the type II cytokine IL-4 was assessed by ELIspot in order to measure the induction of T_H2-type T cell responses.

Flow cytometric analysis of antigen induced expression of the cell marker CD154, which is also called CD40 ligand (CD40L), was assessed in $CD4^+$ T cells purified from lungs and spleens of immunized animals. Expression of CD154 on $CD4^+$ T cells is an important effector function of this cell type, as CD40 signaling has been implicated in the activation and differentiation of APCs including DCs [87, 88], B cells [83] and macrophages [84, 86]. Therefore CD40-CD40L interaction plays a central role in almost all immunological processes. Factors that influence the expression of CD154 on T cells will for this reason influence the activation and differentiation of APCs, a fundamental process in the development of an effective adaptive immune response. Antigen specific ELISA was chosen for the assessment of serum IgG and serum IgA titers which were induced by intra nasal and parental immunization.

4.2.1 Antigen induced IFN-γ responses

Immunizations were performed as described in section 6.6, materials and methods. IFN-γ responses to candidate antigens CP0529 and CP0177-1, 10 days post 3rd intra nasal immunization, following *ex vivo* restimulation with the indicated amounts of protein, are shown in figure 5. The results demonstrate the absence of immunogenicity for candidate CP0529 in terms of IFN-γ response following i.n. delivery of the antigen, either with or without adjuvant. In contrast experimental groups immunized with the protein CP0177-1 in combination with IC31® showed an intense IFN-γ response to restimulation with the antigen, while IFN-γ secretion was not restimulated in the group immunized with antigen alone. Regarding the recombinant proteins CP0020-1

and CP0504, as well as candidates CP0409 and CP0879, *ex vivo* restimulation induced IFN-γ responses are shown in figures 6 and 7 respectively.

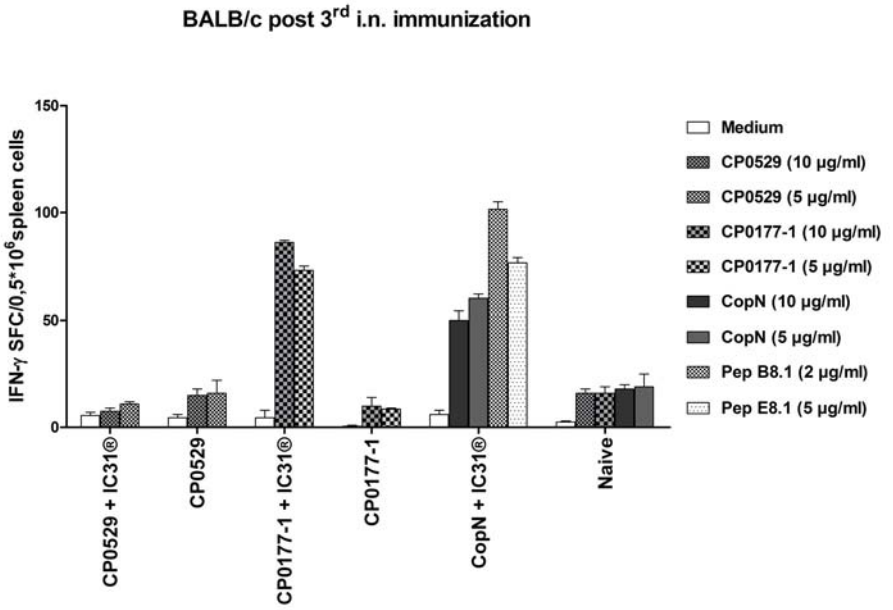

Figure 5: IFN-γ ELIspot; CP0529, CP0177-1: Mice were immunized 3 times i.n. in a distance of each 14 days. 10 days post 3rd immunization 3 mice per group were sacrificed and IFN-γ secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells).

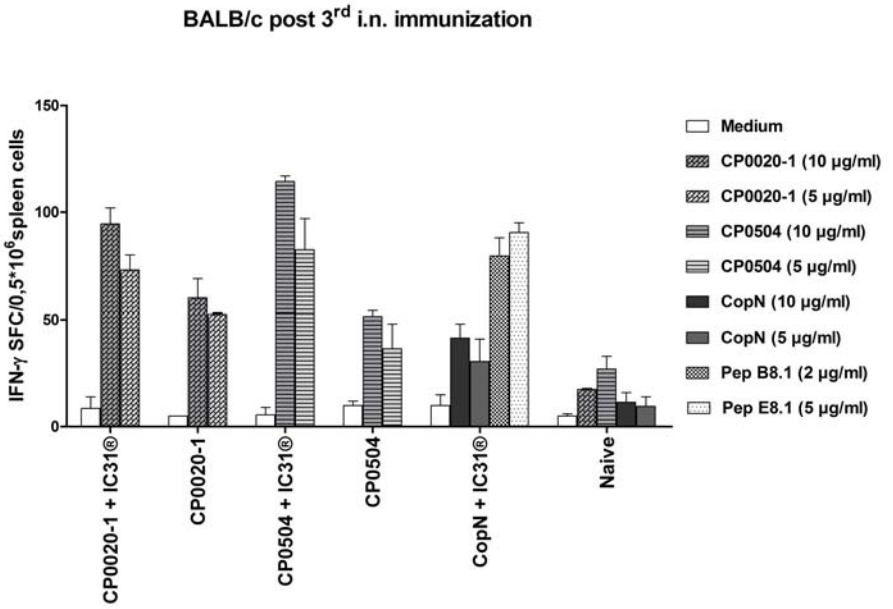

Figure 6: IFN-γ ELIspot; CP0020-1, CP0504: Mice were immunized 3 times i.n. in a distance of each 14 days. 10 days post 3rd immunization 3 mice per group were sacrificed and IFN-γ secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells).

Interestingly, candidate proteins CP0020-1 and CP0504 showed a strong response in terms of IFN-γ production when administered intra nasally

to mice, either in combination with IC31® or when the antigen was delivered alone, while the production of IFN-γ was more pronounced in groups immunized in combination with the adjuvant. Furthermore CP0020-1 showed the tendency to a more balanced response in groups which had received this antigen, while IFN-γ response induced by CP0504 *ex vivo* restimulation was more differentiated in experimental groups that had been immunized with this protein.

The analysis of IFN-γ responses, induced by intra nasal immunization with the recombinant protein CP0879, revealed a strong antigen specific response which was dependent on the adjuvant IC31® (figure 7). Furthermore, the usage of CP0879 as an irrelevant stimulus in naïve and IC31®-alone groups showed a relatively high number of IFN-γ SFC. In contrast, groups immunized with CP0409 responded to *ex vivo* restimulation equally, indicating independence of immunogenicity on the adjuvant in intra nasal immunizations. However, IFN-γ responses to CP0409 were weak compared to CP0879 indicating poor general immunogenicity of CP0409. Additionally there is to add that immunogenicity in terms of IFN-γ secretion of CP0879 and CP0409 were only assessed 10 days after 2nd intra nasal and intra peritoneal immunizations due to company internal reasons. What the results would have looked like after 3 immunizations can only be speculated about, especially when keeping the depot forming property of IC31® in mind which could potentially lead to a more pronounced, delayed adjuvantive effect after 3 doses.

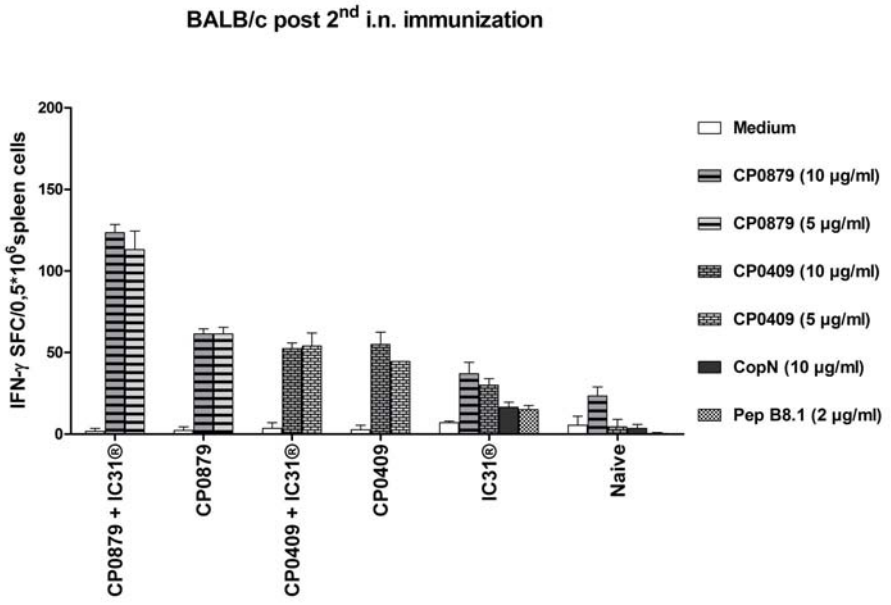

Figure 7: IFN-γ ELIspot; CP0879, CP0409: Mice were immunized 2 times i.n. in a distance of each 14 days. 10 days post 2nd immunization 3 mice per group were sacrificed and IFN-γ secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells).

A significant increase of IFN-γ production, when comparing *ex vivo* restimulated responses post 2nd with post 3rd immunization time points, was especially observed in groups immunized with IC31®, but was less pronounced in groups immunized with protein alone (own observations).

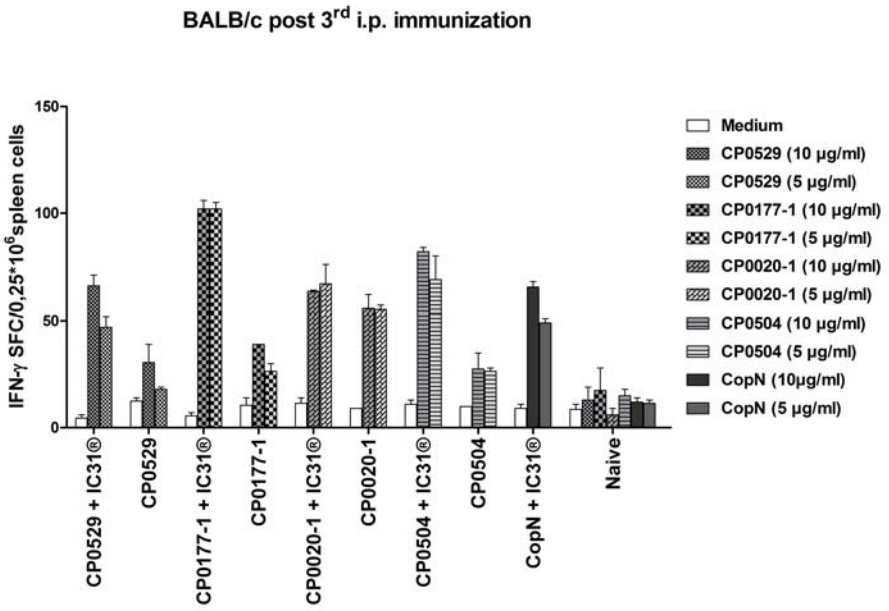

Figure 8: IFN-γ ELIspot; CP0529, CP0177-1, CP0020-1, CP0504: Mice were immunized 3 times i.p. in a distance of each 14 days. 10 days post 3rd immunization 3 mice per group were sacrificed and IFN-γ secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells)

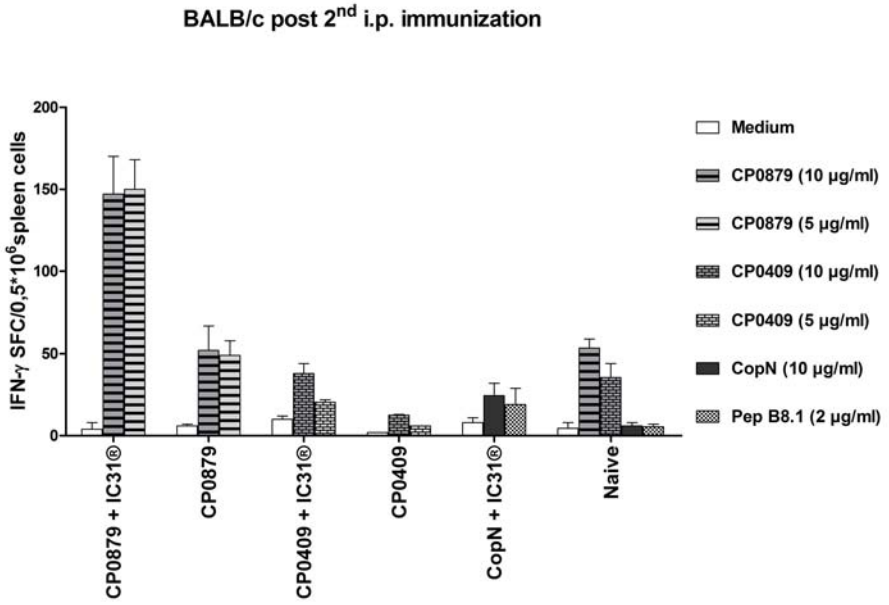

Figure 9: IFN-γ ELIspot; CP0879, CP0409: Mice were immunized 2 times i.p. in a distance of each 14 days. 10 days post 3rd immunization 3 mice per group were sacrificed and IFN-γ secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD SFC (spot forming cells).

In general, intra peritoneal immunizations with the selected antigens, either with or without adjuvant, led to similar responses regarding IFN-γ production as the ones obtained by intra nasal immunizations (figure 8 and figure 9).

The protruding differences were a universally stronger stimulation of IFN-γ production in terms of SFC, immunogenicity of CP0529 which was more pronounced in the group immunized in combination with IC31® and which was not observed after intra nasal immunizations as well as reduced immunogenicity of CP0409 when administered without adjuvant.

4.2.2 Antigen induced IL-4 responses

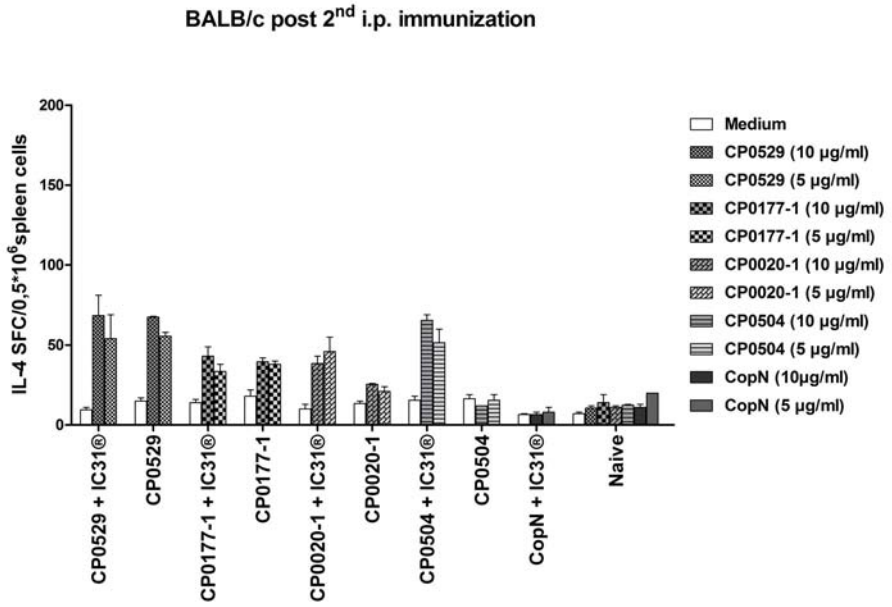

Figure 10: IL-4 ELIspot; CP0529, CP0177-1, CP0020-1, CP0504 post 2nd immunization; Mice were immunized 2 times i.p. in a distance of each 14 days. 10 days post 2nd immunization 2 mice per group were sacrificed and IL-4 secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells).

IL-4 responses to *ex vivo* restimulation were only assessed for protein antigens CP0529, CP0177-1, CP0020-1 and CP0504. The results obtained 10 days post 2nd and 10 days post 3rd immunizations are shown in figure 10 A and B. Interestingly candidate CP0529 showed the strongest response of all selected antigens in terms of IL-4 production, but only when administered without the adjuvant IC31®, indicating the induction of a strong T_H2-type response to this antigen, which is further supported by the low levels of IFN-γ production in response to CP0529. Regarding the adjuvantive effect of IC31® on IL-4 production, CP0177-1 was the only antigen showing an increased IL-4 response comparing groups immunized with and without adjuvant.

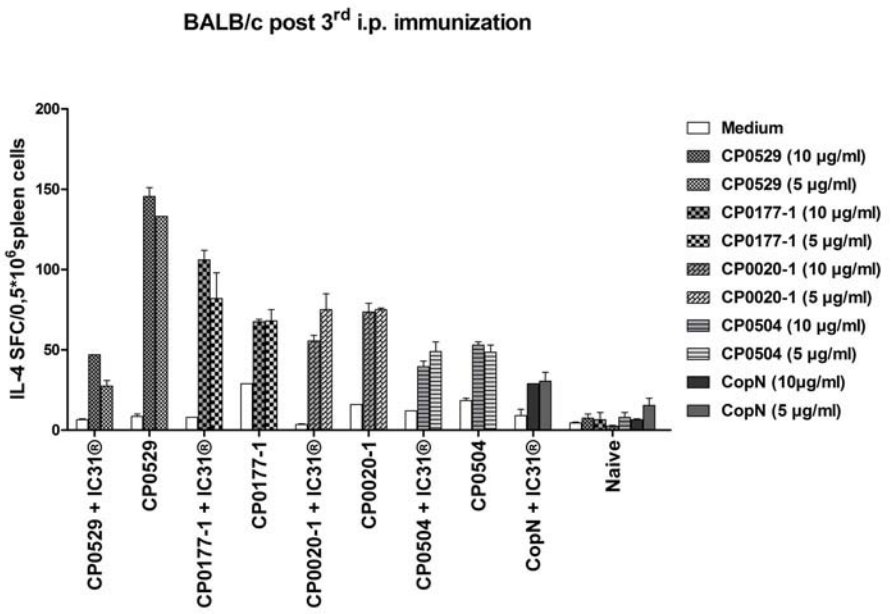

Figure 11: IL-4 ELIspot; CP0529, CP0177-1, CP0020-1, CP0504 post 3rd immunization. Mice were immunized 3 times i.p. in a distance of each 14 days. 10 days post 3rd immunization 3 mice per group were sacrificed and IL-4 secretion by splenic lymphocytes in response to *ex vivo* restimulation was assessed. Mean ± SD. SFC (spot forming cells).

While candidates CP0504 and CP0020-1 showed an elevated IL-4 response in groups immunized with adjuvant after 2 immunizations, this effect was not observed post 3rd immunization, where IL-4 levels in each both experimental groups showed equal responses.

Together these results strongly support the dominating T_H1 driving activity in the mixed T_H1/T_H2 adjuvantive effect of IC31®, as well as the effectivity in eliciting a systemic immune response by this adjuvant either when administered intra nasally or intra peritoneal.

4.2.3 Antigen-dependent induction of CD154 expression

As mentioned in the beginning, interaction between CD154 and CD40 on APCs is a fundamental process in the generation of an effective antigen specific adaptive immune response through the activation and induction of proliferation of DCs, B cells and macrophages. In order to measure the specifically induced upregulation of CD154 cell marker expression by $CD4^+$ T cells by antigen restimulation following intra nasal immunization, flow cytometric analysis was conducted. The procedures of cell preparation, CD154 and CD4 specific staining as well as *ex vivo* restimulation are described in sections 6.10, 6.15 and 6.16, materials and methods.

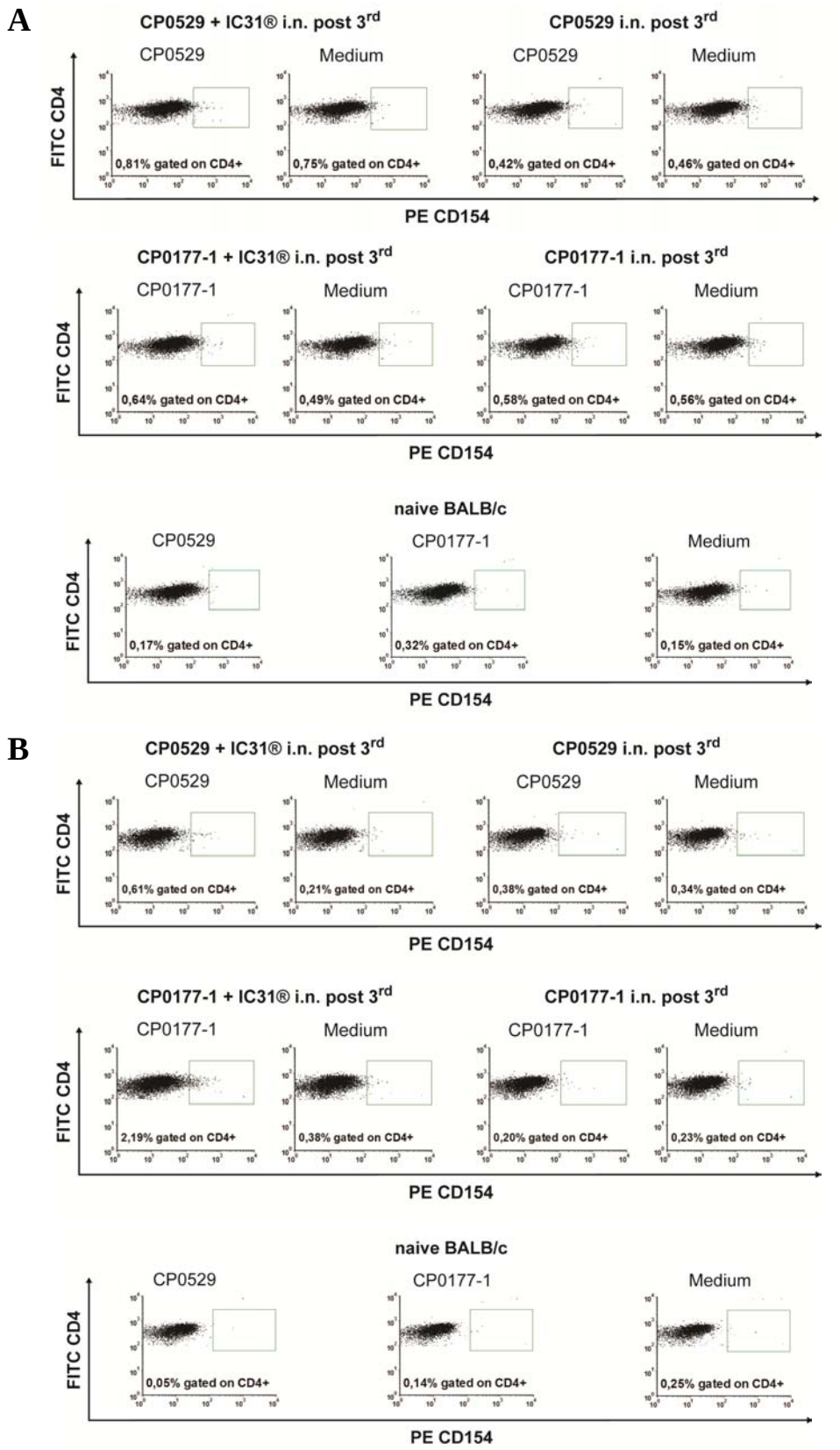

Figure 12: CD154 FCM 10 days post 3rd i.n. immunization from **A**: splenic lymphocytes; **B**: lung-derived lymphocytes: Total spleen cells/lung cells were restimulated *ex vivo* for 6 hours with 10 µg/ml of antigen prior to acquisition. Different experimental groups and stimuli are indicated on top of each dot blot. Events shown were gated from FCS/SSC (lymphocytes) and the $CD4^+$ T cell population (FITC). The percentages of CD4/CD154 double positive T cells in the $CD4^+$ T cell population are shown on the bottom of each dot blot.

$CD4^+$ T cells derived from spleens and lungs, representative for systemic and local induction of basic mechanisms in adaptive immune responses respectively, were analyzed from intra nasally immunized BALB/c mice.

Figure 11A shows the results from CD154 FCM analysis of splenic $CD4^+$ T cells; figure 11B the results of lung-derived $CD4^+$ T cells for the candidate antigens CP0529 and CP0177-1.

Upregulation of CD154 on splenic $CD4^+$ T cells following restimulation was neither observed for CP0529 nor CP0177-1 in groups immunized with the respective antigen. Immunization with CP0177-1 in combination with IC31® in contrast led to a slight increase of CD154 expression following restimulation when compared to the respective medium controls: 1.31 fold for CP0177-1, indicating a systemic response to CP0177-1 following intra nasal immunization in combination with IC31®.

In respect to the upregulation of CD154 at local compartments a different picture was obtained. Again no elevated CD154 response were seen in groups immunized with the antigen (CP0529, CP0177-1) alone, but a significant increase of CD154 expression was observed in groups immunized in combination with the adjuvant IC31® for both antigens. CP0529 induced upregulation of CD154 was 2.90 fold, while the increase in CD154 expression induced by restimulation with CP0177-1 was 5.76 fold in comparison to the medium control.

Figure 13A and B show the antigen specific upregulation of CD154 by candidates CP0020-1 and CP0504. Interestingly *ex vivo* restimulation with CP0020-1 led to a substantial increase in CD154 expression in both $CD4^+$ T cells derived from murine spleens and lungs. Additionally, upregulation of CD154 expression was observed in groups immunized either with the antigen alone or in combination with IC31®: spleen with IC31®: 1.91 fold; spleen with-out IC31® 2.14 fold; lung with IC31®: 5.88 fold; lung without IC31®: 2.80 fold (corresponding to the respective medium control).

Notable upregulation of CD154 in response to CP0504 was only observed in groups immunized with the antigen in combination with IC31®. The increase was 4.79 fold respective to the medium control. Though slight CD154 upregulation was also observed in other groups: spleen with IC31®: 1.22 fold; spleen with-out IC31®: 1.35 fold; lung without IC31®: 1.43 fold.

Regarding the recombinant *Chlamydia* proteins CP0879 and CP0409, antigen induced CD154 upregulation on $CD4^+$ T cells was only assessed post 2^{nd} intra nasal immunization (figure 14A and B). Restimulation with CP0879 showed a considerable increase of CD4/CD154 double-positive cells in spleens (1.80 fold) and lungs (1.95 fold) of those groups immunized with adjuvanted protein, whereas groups immunized without IC31® showed a smaller increase

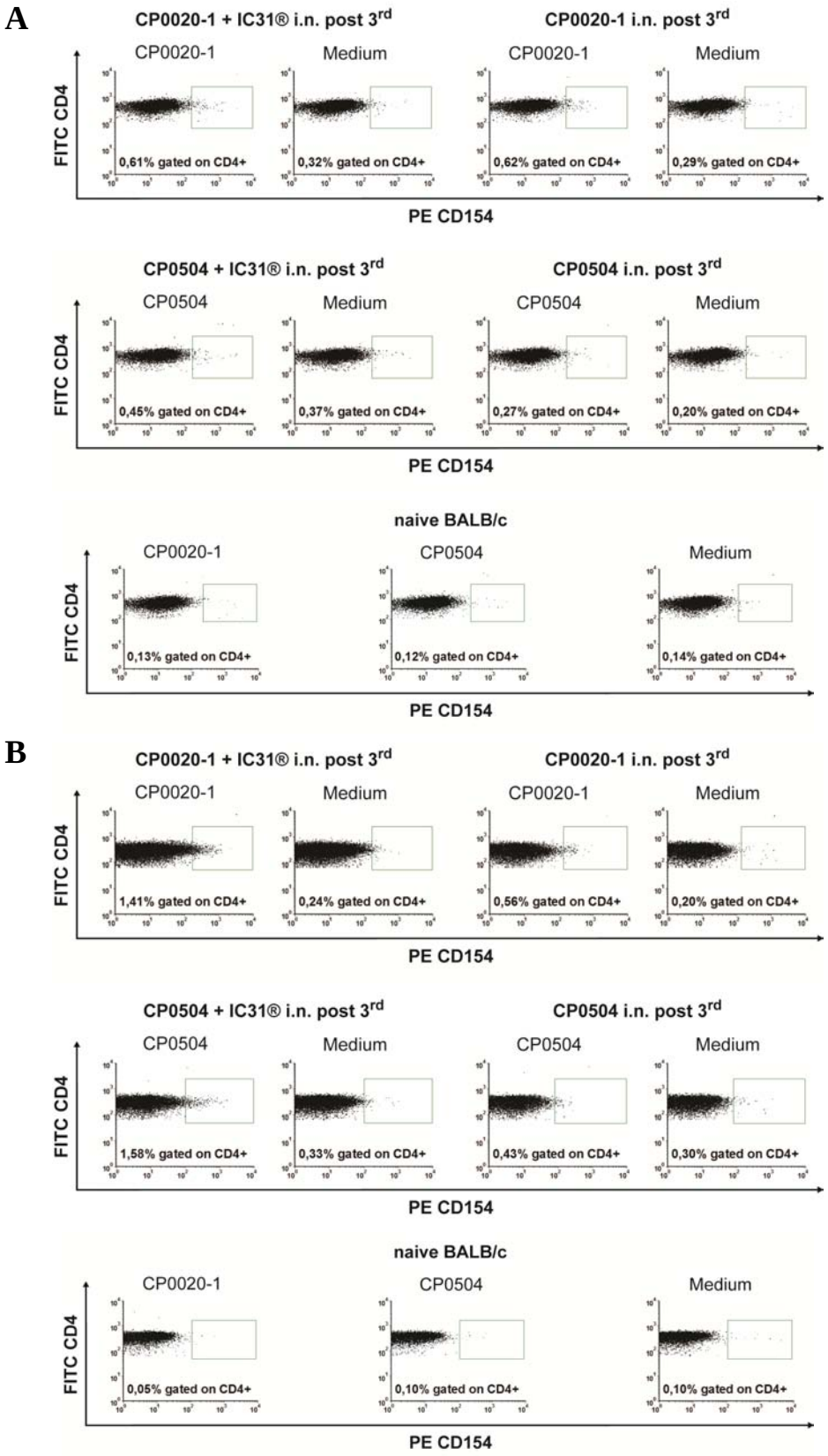

Figure 13: CD154 FCM 10 days post 3rd i.n. immunization from **A**: splenic lymphocytes; **B**: lung-derived lymphocytes: Total spleen cells/lung cells were restimulated *ex vivo* for 6 hours with 10 µg/ml of antigen prior to acquisition. Different experimental groups and stimuli are indicated on top of each dot blot. Events shown were gated from FCS/SSC (lymphocytes) and the $CD4^+$ T cell population (FITC). The percentages of CD4/CD154 double positive T cells in the $CD4^+$ T cell population are shown on the bottom of each dot blot.

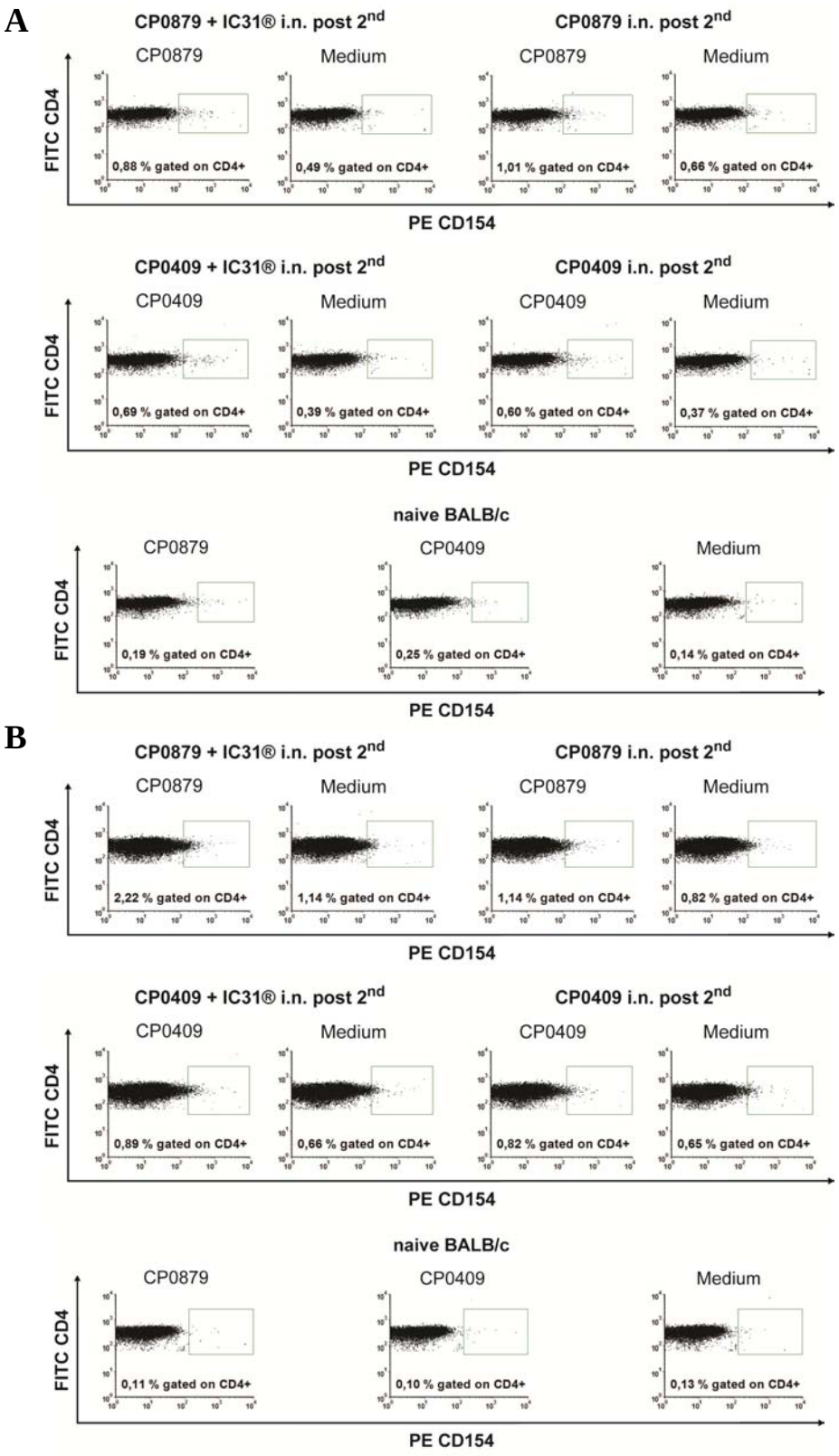

Figure 14: CD154 FCM 10 days post 3rd i.n. immunization from **A**: splenic lymphocytes; **B**: lung-derived lymphocytes: Total spleen cells/lung cells were restimulated *ex vivo* for 6 hours with 10 µg/ml of antigen prior to acquisition. Different experimental groups and stimuli are indicated on top of each dot blot. Events shown were gated from FCS/SSC (lymphocytes) and the $CD4^+$ T cell population (FITC). The percentages of CD4/CD154 double positive T cells in the $CD4^+$ T cell population are shown on the bottom of each dot blot.

in terms of CD154 upregulation on $CD4^+$ T cells: spleen without IC31®: 1.53 fold; lung without IC31®: 1.39 fold.

CP0409 restimulation showed positive responses in terms of CD154 upregulation in all tested groups. Though, the differences between groups immunized without adjuvant compared to those which received antigen and adjuvant were less pronounced: spleen with IC31®: 1.78 fold; spleen with-out IC31® 1.62 fold; lung with IC31®: 1.35 fold; lung without IC31®: 1.26 fold.

Table 2 summarizes the results obtained from CD154 flow cytometric analysis experiments.

Organ	Immunization	CP0529	CP0177-1	CP0020-1	CP0504	CP0879	CP0409
Spleen	Antigen + IC31®	-	1.31	1.91	1.22	1.80	1.78
	Antigen	-	-	2.14	1.35	1.53	1.62
Lung	Antigen + IC31®	2.90	5.76	5.88	4.79	1.95	1.35
	Antigen	1.12	-	2.80	1.43	1.39	1.26

Table 2: Summary: Antigen induced upregulation of CD154 on $CD4^+$ T cells following intra nasal immunization and *ex vivo* restimulation (3 immunizations: CP0529, CP0177-1, CP0020-1, CP0504; 2 immunizations: CP0879, CP0409); numbers are shown as x-fold increase of % of CD4/CD154 double-positive cells in comparison to the respective medium control.

4.2.4 Antigen-specific immunoglobulin responses

Antigenicity of selected *Chlamydia pneumoniae* candidate proteins was assessed by Enyme-linked Immunosorbent Assay (ELISA) using sera obtained from mice that were immunized intra nasally or intra peritoneal. The applied methods are described in section 6.14, materials and methods.

In order to obtain a comprehensive picture of antigen induced immunoglobulin production both, total serum IgG titers as well as serum IgA titers were assessed. Prior to the assessment of immunoglobulin titers by ELISA each recombinant protein was titrated using serum from mice immunized with the relevant antigen, serum from mice immunized with an irrelevant antigen as well as sera from naïve mice as controls. The optimal coating concentration was defined as the concentration of recombinant protein

which allowed the best differentiation between sera of mice immunized with the relevant antigen in comparison to sera from control groups (not shown).
The results obtained from ELISA for candidate antigens CP0529, CP0177-1, CP0020-1 and CP0504 respectively, are shown in figure 15A-D.

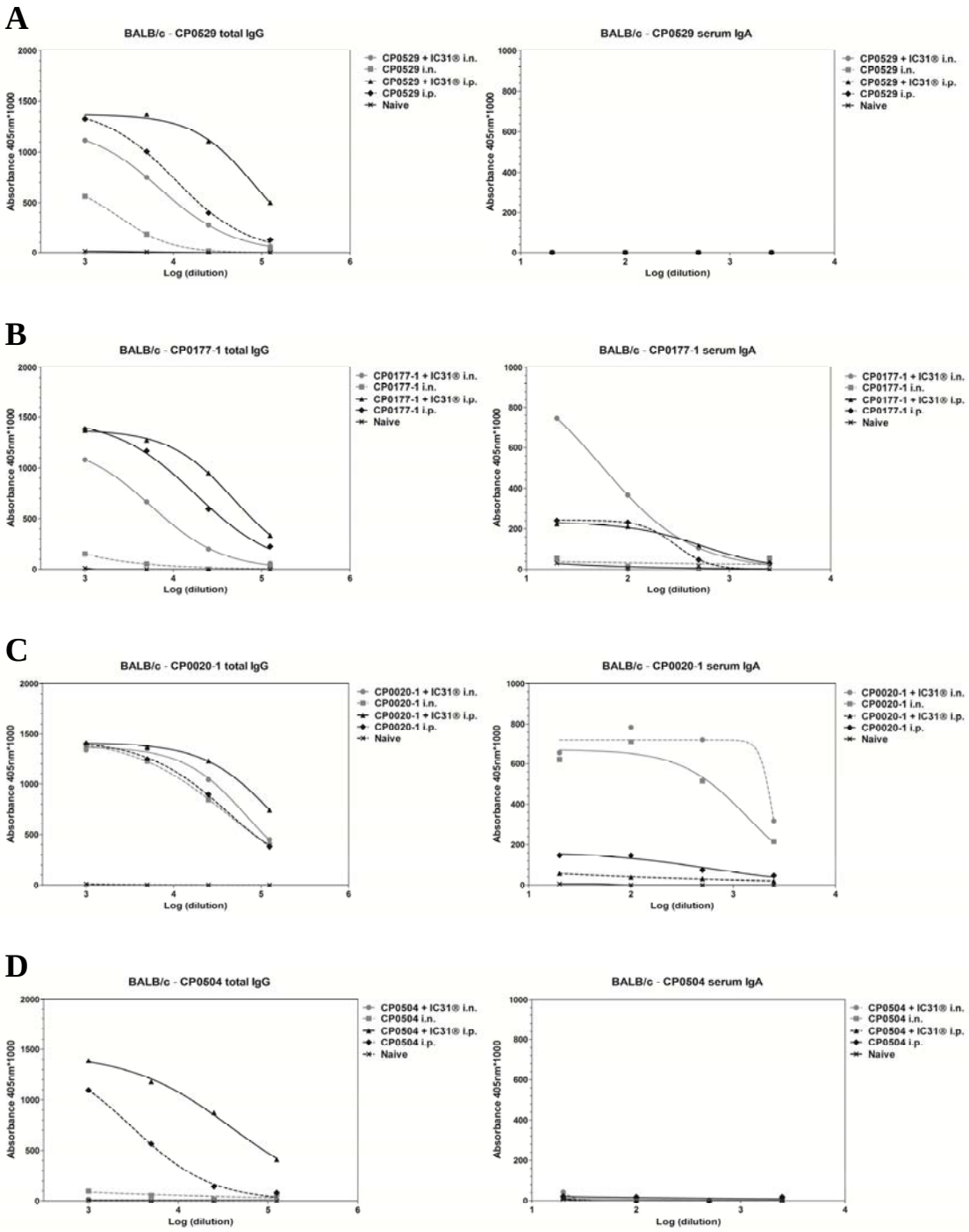

Figure 15: A: CP0529; **B**: CP0177-1; **C**: CP0020-1; **D**: CP0504; ELISA of serum from BALB/c mice obtained 10 days post 3rd intra nasal/intra peritoneal immunization. Total serum IgG titers are shown on the left, serum IgA titers on the right. Pooled serum samples of each 3 mice were used and serially diluted as shown along the X-axis. The results were expressed as OD_{405nm}*1000 values as shown along the Y-axis. Each curve is shown as nonlinear regression over background subtracted individual values.

As can be seen in figure 15A, i.p. immunizations with candidate CP0529 elicited strong IgG responses, while antibody titers where higher in the group immunized with IC31® as an adjuvant. As well as i.p. immunization, i.n. immunizations induced antigen specific IgG production in both groups, but at a lower level. No IgA response was detected in any group of mice immunized with CP0529.

CP0177-1 specific IgG antibodies where detected at the highest level in the group immunized i.p. in combination with IC31®, followed by the group of mice immunized i.p. with the antigen alone and followed by the experimental group which had received CP0177-1 plus IC31® intra nasally. Low levels of IgG where furthermore detected in mice immunized i.n. with CP0177-1 alone.

CP0177-1 specific serum IgA was found in groups immunized i.n. with adjuvanted antigen and at lower but similar levels in groups immunized either with CP0177-1 alone or in combination with IC31® via the i.p. route.

Candidate CP0020-1 clearly showed the overall highest IgG titers indicating high antigenicity of this protein in both routes of immunization while the adjuvant IC31® additionally showed an enhancing effect.

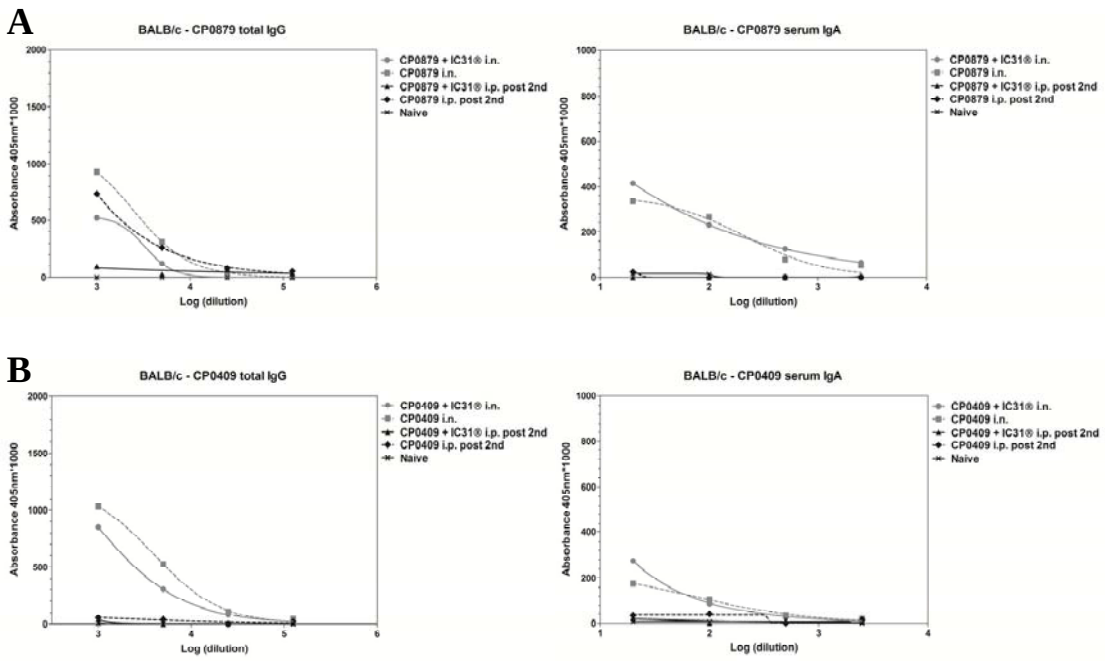

Figure 26: A: CP0879; **B**: CP0409; ELISA of serum from BALB/c mice obtained 10 days post 3rd intra nasal and 10 days post 2nd intra peritoneal immunization. Total serum IgG titers are shown on the left, serum IgA titers on the right. Pooled serum samples of each 3 mice were used and serially diluted as shown along the X-axis. The results were expressed as OD_{405nm}*1000 values as shown along the Y-axis. Each curve is shown as nonlinear regression over background subtracted individual values.

Furthermore, potent IgA induction was observed in groups that had received CP0020-1 i.n. while low IgA titers could be detected in i.p. immunized groups. Interestingly intra nasal immunization with CP0020-1 alone showed a stronger serum IgA induction than immunization with IC31®-adjuvanted protein, while in contrast serum IgA levels where higher in the i.p. immunized that also received the adjuvant than in the group which was immunized with CP0020-1 alone.

In the case of CP0504, antigen specific reaction by IgG antibodies was detected in i.p. immunized groups showing higher immunoglobulin titers in mice immunized with antigen and adjuvant. Low CP0504 specific serum IgG titers where furthermore seen in mice immunized i.n. with CP0504 protein alone, but not in the group where IC31® was included. Furthermore no antigen specific serum IgA was induced neither by i.p. immunization nor i.n. immunization.

Figures 16A and B show the results for candidate antigens CP0879 and CP0409 respectively. ELISA from sera of BALB/c mice immunized 2 times i.p. with CP0879 alone showed a specific IgG response in contrast to the group immunized with adjuvanted protein. IgA response to CP0879 after 2 i.p. injections was absent. Intra nasal immunization, gave rise to both IgG and IgA responses, where IgG responses where stronger in the group immunized with the antigen alone.

Regarding the induction of humoral response in the case of CP0409, only sera obtained from intra nasally immunized mice contained antigen specific IgG and IgA. Similar to the case of CP0879 IgG responses where higher in the groups immunized with the protein alone whereas serum IgA titers were higher in the group immunized with adjuvanted protein.

4.3 *Cpn* K6 infection-primed immune responses

Two different mouse strains, BALB/c and C57BL/6, were used here in order to assess T cell responses and humoral immunogenicity to the selected *Cpn* candidate antigens by infection-primed immune responses. The results should give an idea about the recognition of these antigens during a simulated, natural *C. pneumoniae* infection in mice, as the AIP® technology only gives information about the antigenicity of these candidates during *Cpn* infection in humans.

It should be noted here, that BALB/c ($H2^d$) mice have been reported to preferentially develop a T_H2-type immune response and to be more susceptible to *Cpn* infection/intracellular pathogens in general, which stands in contrast to

observations made with C57BL/6 ($H2^b$). The reason for these observations may be due to the greater $CD4^+CD25^+$ regulatory T cell population of BALB/c mice, as found in a *Leishmania major* infection model [89].

T cell responses were assessed from spleen cells derived from infected/reinfected mice by cultured IFN-γ ELIspot, where infection primed T cells specifically reacting to an antigen by IFN-γ secretion, were expanded *in vitro* over a period of 48 hours followed by the standard procedure of IFN-γ ELIspot.

Humoral responses induced by *C. pneumoniae* infection were assessed by ELISA using sera from infected/reinfected animals. The applied methods are described in section 6.7, 6.11, 6.12 and 6.14, materials and methods. Figures 17 and 18 respectively show infection-primed T cell responses, which were obtained by cultured IFN-γ ELIspots, against the selected *Cpn* antigens in BALB/c and C57BL/6 mice.

IFN-γ responses of spleen cells that were derived from infected/ reinfected BALB/c mice clearly showed that 5 out of the 6 selected *Cpn* antigens were recognized by T cells, inducing IFN-γ production, during infection. These antigens were: CP0529, CP0177-1, CP0020-1, CP0504 and CP0409.

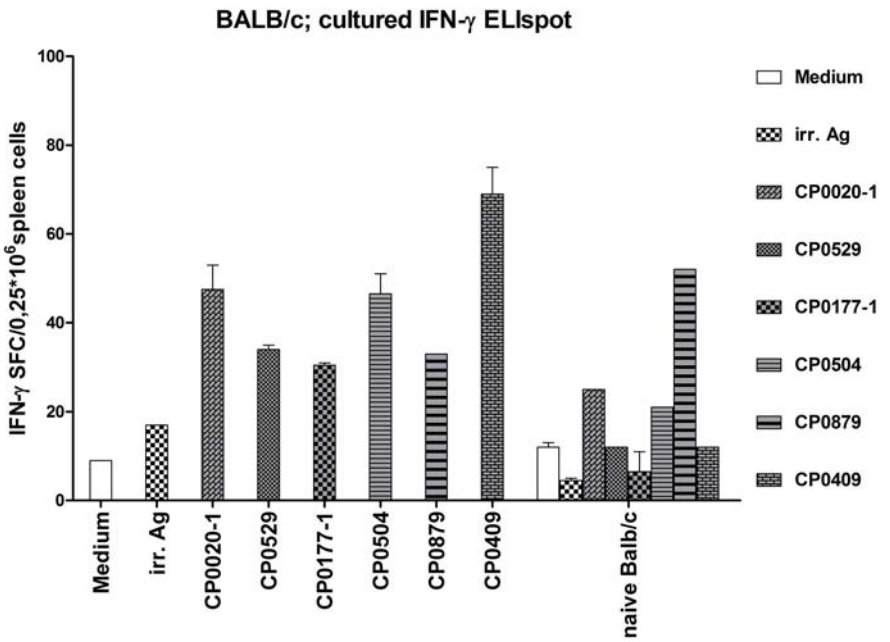

Figure 17: Cultured IFN-γ ELIspot from spleen cells derived 12 days post reinfection from *Cpn* K6 infection primed BALB/c mice: Total spleen cells of each experimental group (indicated along the X-axis) where cultured *ex vivo* for 48 hours in presence of 5 µg/ml of the respective *Cpn* antigen. Subsequent cells were transferred onto ELIspot plates and restimulated for 24 hours with 2 µg/ml of the respective *Cpn* antigen. Mean ± SD. SFC (Spot Forming Cells).

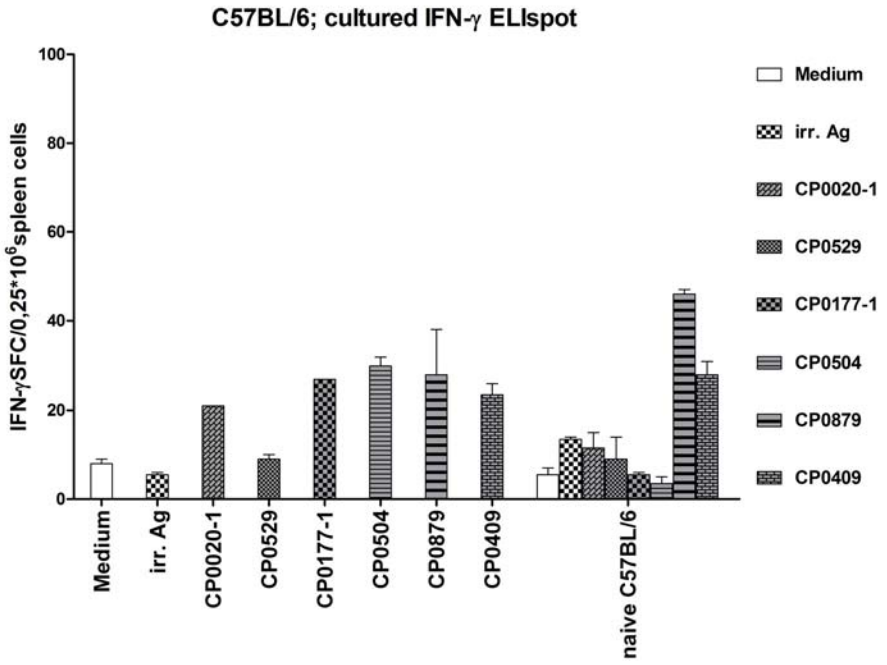

Figure 18: Cultured IFN-γ ELIspot from spleen cells derived 12 days post reinfection from *Cpn* K6 infection primed C57BL6/c mice: Total spleen cells of each experimental group (indicated along the X-axis) where cultured *ex vivo* for 48 hours in presence of 5 µg/ml of the respective *Cpn* antigen. Subsequent cells were transferred onto ELIspot plates and restimulated for 24 hours with 2 µg/ml of the respective *Cpn* antigen. Mean ± SD. SFC (Spot Forming Cells).

In contrast, the results obtained from C57BL/6 mice indicated that 3 out of 6 candidates were recognized by this mouse strain during *Cpn* infection, when compared to the responses of the naïve control group. In this case the proteins which were recognized are CP0504, CP0177-1 and CP0020-1, indicating the predominant induction of a T_H2-type response to *Cpn* antigens CP0529 and CP0409 during *Cpn* infection.

As mentioned in the beginning of this section, infection primed humoral responses were assessed by ELISA. This experiment resembled a repetition of the results obtained from the screening during the AIP®, but in mice. The results are shown in figures 19A-C (C0529, CP0177-1, CP0020-1 respectively) and figures 20D-F (CP0504, CP0879, CP0409 respectively).

As in immunization studies, also here, antigen specific total serum IgG and IgA titers were assessed. In general, *Cpn* K6 infection elicited specific IgG antibody responses to all selected candidate antigens in BALB/c and C57BL/6 mice, while the later mouse strain clearly showed the lower titers.

Infection-primed serum IgA responses were detected for candidates CP0177-1, CP0020-1, CP0504, CP0879 and CP0409, while IgA antibodies against candidates CP0504 and CP0879 were only induced in C57BL/6 mice. Candidate CP0020-1, as already observed in immunization studies, was

predominantly recognized in terms of total serum IgG and serum IgA antibody titers in both BALB/c and C57BL/6 mice.

Furthermore, variations regarding the different mouse strains, except the overall lower antigen specific IgG titers in C57BL/6 mice compared to BALB/c mice, were especially seen in serum IgA responses.

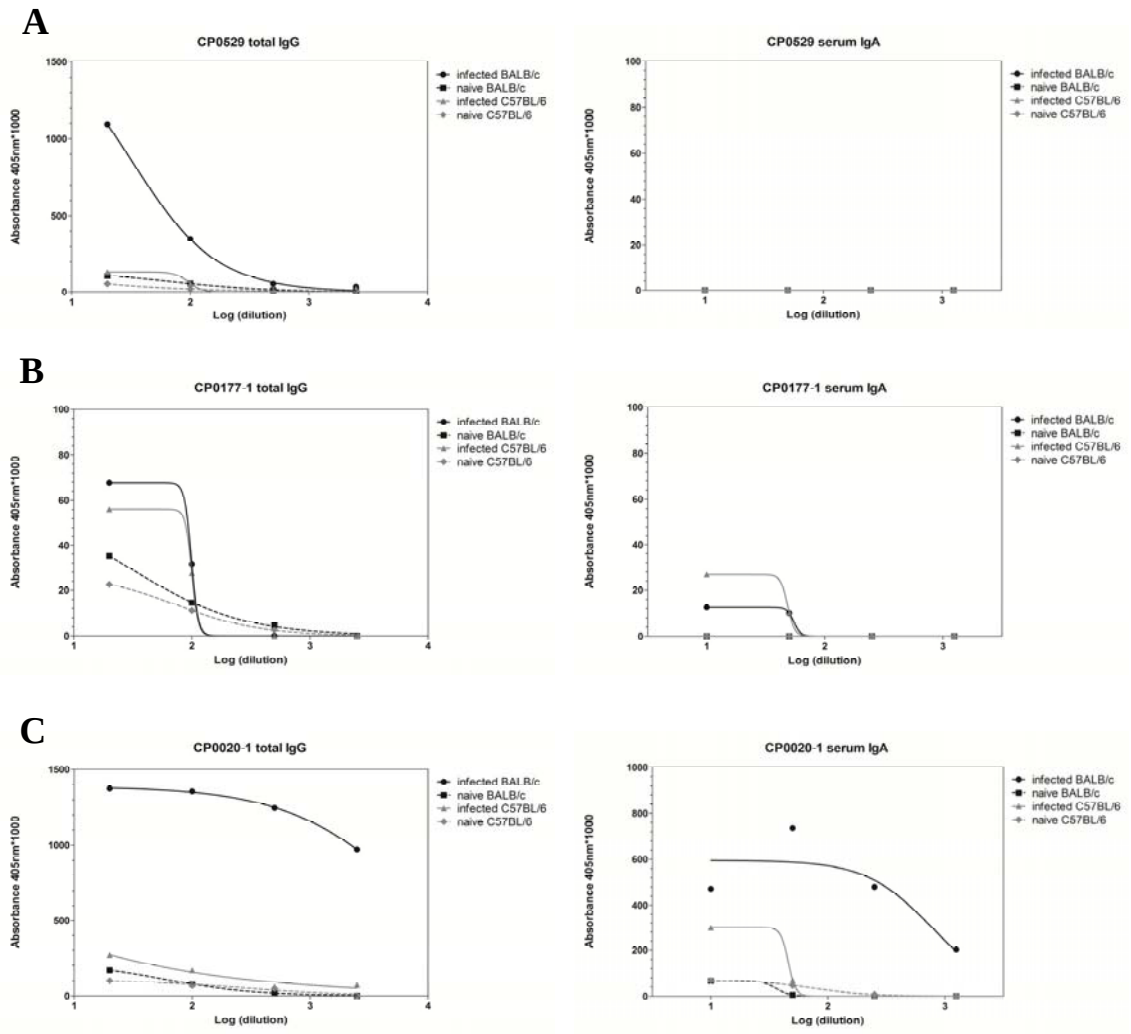

Figure 19: A: CP0529; **B**: CP0177-1; **C**: CP0020-1; ELISA of serum from BALB/c and C57BL/6 mice obtained 12 days post intra nasal reinfection with *Cpn* K6. Total serum IgG titers are shown on the left, serum IgA titers on the right. Pooled serum samples of each 5 mice were used and serially diluted as shown along the X-axis. The results were expressed as OD_{405nm}*1000 values as shown along the Y-axis. Each curve is shown as nonlinear regression over background subtracted individual values.

Here the majority of antigens (CP0177-1, CP0504, CP0879 and CP0409) led to the generation of higher serum IgA titers in C57BL/6 mice. The exceptions were CP0529 (which did not elicit any IgA response during *Cpn* infection) and CP0020-1 which elicited higher serum IgA titers in BALB/c mice than in

C57BL/6 mice. However, *Cpn* infection-induced anti-CP0020-1 serum IgA antibody titers surpassed all detected serum IgA titers generated against the other candidate proteins.

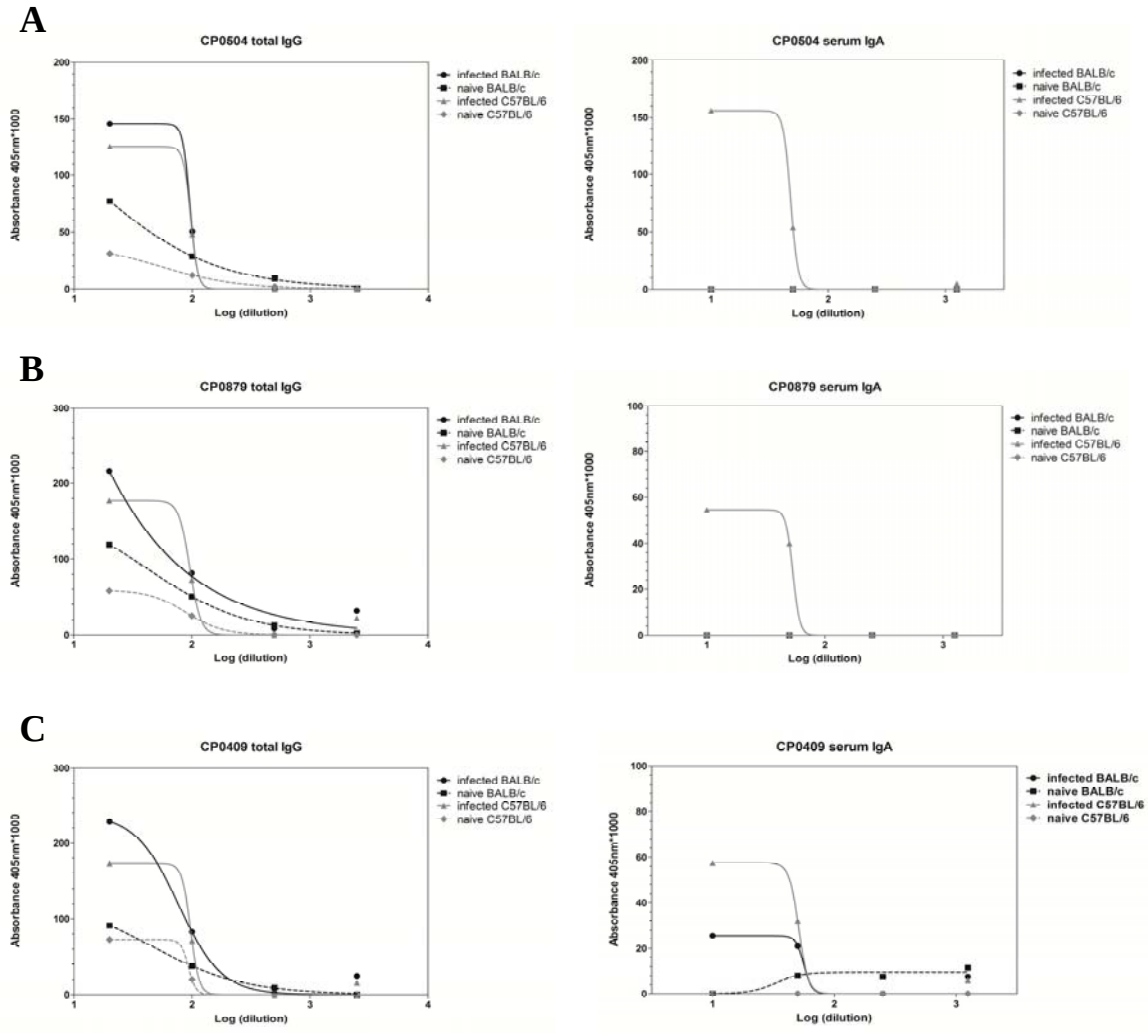

Figure 20: A: CP0504; **B**: CP0879; **C**: CP0409; ELISA of serum from BALB/c and C57BL/6 mice obtained 12 days post intra nasal reinfection with *Cpn* K6. Total serum IgG titers are shown on the left, serum IgA titers on the right. Pooled serum samples of each 5 mice were used and serially diluted as shown along the X-axis. The results were expressed as OD_{405nm}*1000 values as shown along the Y-axis. Each curve is shown as nonlinear regression over background subtracted individual values.

4.4 *Chlamydia pneumoniae* K6 challenge studies

As a central part of this study, dealing with potential subunit-vaccine candidate proteins, immunization-induced protection against *C. pneumoniae* infection was assessed in a BALB/c mouse model. The "gold-standard" for the estimation of reduction of bacterial loads in murine lungs, is the evaluation of Inclusion Forming Units (IFU) by *in vitro* infection of HEp-2 cells with different dilutions of lung homogenates derived from challenged animals, followed by immunofluorescent staining of the formed chlamydial inclusions. Sections 6.6, 6.7 and 6.9, material and methods, give a more detailed description of the applied techniques for the evaluation of protection.

BALB/c mice where sacrificed 10 days post intra nasal infection with each $1*10^6$ IFUs of *Cpn* K6 strain. Each mouse had received 3 intra nasal vaccinations with the respective recombinant *Cpn* antigen (40 µg/mouse/dose) either in combination with IC31® or without adjuvant, prior to intra nasal bacterial challenge. The experimental groups consisted of each 5 female BALB/c mice, and were kept under specified-pathogen-free (SPF) conditions prior to infection with *Cpn* K6 and group wise, in separated and ventilated cages, post infection. Figures 21, 22 and 23 show the results of immunization-challenge studies including the antigens CP0529 and CP0177-1, CP0020-1 and CP0504, CP0879 and CP0409 respectively.

Immunizations with candidate CP0529, either in combination with IC31® and when administered without adjuvant, led to significant decreases of bacterial loads in murine lungs following 3 intra nasal vaccinations. Keeping the previous results from immunization studies in mind, the mechanism of reduction of bacterial loads by immunization with CP0529 seemed to be the result of a strong but not fully protective T_H2-type immune response to this antigen.

Regarding the antigen CP0177-1 a strong protective effect was observed in the group immunized in combination with IC31®, as can be seen in the highly reduced number of IFUs. Unsurprisingly this effect was not observed in the group immunized with CP0177-1 alone, as immunization primed immune responses did only lead to high levels of IFN-γ secreting T cells as well as CD154 induction on $CD4^+$ T cells in mice immunized with IC31®-adjuvanted CP0177-1.

Interestingly CopN and MOMP adjuvanted with IC31® led to a substantial reduction of IFUs, which is consistent with previous studies dealing with these two antigens (see introduction), and supporting the dominating T_H1-

driving adjuvantive effect of IC31® that potentially induces the establishment of a protective immune response against infection with *Cpn*.

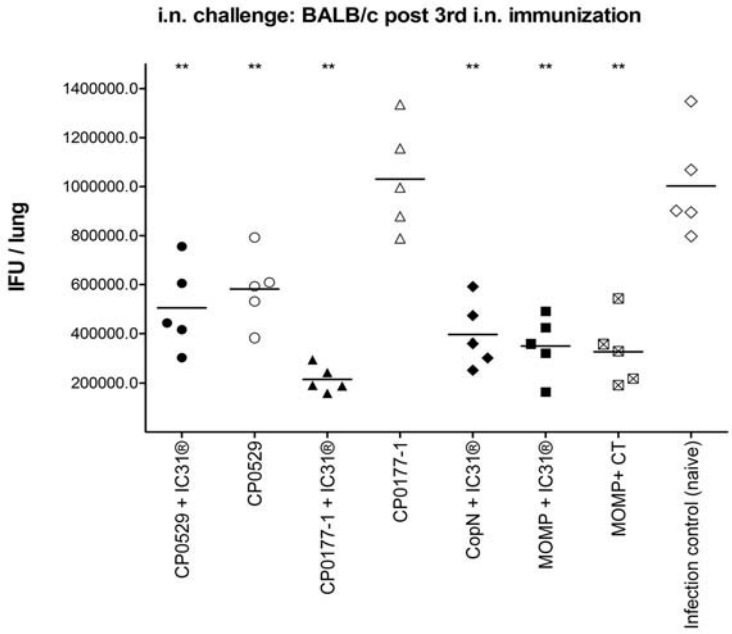

Figure 21: CP0529, CP0177-1: Assessment of IFUs 10 days post i.n. challenge: BALB/c mice were infected i.n. with each $1*10^6$ IFUs 10 days following 3rd i.n. vaccination with each 40 µg of antigen per mouse including or not including IC31® as an adjuvant. As control groups served mice immunized 3 times intra nasally with IC31®-adjuvanted CopN as well as MOMP, MOMP adjuvanted with CT and naïve mice as infection control. * = $p < 0.05$; ** = $p < 0.01$ (Mann-Whitney-U-Test).

Candidate CP0020-1 already showed a potent induction of T_H2-type responses during immunization studies. However, in contrast to CP0529, i.n. immunization with CP0020-1 (with and without IC31®) resulted in intense T cell responses, including the secretion of the type I cytokine IFN-γ.

Regarding reduction of bacterial loads, a substantial effect was seen in those groups of mice that had received CP0020-1. CP0020-1 was also included in the 3rd round of immunization-challenge studies (figure 23). In contrast to the 2nd (figure 21) round, here the reduction of IFUs was stronger in the group immunized in combination with the adjuvant IC31®. Though, differences between these experimental groups were not significant.

Reduction of IFUs in the case of immunizations with CP0504, showed a significant effect in combination with IC31® and a minor, insignificant effect with the protein alone. This might be due to the fact that CP0504 was not able to elicit neither serum IgG nor IgA responses when it was administered intra nasally, even when combined with IC31®.

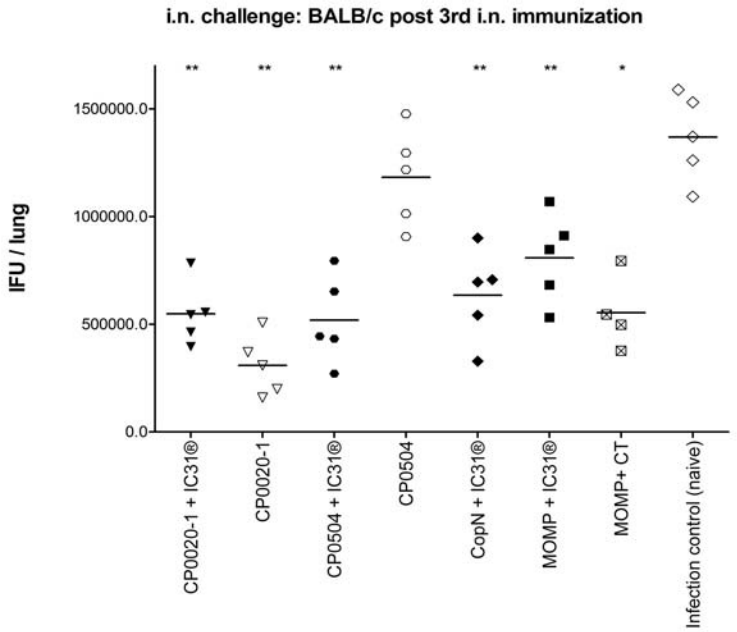

Figure 22: CP0020-1, CP0504: Assessment of IFUs 10 days post i.n. challenge: BALB/c mice were infected i.n. with each $1*10^6$ IFUs 10 days following 3rd i.n. vaccination with each 40 µg of antigen per mouse including or not including IC31® as an adjuvant. As control groups served mice immunized 3 times intra nasally with IC31®-adjuvanted CopN as well as MOMP, MOMP adjuvanted with CT and naïve mice as infection control. * = $p < 0.05$; ** = $p < 0.01$ (Mann-Whitney-U-Test).

Furthermore, T cell responses in terms of IFN-γ secretion and CD154 upregulation where only strong when CP0504 was delivered in combination with IC31®.

Finally, neither of the two antigens CP0879 and CP0409 showed a significant reduction of bacterial loads following 3 intra nasal immunizations even not, when immunizations where performed with adjuvanted recombinant protein. In the case of CP0409, this result was not surprising, given the low immunogenicity of this antigen.

CP0879 in contrast, showed good immunogenicity, especially in combination with the adjuvant IC31®, but as infection-primed immune response studies showed, CP0879 might not be at all, or only in low levels, accessible to T cells of the immune system of the host, which could explain the obtained results in immunization–challenge studies for this recombinant *Cpn* antigen.

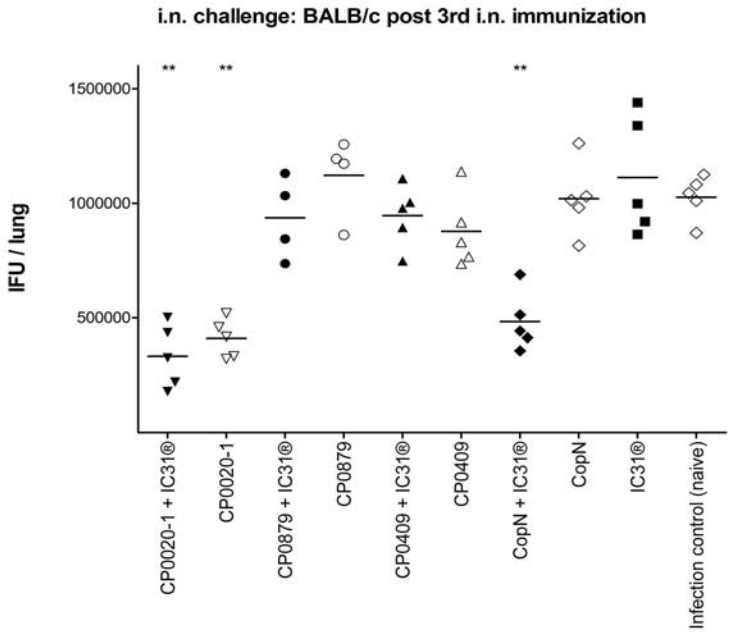

Figure 23: CP0020-1, CP0879, CP0409: Assessment of IFUs 10 days post i.n. challenge: BALB/c mice were infected i.n. with each $1*10^6$ IFUs 10 days following 3rd i.n. vaccination with each 40 µg of antigen per mouse including or not including IC31® as an adjuvant. As control groups served mice immunized 3 times intra nasally with IC31®-adjuvanted CopN, IC31® alone and naïve mice as infection control. * = $p < 0.05$; ** = $p < 0.01$ (Mann-Whitney-U-Test).

Importantly, intra nasal immunizations with the adjuvant IC31® alone did not show to have a significant effect in reducing bacterial burdens or in deteriorating infection in the lungs of *C. pneumoniae*-challenged mice.

4.5 Characterization of T cell responses using peptide libraries

Based on immunogenicity and efficacy data the two top protective antigens, CP0177-1 and CP0020-1, were selected in order to further characterize T cell responses using peptide libraries consisting of 15-mers, overlapping by 10 residues and spanning the entire sequence of these recombinant proteins. For CP0177-1 a peptide library of 76 15-mers was generated covering the 382 residues. In the case of CP0020-1, which consists of 466 residues, 86 15-mers were generated. Peptides of the respective recombinant proteins were denominated according to their position inside the synthesis plate (A1, A2, A3…A12, B1…) until the last position.

As the first part of T cell response characterization and epitope mapping, the entire peptide libraries were used for *ex vivo* restimulation of spleen cells derived from immunized BALB/c mice. Immunizations were performed s.c. and i.d., at the tail base with 100 µl of vaccine per injection, either with a total of 40 µg recombinant protein alone or in combination with IC31® (100/4) as an adjuvant.

In addition, peptide specific induction of lymphocyte proliferation was assessed by flow cytometric analysis using carboxyfluorescein diacetate succinimidyl ester (CFSE) combined with selective staining of $CD3^+$ T cells and $CD4^+$ T cells. The application of this method enabled the differentiation between proliferating $CD3^+$ T cell- and $CD4^+$ T cell populations, thereby providing particularized information about the responding T cell sub-population.

The second part of T cell response characterization involved peptide library screening using spleen cells derived from *Cpn* K6 infected/reinfected BALB/c and C57BL/6 mice, giving information about epitopes to which infection primed T cells of the respective mouse strain react. Naïve mice served as control groups in all screening experiments. Each experimental group consisted of 5 female mice.

4.5.1 Characterization of T cell responses using the CP0177-1 peptide library

The results of CP0177-1 T cell epitope mapping by IFN-γ ELIspot of spleen cells derived from s.c. + i.d. immunized animals are shown in figure 24A-C. No protruding signal was obtained in this screening round, though restimulation with 10 µg/ml of the whole protein resulted in 120 IFN-γ

SFC/0.5*10^6 in the group immunized with the adjuvanted antigen and 45 IFN-γ SFC/0.5*10^6 in the group immunized with CP0177-1 alone (not shown).

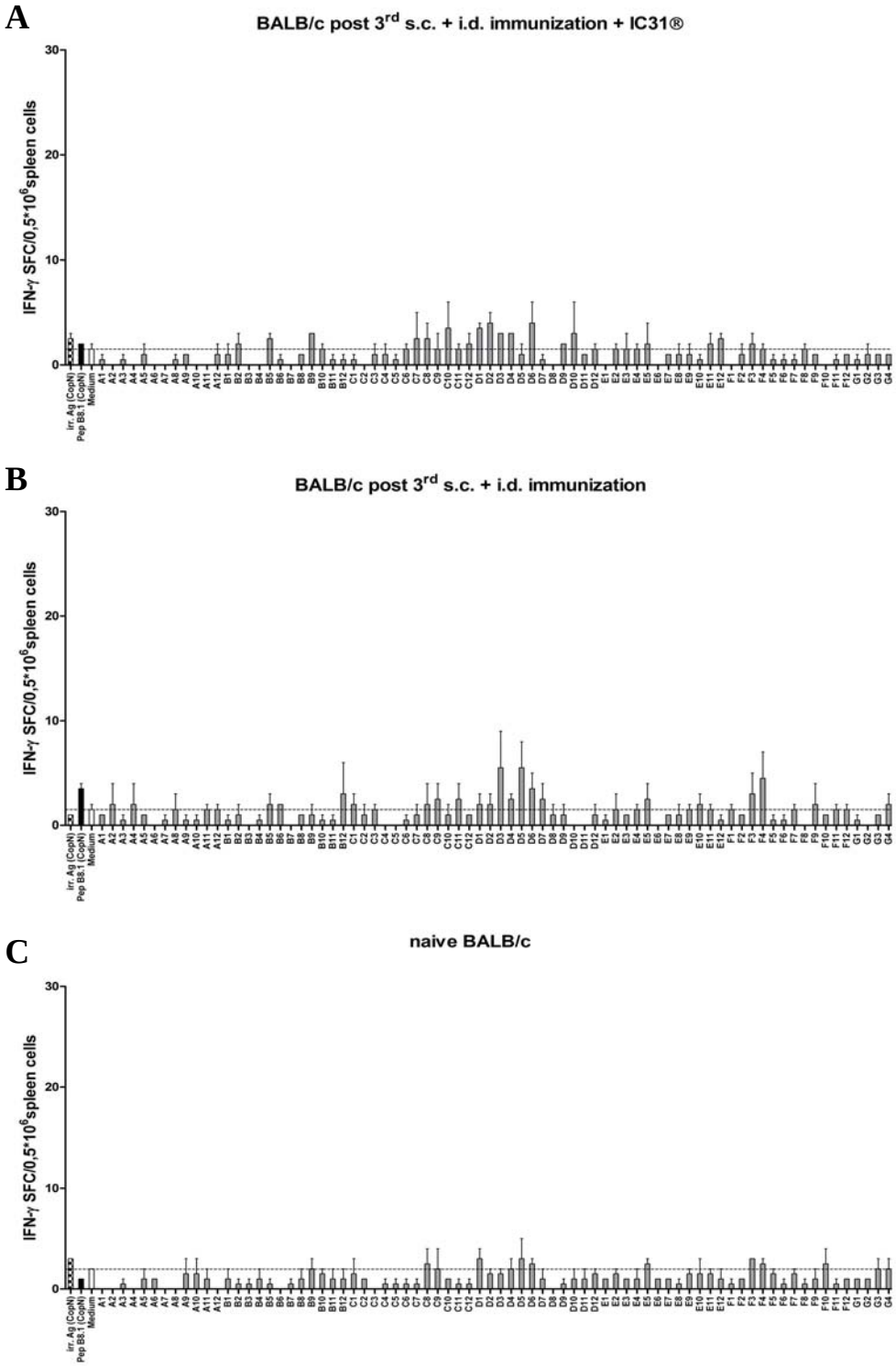

Figure 24: IFN-γ ELIspot 10 days post 3rd immunization: CP0177-1 peptide library screening using spleen cells derived from BALB/c mice immunized 3 times s.c. and i.d. with **A:** 40 µg CP0177-1 + IC31® (100/4); **B**: 40 µg CP0177-1; **C**: naïve control group; in a distance of 14 days. *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for CopN and 2 µg/ml for peptide B8.1 (CopN). Mean ± SD. SFC (Spot Forming Cells).

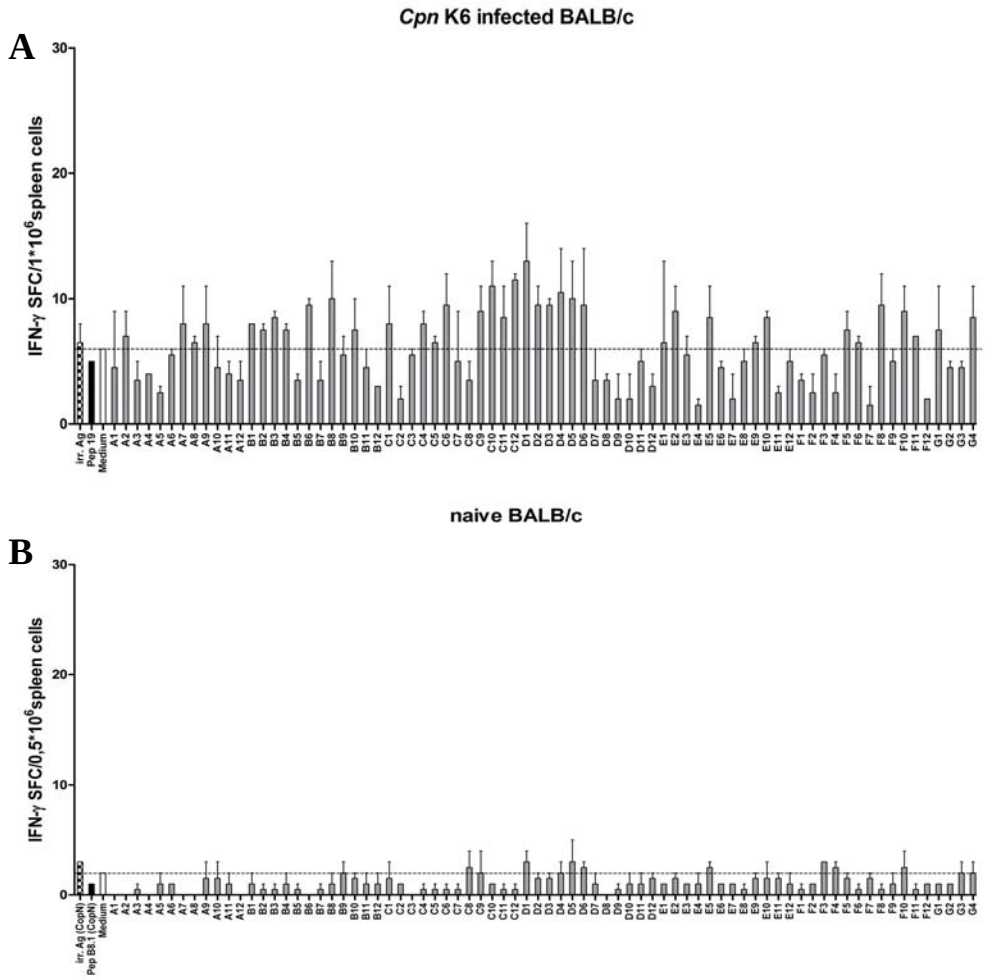

Figure 25: IFN-γ ELIspot 12 days post reinfection: CP0177-1 peptide library screening using spleen cells derived from infected/reinfected BALB/c mice; **A:** infected/reinfected BALB/c **B**: naïve control group; *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer, as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for BAP038 and 5 µg/ml for peptide 19. Mean ± SD. SFC (Spot Forming Cells).

However, a cluster of IFN-γ responses, ranging from 15-mer D1 to D6 was observed in both groups, indicating the presence of stimulatory properties in terms of IFN-γ secretion inside this cluster. Elevated IFN-γ levels in response to these peptides were additionally observed in spleen cells derived from *Cpn* K6 infected BALB/c but not C57BL/6 mice (figures 25 and 26).

In dissent, stimulatory characteristics for these 15-mers were not seen in proliferation assays with the only exception of peptide D1 (SILNFLKNPAVQ QKM), which also showed the strongest IFN-γ response in ELIspots with spleen cells derived from infected/reinfected BALB/c mice (figure 25A and 28A).

In CFSE-$CD3^+$-$CD4^+$ FCM analysis, further induction of proliferation was only observed in groups restimulated with the whole protein CP0177-1 and in response to restimulation with peptides G2 (GATVVPNVNVNLGGI)

or G3 (PNVNVNLGGILEHHH) (figures 27 and 28). Though, neither of the peptides led to significant increases in IFN-γ secretion following *ex vivo* restimulation of spleen cells derived from immunized animals.

Interestingly, restimulation of spleen cells, derived from mice immunized with adjuvanted CP0177-1, with 10 µg/ml of the whole protein induced strong proliferation, not only in the $CD4^+$ T cell population, but also in a $CD3^+$ T cell population that was not $CD4^+$, leaving $CD8^+$ T cells as candidates. However, this response was not observed in the experimental group immunized with the antigen alone, where mainly $CD4^+$ T cells responded by proliferation upon restimulation and proliferative responses observed in the $CD3^+$ T cell population of the naïve group surpassed the ones seen in the group immunized with CP0177-1 alone (figure 27 A).

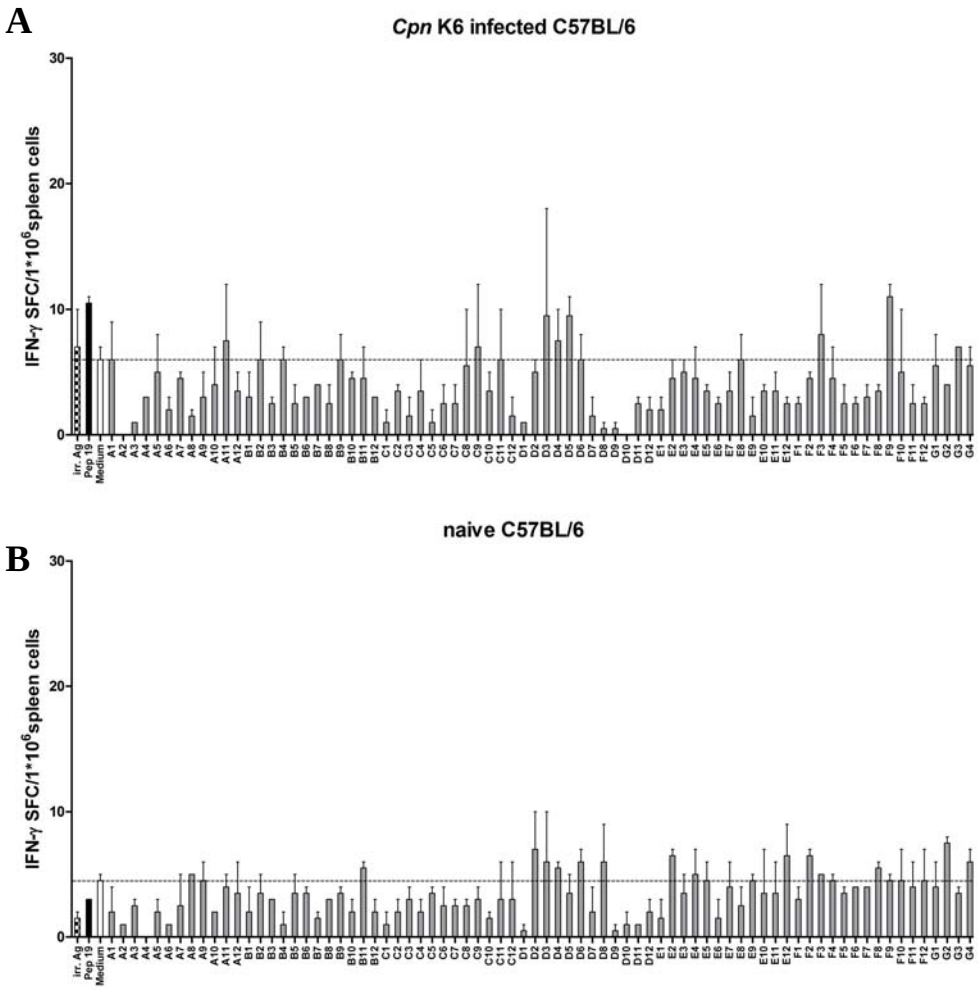

Figure 26: IFN-γ ELIspot 12days post reinfection: CP0177-1 peptide library screening using spleen cells derived from infected/reinfected C57BL/6 mice; **A:** infected/reinfected C57BL/6 **B**: naïve control group; *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer, as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for BAP038 and 5 µg/ml for peptide 19. Mean ± SD. SFC (Spot Forming Cells).

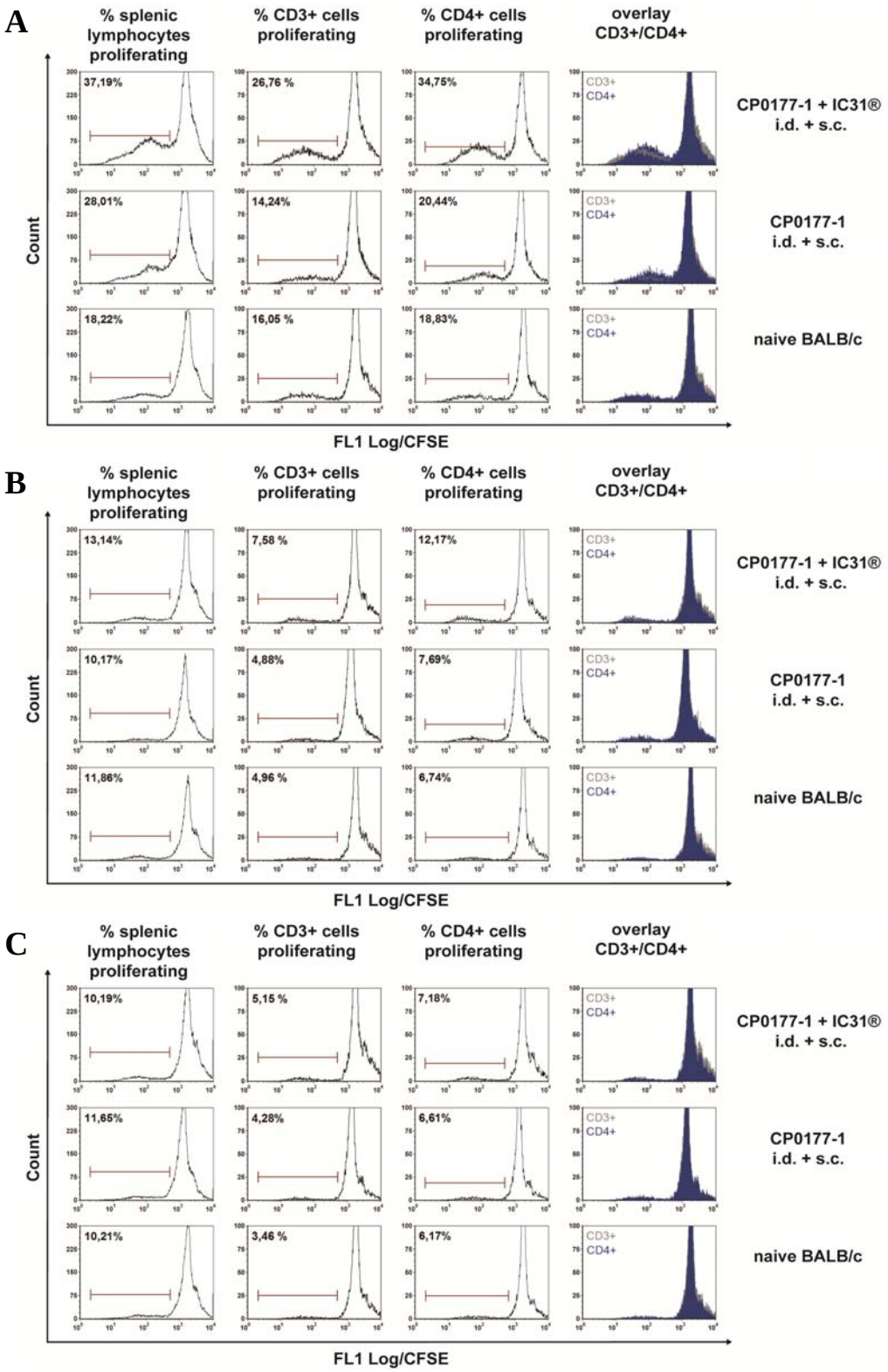

Figure 27: CFSE-CD3$^+$-CD4$^+$ differential proliferation screening. Spleen cells of CP0177-1 s.c. + i.d. immunized BALB/c mice 10 days post 3rd immunization: **A**: Restimulation with 10 µg/ml CP0177-1; **B**: Restimulation with 10 µg/ml irrelevant Peptide B8.1 (CopN); **C**: Restimulation with TCM; Experimental groups are indicated on the right. Histograms are shown from FSC/SSC gated lymphocytes, APC gated CD3$^+$ T cells and PE-Cy7 gated CD4$^+$ T cells as indicated on the top of each row. Percent of proliferating cells of the respective lymphocyte population are shown in the upper left quadrant of each histogram.

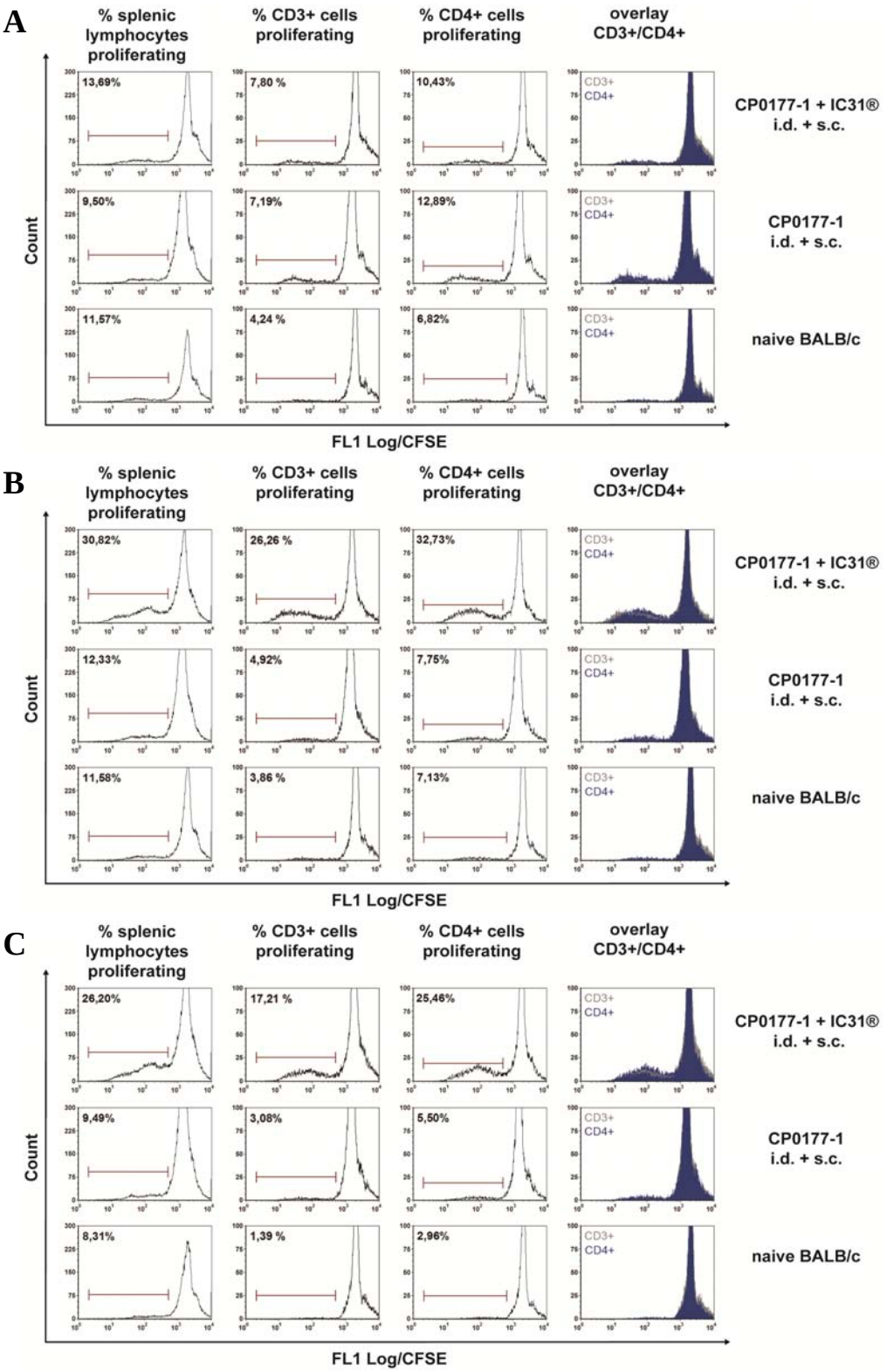

Figure 28: CFSE-CD3$^+$-CD4$^+$ differential proliferation screening. Spleen cells of CP0177-1 s.c. + i.d. immunized BALB/c mice 10 days post 3rd immunization: **A:** Restimulation with 10 µg/ml D1 (SILNFLKNPAVQQKM) **B**: Restimulation with 10 µg/ml G2 (GATVVPNVNVNL GGI); **C**: Restimulation with 10 µg/ml G3 (PNVNVNLGGILEHHH); Experimental groups are indicated on the right. Histograms are shown from FSC/SSC gated lymphocytes, APC gated CD3$^+$ T cells and PE-Cy7 gated CD4$^+$ T cells as indicated on the top of each row. Percent of proliferating cells of the respective lymphocyte population are shown in the upper left quadrant of each histogram.

4.5.2 Characterization of T cell responses using the CP0020-1 peptide library

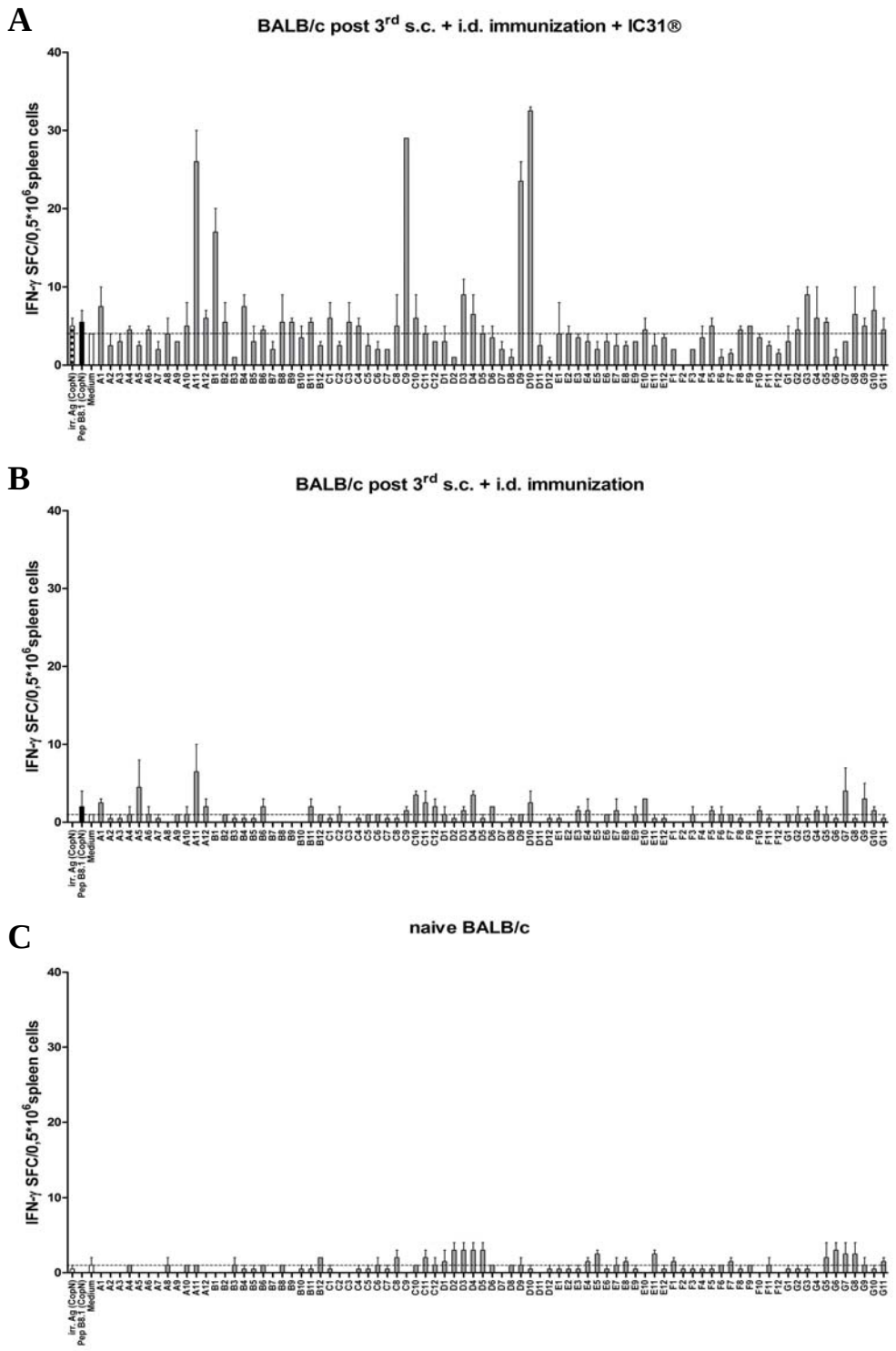

Figure 29: IFN-γ ELIspot 10 days post 3rd immunization: CP0020-1 peptide library screening using spleen cells derived from BALB/c mice immunized 3 times s.c. and i.d. with **A:** 40 µg CP0020-1 + IC31® (100/4); **B**: 40 µg CP0020-1; **C**: naïve control group; in a distance of 14 days. *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for CopN and 2 µg/ml for peptide B8.1 (CopN). Mean ± SD. SFC (Spot Forming Cells).

In contrast to CP0177-1, CP0020-1 peptide library screening led to more distinguished results. As IFN-γ ELIspots from immunized BALB/c mice showed, five peptides namely A11, B1, C9, D9 and D10 stimulated a significant increase in IFN-γ secretion compared to the respective control (figure 29A-C). Though, positive stimulation was only observed in the experimental group immunized with adjuvanted CP0020-1, with the exception of peptide A11 which also showed to elicit elevated IFN-γ secretion in the group immunized with CP0020-1 alone.

Responses in IFN-γ ELIspot to restimulation with 10 µg/ml of the whole protein where 200 SFC/0.5*10^6 and 91 SFC/0.5*10^6 in the group immunized with IC31® as an adjuvant and the group which received CP0020-1 alone respectively (not shown).

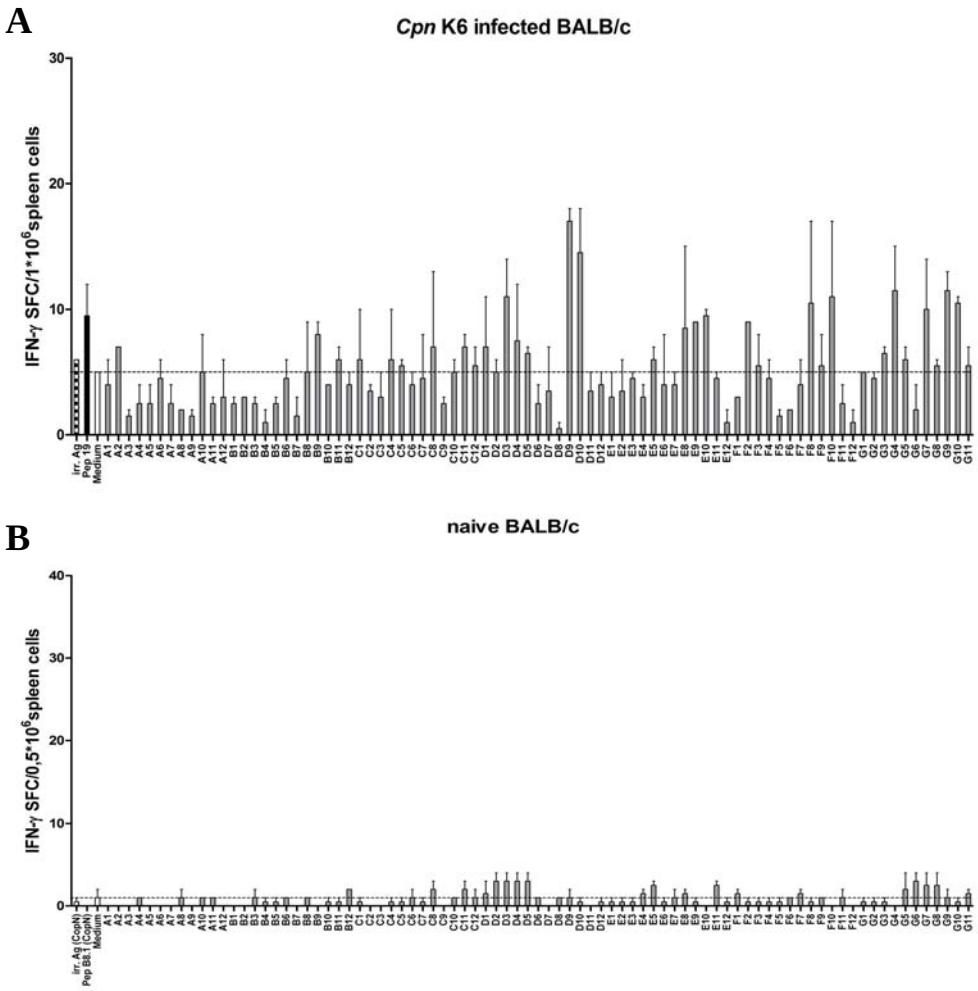

Figure 30: IFN-γ ELIspot 12 days post reinfection: CP0020-1 peptide library screening using spleen cells derived from infected/reinfected BALB/c mice; **A:** infected/reinfected BALB/c **B**: naïve control group; *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer, as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for BAP038 and 5 µg/ml for peptide 19. Mean ± SD. SFC (Spot Forming Cells).

Interestingly, peptides D9 and D10 also showed to be the strongest peptides stimulating IFN-γ secretion in ELIspots, using spleen cells from *Cpn* K6 infected/reinfected BALB/c mice, indicating that these peptides were also recognized during *C. pneumoniae* infection. In dissent, IFN-γ ELIspots of spleen cells from infected/reinfected C57BL/6 mice showed to respond in highest levels to restimulation with peptides D3 (LAGKYGNQATLTQTF) and D4 (GNQATLTQ TFADGRV).

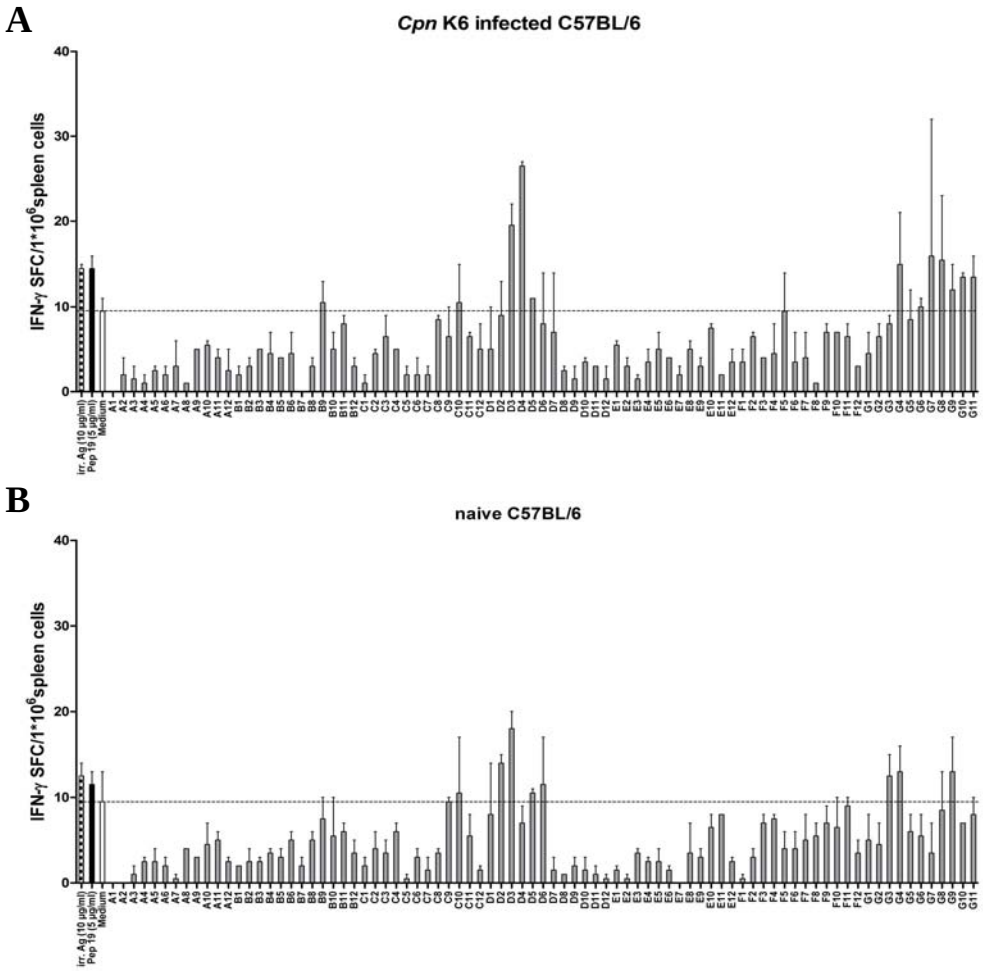

Figure 31: IFN-γ ELIspot 12 days post reinfection: CP0020-1 peptide library screening using spleen cells derived from infected/reinfected C57BL/6 mice; **A:** infected/reinfected C57BL/6 **B**: naïve control group; *Ex vivo* restimulation was conducted with 5 µg/ml of the respective 15-mer, as shown along the X-axis. Concentrations of irrelevant antigens were 10 µg/ml for BAP038 and 5 µg/ml for peptide 19. Mean ± SD. SFC (Spot Forming Cells).

Regarding the induction of lymphocyte proliferation upon *ex vivo* restimulation, FCM analysis showed that especially peptides A11, D9 and D10 elicited cellular responses (figure 33A-C). Primarily $CD4^+$ T cells were found to react by proliferation in response to restimulation with these peptides, whereas peptide D10 clearly showed the strongest inductive potential.

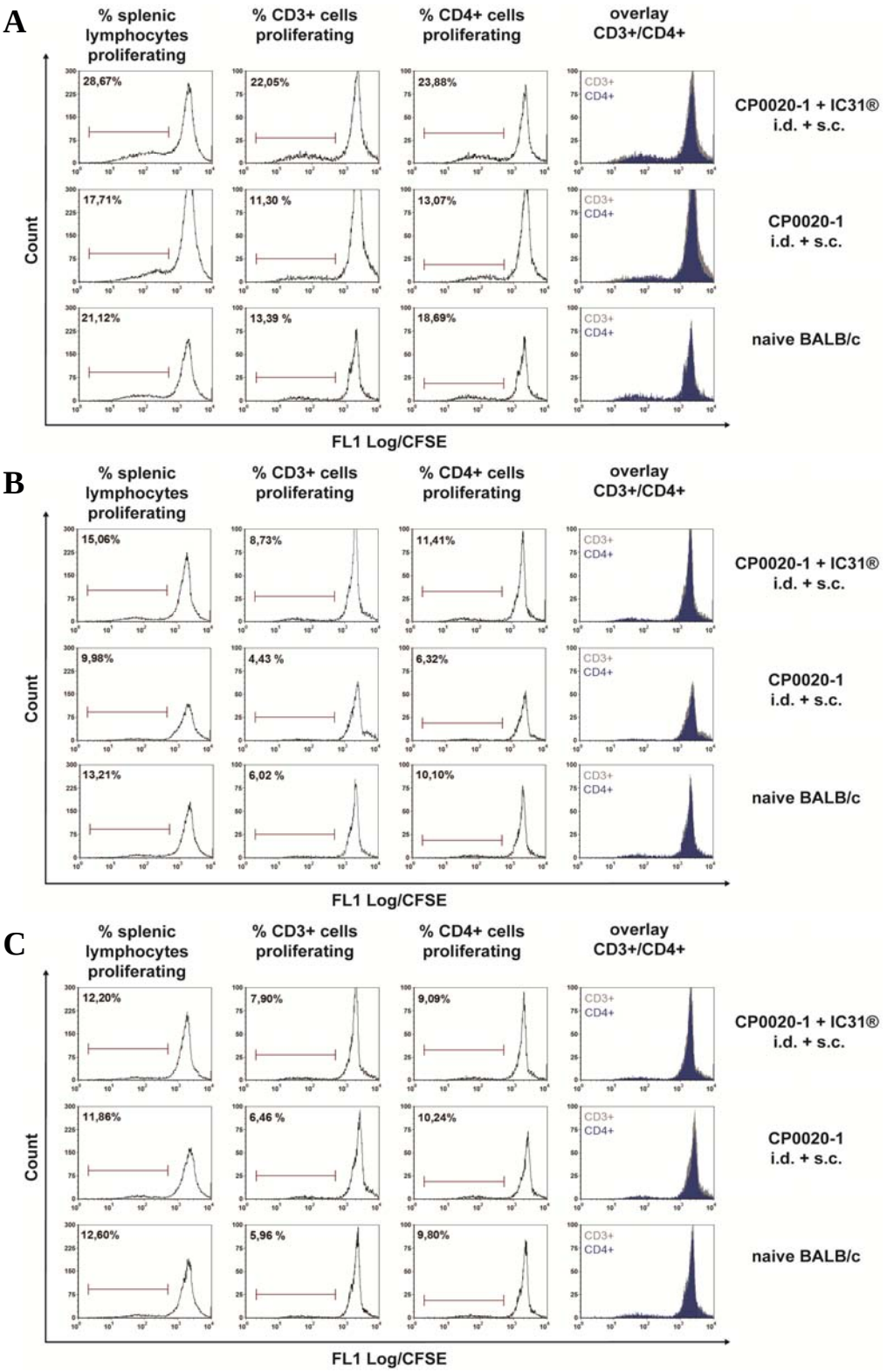

Figure 32: CFSE-CD3$^+$-CD4$^+$ differential proliferation screening. Spleen cells of CP0020-1 s.c. + i.d. immunized BALB/c mice 10 days post 3rd immunization: **A**: Restimulation with 10 µg/ml CP0020-1 **B**: Restimulation with 10 µg/ml irrelevant peptide B8.1 (CopN); **C**: Restimulation with TCM. Experimental groups are indicated on the right. Histograms are shown from FSC/SSC gated lymphocytes, APC gated CD3$^+$ T cells and PE-Cy7 gated CD4$^+$ T cells as indicated on the top of each row. Percent of proliferating cells of the respective lymphocyte population are shown in the upper left quadrant of each histogram.

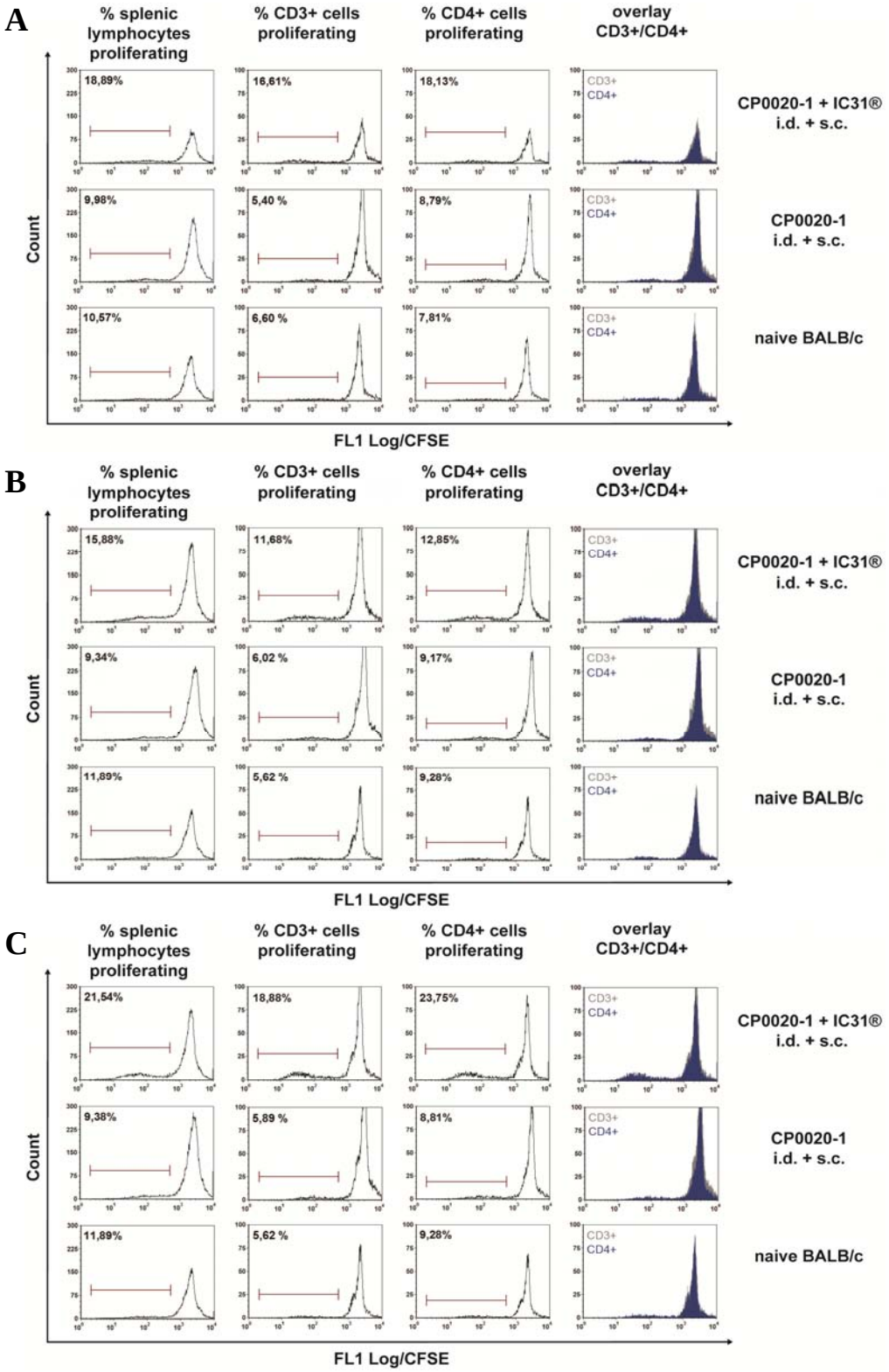

Figure 33: CFSE-CD3$^+$-CD4$^+$ differential proliferation screening. Spleen cells of CP0020-1 s.c. + i.d. immunized BALB/c mice 10 days post 3rd immunization: **A**: Restimulation with 10 µg/ml A11 (LHKYEVERATVAKSI); **B**: Restimulation with 10 µg/ml D9 (QGVLTPEQFTIF AEI); **C**: Restimulation with 10 µg/ml D10 (PEQFTIFAEIATELQ). Experimental groups are indicated on the right. Histograms are shown from FSC/SSC gated lymphocytes, APC gated CD3$^+$ T cells and PE-Cy7 gated CD4$^+$ T cells as indicated on the top of each row. Percent of proliferating cells of the respective lymphocyte population are shown in the upper left quadrant of each histogram.

Additionally peptides A12, B1, G3, G8 and G9 elicited proliferative responses (not shown), while B1 and G3 where also found to give positive signals in IFN-γ ELIspot from immunized animals and G9 in IFN-γ ELIspot from infected /reinfected BALB/c mice, but not vice versa.

Restimulation with the whole protein CP0020-1 in proliferation experiments resulted in proliferative responses in the $CD4^+$ T cell population as well as in a $CD3^+$ $CD4^-$ T cell population. This induction of most probably $CD8^+$ T cells, was not only observed in the group immunized with CP0020-1 adjuvanted by IC31® but also in the group that was immunized with the recombinant antigen alone (figure 32A-C).

4.6 Immunofluorescence microscopy

With the purpose to localize the selected *Cpn* antigens, sera from immunized BALB/c mice were used for the detection of proteins in *Cpn* K6 infected HEp-2 cells using fluorochrome-labeled anti-mouse antibodies. Supplementary, anti-Cp0585 antibodies were used for the visualization if the inclusion membrane, as protein Cp0585 is a well characterized antigen present in the inclusion of *Cpn.*

As shown in figure 34A and B anti-CP0529 sera reacted with *Cpn* RBs at 44 hours post infection. No reaction of anti-CP0529 sera was observed with *Cpn* EBs at this time point.

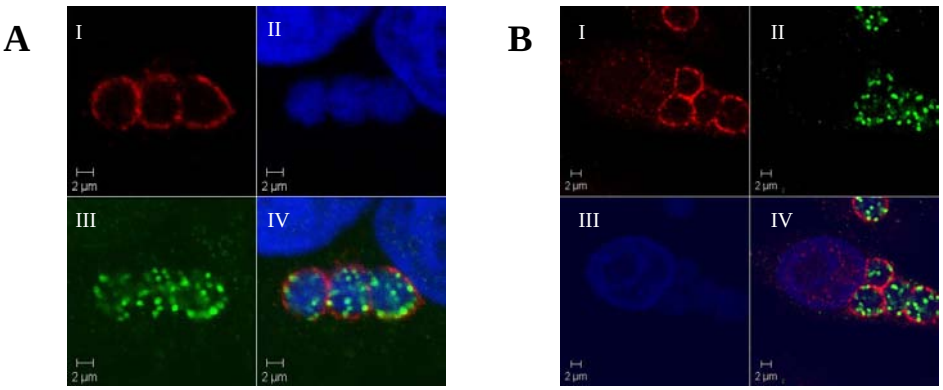

Figure 34: CP0529 Immunofluorescence; **A**: HEp-2 cells 44 hours post infection; **AI**: anti-Cp0585; **AII**: DAPI; **AIII**: anti-CP0529 serum; **AIV**: overlay; **B**: HEp-2 cells 44 hours post infection; **BI**: anti-Cp0585; **BII**: anti-CP0529 serum; **BIII**: DAPI; **BIV**: overlay; $3*10^5$ HEp-2 cells were plated on 13 mm diameter glass slides in 24-well plates and incubated for 20 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 44 hours followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0529 40 µg/mouse + IC31® and collected by terminal bleeding 10 days post 3rd i.p. immunization.

The respective control is shown in figure 40A, where unspecific reactivity and a tendency of these antibodies to oligomerization were observed.

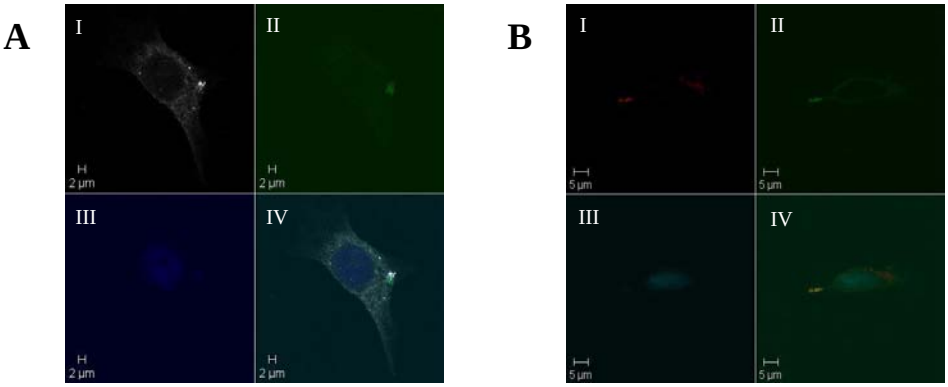

Figure 35: CP0177-1 Immunofluorescence; **A**: HEp-2 cells 1 hour post infection; **AI**: anti-Cp0585 (white); **AII**: anti-CP0177-1 serum; **AIII**: DAPI; **A-IV**: overlay; **B**: HEp-2 cells 1 hour post infection; **BI**: anti-Cp0585; **BII**: anti-CP0177-1 serum; **BIII**: DAPI; **BIV**: overlay; $1.5*10^5$ HEp-2 cells were plated on 13 mm diameter glass slides in 24-well plates and incubated for 21 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 1 hour followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0177-1 40 µg/mouse + IC31® and collected by terminal bleeding 10 days post 3rd i.n. immunization.

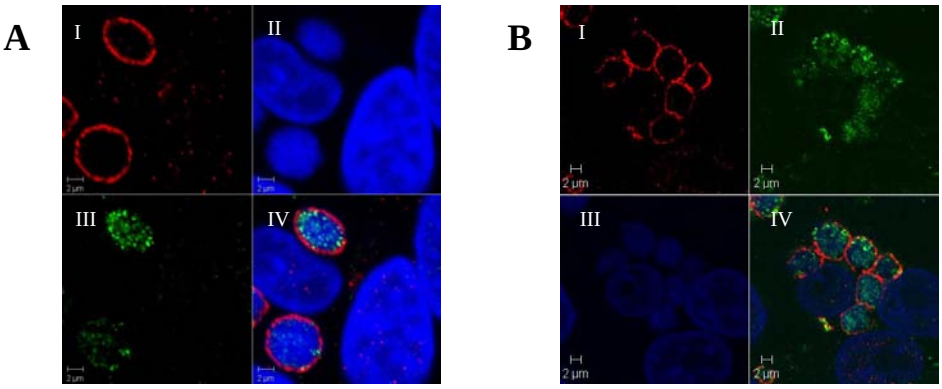

Figure 36: CP0020-1 Immunofluorescence; **A**: HEp-2 cells 44 hours post infection; **AI**: anti-Cp0585; **AII**: DAPI; **AIII**: anti-CP0020-1 serum; **AIV**: overlay; **B**: HEp-2 cells 44 hours post infection; **BI**: anti-Cp0585; **BII**: anti-CP0020-1 serum; **BIII**: DAPI; **BIV**: overlay; $3*10^5$ HEp-2 cells were plated on 13mm diameter glass slides in 24-well plates and incubated for 20 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 44 hours followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0020-1 40 µg/mouse + IC31® and collected by terminal bleeding 10 days post 3rd i.p. immunization.

Sera derived from mice immunized with the recombinant *C. pneumoniae* antigen CP0177-1 did not show any reactivity at 44 h post infection (not shown). According to earlier studies dealing with CP0177 orthologs, in addition to the 44 hours time point, immunofluorescent staining of infected HEp-2 cells was additionally performed at 1 hour post infection. Reaction of sera derived from animals, immunized 3 times i.p. with CP0177-1 in combination with IC31®, clearly showed to react with *Cpn* EBs at the earlier time point, which was corresponding to previous investigations [79, 90].
Figure 35A+B show *Cpn* EBs visualized by anti-CP0177-1 reactivity located at the putative entry site of the *Chlamydia*. Interestingly, a co localized signal for Cp0585 was observed indicating the formation of the early inclusion.

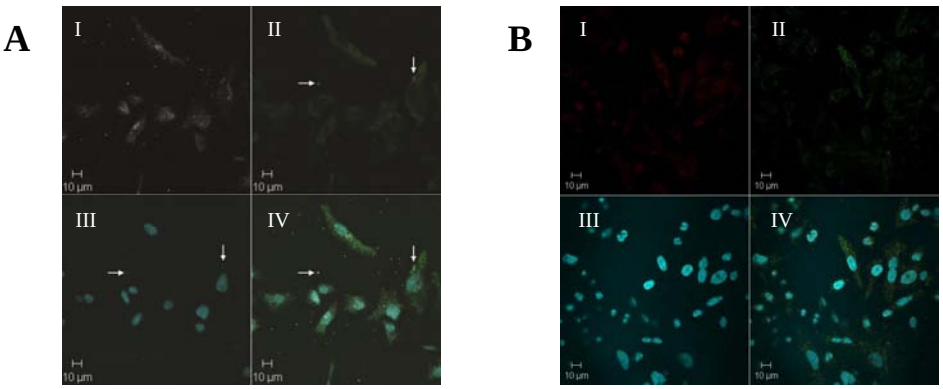

Figure 37: CP0504 Immunofluorescence; **A**: HEp-2 cells 1 hour post infection; **AI**: anti-Cp0585 (white); **AII**: anti-CP0504 serum; **AIII**: DAPI; **AIV**: overlay; **B**: uninfected HEp-2 cells; **BI**: anti-Cp0585 (white); **BII**: anti-CP0504 serum; **BIII**: DAPI; **BIV**: overlay; $1.5*10^5$ HEp-2 cells were plated on 13mm diameter glass slides in 24-well plates and incubated for 21 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 1 hour followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0504 40 µg /mouse + IC31® and collected by terminal bleeding 10 days post 3rd i.n. immunization.

Sera of CP0020-1 immunized BALB/c mice showed to react with *Cpn* RBs at 44 hours post infection (figure 36A+B). Furthermore reaction with *Cpn* EBs was observed at this time point, but seemed less strong than seen for RBs.
Immunofluorescent control straining of uninfected HEp-2 cells using sera derived from CP0177-1 and CP0020-1 immunized animals are shown in figure 40B+C respectively.

The signals obtained with anti-CP0504 and anti-CP0879 sera both suggest a specific reaction with *Cpn* EBs at 1 hour post infection, while unspecific reactivity seemed stronger with sera from CP0879 immunized

animals. No specific signal was obtained for either antigen at 44 hours post infection using the same sera, which excludes the presence of these antigens on *Cpn* RBs (not shown).

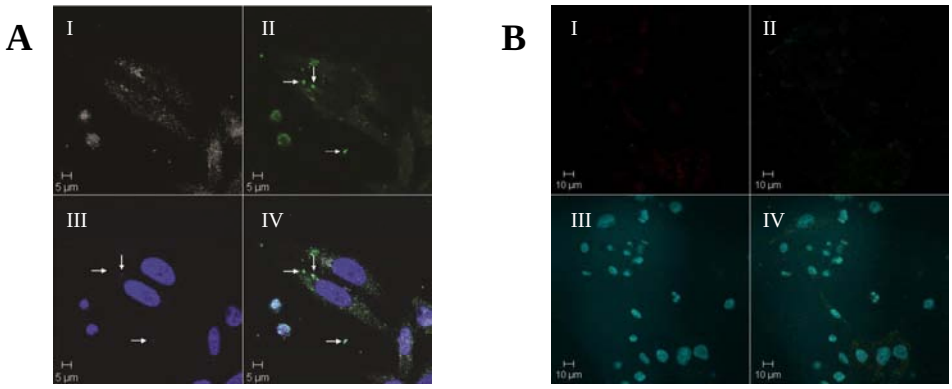

Figure 38: CP0879 Immunofluorescence; **A**: HEp-2 cells 1 hour post infection; **AI**: anti-Cp0585 (white); **AII**: anti-CP0879 serum; **AIII**: DAPI; **AIV**: overlay; **B**: uninfected HEp-2 cells; **BI**: anti-Cp0585 (white); **BII**: anti-CP0504 serum; **BIII**: DAPI; **BIV**: overlay; $1.5*10^5$ HEp-2 cells were plated on 13mm diameter glass slides in 24-well plates and incubated for 21 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 1 hour followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0879 40 µg /mouse + IC31® and collected by terminal bleeding 6 days post 3rd i.n. immunization.

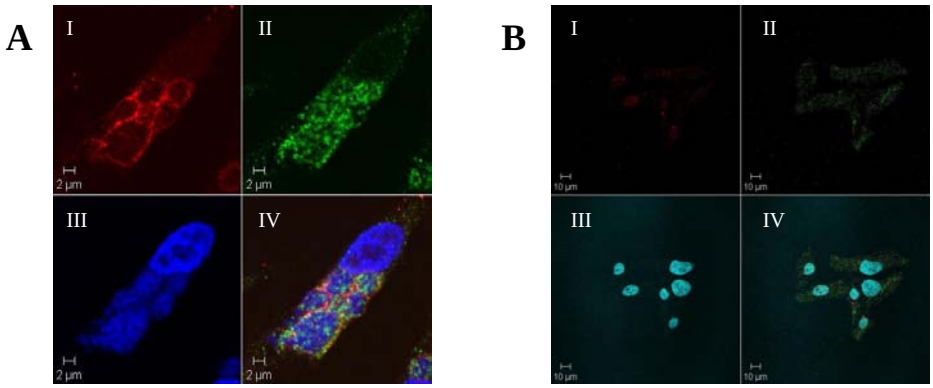

Figure 39: CP0409 Immunofluorescence; **A**: HEp-2 cells 44 hours post infection; **AI**: anti-Cp0585 (red); **AII**: DAPI; **AIII**: anti-CP0409 serum; **AIV**: overlay; **B**: uninfected HEp-2 cells; **BI**: anti-Cp0585 (red); **BII**: anti-CP0409 serum; **BIII**: DAPI; **BIV**: overlay; $3*10^5$ HEp-2 cells were plated on 13mm diameter glass slides in 24-well plates and incubated for 21 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 44 hours followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with CP0020-1 40 µg/mouse + IC31® and collected by terminal bleeding 6 days post 3rd i.n. immunization.

CP0409 was found to be located on both *Cpn* RBs and *Cpn* EBs, as signals obtained at 44 hours post infection did show (figure 39A), though reactivity with EBs seemed inferior. Furthermore, weak unspecific reactivity of

these sera, located at the cytoplasm of HEp-2 cells, was observed in uninfected samples (figure 39B).

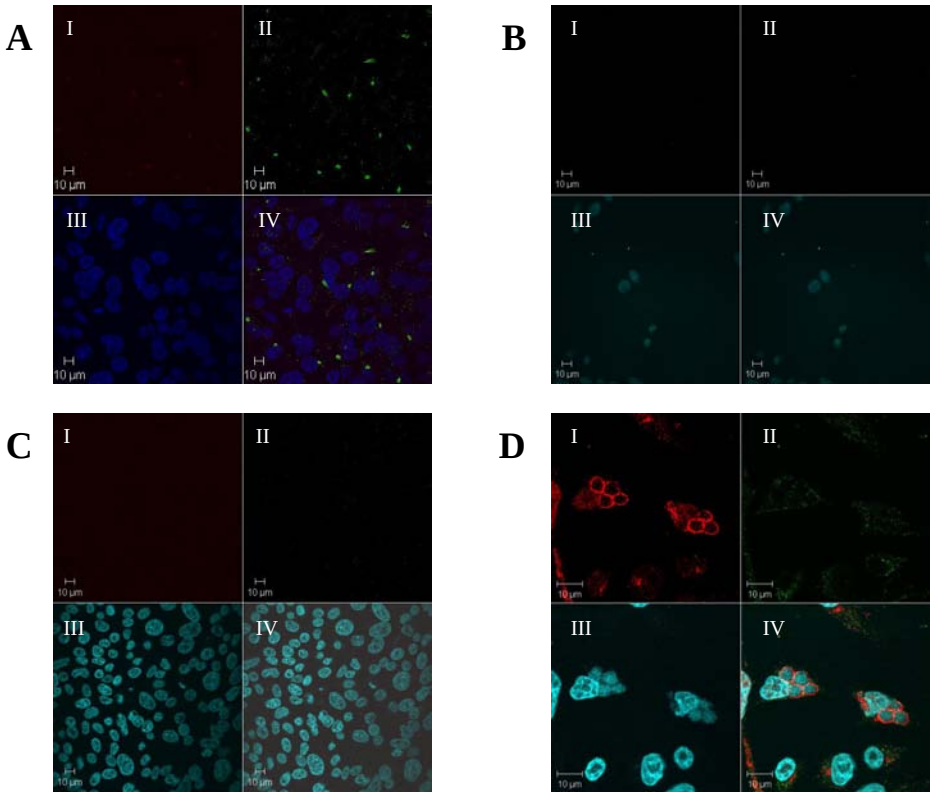

Figure 40: CP0529, CP0177-1, CP0020-1, Adjuvant controls; Immunofluorescence; **A**: uninfected HEp-2 cells; **AI**: anti-Cp0585 (red); **AII**: anti-CP0529 serum; **AIII**: DAPI; **AIV**: overlay; **B**: uninfected HEp-2 cells; **BI**: anti-Cp0585 (red); **BII**: anti-CP0177-1 serum; **BIII**: DAPI; **BIV**: overlay; **C**: uninfected HEp-2 cells; **CI**: anti-Cp0508; **CII**: anti-CP0020-1 serum; **CIII**: DAPI; **CIV**: overlay; **D**: HEp-2 cells 44 hours post infection; **DI**: anti-Cp0508; **DII**: anti-IC31® serum; **DIII**: DAPI; **DIV**: overlay; **A, C**: $3*10^5$ HEp-2 cells were plated on 13mm diameter glass slides in 24-well plates and incubated for 20 hours. Immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI was performed after another incubation period of 44 hours. **B**: $1.5*10^5$ HEp-2 cells were plated on 13 mm diameter glass slides in 24-well plates and incubated for 21 hours. Immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI was performed after another incubation period of 1 hour. **D**: $1.5*10^5$ HEp-2 cells were plated on 13 mm diameter glass slides in 24-well plates and incubated for 21 hours before infection with *Cpn* K6 at a MOI of 2. Subsequently HEp-2 cells were incubated for 44 hours followed by immunofluorescent staining of the inclusion membrane, the respective antigen and DNA staining with DAPI. Sera were derived from mice immunized 3 times with **A**: CP0529 40 µg/mouse + IC31® i.p.; **B**: CP0177-1 40 µg/mouse + IC31® i.n.; **C**: CP0020-1 40 µg/mouse + IC31® i.p.; **D**: IC31® (20/0.8) only i.n. All sera were obtained 10 days post 3rd immunization by terminal bleeding.

Figure 40D shows an immunofluorescent staining of *Cpn* K6 infected HEp-2 cells using sera from BALB/c mice immunized 3 times i.n. with the adjuvant IC31® alone. Slight unspecific reactivity was observed in the green (anti-IC31®) channel.

In general, immunizations of mice with the diverse *Cpn* antigens in combination with IC31® resulted in superior intensities of signals in immunofluorescence microscopy compared to signals obtained from sera of mice immunized with the antigen alone. This was especially observed when sera from intra nasal immunizations were used as primary antibodies.

Exceptions were candidate antigens CP0409 and CP0020-1, which gave comparable intensities when administered to mice with and without adjuvantation. Furthermore, intra peritoneal immunizations led to a higher specificity of antibody reaction in immunofluorescence assays compared to intra nasal immunizations, as the later showed an overall greater tendency for unspecific reactions (own observations).

5 Discussion

The application of the AIP® technology to various human pathogens has already led in the past to the identification of promising novel subunit vaccine candidates. The results of this study demonstrated that also in the case of *C. pneumoniae* the serological antigen identification on a genome-wide basis is an efficient tool for the discovery of antigenic proteins that are furthermore in the majority highly immunogenic and potentially protective.

Regarding the adjuvantive effect of IC31® in combination with the diverse recombinant *Cpn* antigens, which were selected on the basis of the results obtained by the AIP® technology, the data obtained during this study strongly supports the efficacy and efficiency of this adjuvant in mucosal applications. In general IC31® strongly improved the immunogenicity of the tested recombinant proteins by enhancing the production of the type 1 interferon IFN-γ as well as antigen specific immunoglobulin responses and induction of CD154 cell marker on $CD4^+$ T cells. Importantly the adjuvantive effect of IC31® was observed in local as well as systemic compartments following intra nasal application. Several findings, besides the improved IFN-γ production, support the dominating type 1 immune response-inducing effect of IC31®: No substantial enhancing effect on IL-4 secretion was observed comparing adjuvanted and antigen-alone immunizations. The secretion of IL-4

in response to restimulation of spleen cells derived from mice immunized i.p. with IC31®-adjuvanted CP0529, for which strong T_H2-type stimulatory properties were identified, was even reduced compared to the experimental group that had received the protein antigen alone. Furthermore, the protective effect of those antigens which showed to have a significant influence on the reduction of bacterial loads (CP0529, CP0177-1, CP0020-1, CP0504) in the lungs of *Cpn* K6 challenged animals, where the importance of the induction of type 1 immune responses for protection is generally accepted, was improved by the co administration of the adjuvant IC31®. Though, intern differences at experimental groups immunized with the antigens CP0020-1 and CP0529 where not significant. Additionally, intra nasal immunization of BALB/c mice with the well characterized *Cpn* T cell antigen CopN in combination with IC31® showed to have a substantial reductive impact on the bacterial burden in murine lungs during challenge studies. Importantly, intra nasal administration of IC31® alone did not influence the outcome of challenge experiments nor were any toxic effects observed in experimental groups that had received this adjuvant.

In more detail, each of the 6 selected *Cpn* antigens showed its own pattern of immune responses directed against the particular protein, following immunization with the purified antigen, as well as in infection primed immune response studies. The results of both antigen specific immune responses were correlated to the data obtained in *Cpn* challenge experiments.

CP0529 protein, which was found to be located on *Cpn* RBs but not EBs by immunofluorescence microscopy, was not immunogenic in terms of IFN-γ production as evaluated by ELIspot from murine splenic lymphocytes when delivered via the intra nasal route and additionally failed to elicit systemic immune responses in terms of CD154 upregulation on splenic $CD4^+$ T cells. On the other side, intra nasal administration of this protein antigen led to the upregulation of CD154 in local compartments and elicited strong immunoglobulin responses of the IgG isotype but failed to evoke antigen specific serum IgA. Infection primed immune responses directed against CP0529 revealed a similar picture, as no antigen specific IgA but only IgG could be detected in serum of infected/reinfected BALB/c and at a lower level in C57BL/6 mice. However, i.n. immunization of BALB/c mice with CP0529 did show to have a significant but modest effect on the number of IFUs in lungs derived from *Cpn* challenged animals, most probably induced by a strong local T_H2-type response. Altogether CP0529 is not a preferable vaccine candidate.

This statement is also true for candidates CP0409, CP0879 and candidate CP0504. CP0409 showed a general low immunogenicity as well as

an in this case undesired preferential induction of T_H2-type immune responses. Similar to CP0529, CP0409 was recognized during *Cpn* infection in BALB/c but not in C57BL/6 mice as assessed by cultured IFN-γ ELIspot, further supporting the T_H2 driven responses to these antigens. CP0879, a protein most probably located on *Cpn* EBs but not present in *Cpn* RBs did not reduce bacterial loads in lungs of intra nasally challenged BALB/c mice, though vaccination with the adjuvanted recombinant protein showed promising results in cellular and humoral immune response experiments. However, as CP0879 was only recognized in a low amount by antibodies but no antigen specific IFN-γ responses were seen in infection primed immune response studies, most probably this antigen is not able to induce protective T cell responses due to low exposure/accessibility. The unspecific induction of IFN-γ production, in response to restimulation with the whole protein, in these experiments was most probably due to the in comparison high level of LPS (182.58 EU/mg) present in the preparation of CP0879 recombinant protein.

Candidate CP0504, which gave similar results in immunofluorescence microscopy as CP0879, indicating the presence of this protein on *Cpn* EBs, but not RBs, was found to have a significant reductive effect on the bacterial burden in the lungs of *Cpn* K6 challenged animals when administered intra nasally in combination with the adjuvant IC31®. As assessed in infection primed immune response studies, CP0504 was recognized during *Cpn* K6 infection in both investigated mouse strains, indicated by systemic antigen specific IFN-γ responses as well as serum IgG and serum IgA responses. Though the latter was only found in C57BL/6 mice. In general the results obtained for candidate CP0504 during this study were quite encouraging but 2 protein candidates, namely CP0177-1 and CP0020-1, gave superior results in all evaluated criteria.

The conserved hypothetical protein CP0177-1 was identified by aa sequence homology as the *Ct* TARP ortholog. Reaction of anti-CP0177-1 sera with *Cpn* K6 EBs was observed at 1 hour post infection at the putative entry site of the *Chlamydia*, which was consistent with previous studies reporting the presence of TARP on *Cpn* EBs at this time point [79]. The recombinant protein CP0177-1, as assessed in immunization studies, was not immunogenic. In contrast, when CP0177-1 was combined with the adjuvant IC31®, strong immunogenicity in terms of systemic IFN-γ responses as well as local and systemic antigen-induced upregulation of CD154 cell marker was observed following intra nasal delivery. Furthermore, intra nasal immunization of mice with adjuvanted CP0177-1 induced the production of serum IgG and serum IgA antigen specific antibodies. Correlative, CP0177-1 in combination with IC31® led to a dramatic reduction of IFUs in the lungs of challenged mice,

while immunization with the protein alone did not show this effect. Using the CP0177-1 peptide library, for a more precise analysis of T cell responses, did not reveal a protruding immunogenic epitope. However, a cluster of peptides leading to intensified IFN-γ responses in ELIspot from spleen cells of both, immunized and infected/reinfected BALB/c mice, could be identified. A more detailed picture might have been obtained if the concentration of stimuli would have been increased to 10 µg/ml, as in ELIspot assays 5 µg/ml of each peptide were used as stimulus. However, FCM proliferation experiments using CFSE-$CD3^+$-$CD4^+$ staining revealed a strong inductive potential when spleen cells from CP0177-1 immunized animals were restimulated with 10 µg/ml of the whole protein. This potential was more pronounced in the group immunized in combination with the adjuvant IC31® and furthermore showed a greater induction of $CD3^+$ $CD4^-$ T cells, which were most probably $CD8^+$ T cells, in comparison to the experimental group which had received CP0177-1 alone. Peptide D1 (SILNFLKNPAVQQKM) was the only 15-mer identified as a positive stimulator of IFN-γ in ELIspots from immunized and infected/ reinfected BALB/c mice as well as in proliferation experiments, though responses were weak. Interestingly, peptides G2 and G3 showed strong induction of T cell proliferation following restimulation of spleen cells derived from mice immunized with adjuvanted CP0177-1, but did not stimulate IFN-γ responses. This T cell population may be composed of IL-4 secreting $CD4^+$ T cells and/or T-helper 17 cells, a subclass of $CD4^+$ T cells that is characterized by IL-23 and IL-17 secretion but not IFN-γ, and which has been found to be critical to augmenting recruitment of T_H1 cells in *M. tuberculosis* pulmonary infection as well as playing critical roles in mediating host mucosal immunity to a number of other pulmonary pathogens such as the Gram-negative bacteria *P. aeroginosa* and *K. pneumoniae* [92, 93].

Further support for the induction of T_H17 cells in response to CP0177-1 come from cytokine bead assay experiments where upon *in vitro* restimulation of spleen cells, which were derived from BALB/c mice 10 days post 3rd i.p. immunization, a substantial increase of IL-17 in culture supernatants was observed (not shown). IL-17 production in these experiments was even more pronounced in the group immunized with IC31®-adjuvanted CP0177-1, while IC31®-alone immunized and naïve mice did not show this effect. A potential source for the higher induction of IL-17 in experimental groups immunized with adjuvanted antigen are activated DCs that secrete IL-23 and thereby indirectly induce IL-17 production.

Regarding the role of IL-17 in chlamydial infection, various studies demonstrated the importance of this cytokine in initiating protective host immune response mechanisms in a pulmonary *C. muridarum* infection model

by for example promoting type 1 T cell immunity through the modulation of DC function [94-96]. However, further investigations respecting the role of IL-17 in intracellular bacterial infection are necessary in order to obtain a more conclusive picture about the importance of T_H17 cells in protection against *Chlamydia* infection as well as inflammatory pathologies associated with disease caused by *Chlamydia*.

CP0020-1 protein was the second highly immunogenic antigen which additionally revealed a distinctive protective effect in the process of this study. Regarding immunogenicity of CP0020-1, studies in BALB/c mice showed a substantial induction of IFN-γ in systemic compartments following intra nasal delivery of this antigen, as well as strong upregulation of CD154 cell marker on $CD4^+$ T cells derived from lungs and spleens of these animals. Interestingly, immunizations with CP0020-1 elicited strong antibody responses as evaluated in terms of serum IgG and serum IgA titers, which were in comparison less dependent on the adjuvant IC31® than observed with the other investigated antigens, indicating high $CD4^+$ T cell stimulatory properties of this *Cpn* antigen. Respecting i.n. *Cpn* infection primed immune responses, antigen specific IFN-γ responses induced by CP0020-1 were observed in both investigated mouse strains, while IFN-γ levels were clearly higher in BALB/c mice which was most probably due to the strong antigenic properties of CP0020-1. Furthermore, *Cpn* infection evoked high titers of CP0020-1 specific serum IgG and serum IgA. Correlative to the pronounced immune responses observed in experimental groups immunized i.n. with the antigen CP0020-1, either in combination with the adjuvant IC31® or with the protein alone, a substantial reductive effect on the number of IFUs in lungs of challenged BALB/c mice was observed in both groups in two independent experiments. Using sera from immunized BALB/c mice CP0020-1 was localized by immunofluorescence microscopy on *Cpn* RBs and EBs, while reaction seemed weaker with EBs.

As for CP0177-1, a peptide library consisting of 15-mers spanning the entire sequence of the CP0020-1 protein and overlapping by 10 residues was generated in order to evaluate T cell responses in more detail, as well as in order to identify immunogenic epitopes. As found by IFN-γ ELIspot using spleens from s.c. and i.d. immunized BALB/c mice, 5 immunogenic peptides were identified. Out of these 5, only peptide A11 additionally showed to elicit IFN-γ responses in mice immunized with the protein alone, though IFN-γ levels were dramatically lower than observed in mice that had received the adjuvanted protein. However, only peptides D9 (QGVLTPEQFTIFAEI) and D10 (PEQFTIFAEIATELQ) furthermore evoked IFN-γ responses during *Cpn* infection in BALB/c mice. Interestingly, peptides D3 (LAGKYGNQATLTQ

TF) and D4 (GNQATLTQTFADGRV) were immunogenic in infected/ reinfected C57BL/6 mice as evaluated by restimulation with 5 µg/ml of the respective 15-mer in IFN-γ ELIspot. Peptides A11, D9 and D10 were moreover identified as positive stimulators in proliferation assays using BALB/c mice. In respect to the analyzed T cell subpopulations, 15-mers D9 and D10 mainly induced proliferation of $CD4^+$ T cells, while peptide A11 additionally induced proliferation in a $CD3^+$ $CD4^-$ T cell population, which represented most probably $CD8^+$ T cells. Regarding the induction of T cell proliferation by *ex vivo* restimulation with 10 µg/ml of the whole recombinant protein, both $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ responses were observed. In contrast to CP0177-1, the induction of $CD3^+$ $CD4^-$ T cell proliferation was also observed in the group immunized with CP0020-1 alone, indicating $CD8^+$ stimulatory properties of CP0020-1. As with CP0177-1, peptide screening using the CFSE-CD3-CD4 staining revealed 15-mers that stimulated T cell proliferation but which were not identified as stimulators of IFN-γ in ELIspots (A12, B1, G3, G8 and G9; not shown). As described before for protein CP0177-1, this T cell populations may be composed of IL-4 secreting $CD4^+$ T cells and/or T_H17 cells, as also for this antigen, high levels of IL-17 were detected in culture supernatants of T cells derived from spleens of i.p. immunized BALB/c mice after *in vitro* restimulation with CP0020-1 (not shown).

In summary two promising subunit vaccine candidates could be identified in the course of this study, though peptide screenings for candidate CP0177-1 should be repeated using higher concentrations of *in vitro* stimuli in order to obtain a more distinguished idea about IFN-γ responses to immunogenic peptides. Furthermore, as here only partial constructs of the whole proteins were used for the evaluation of immunogenicity and immunization induced protection using IC31® as an adjuvant, subsequent studies should evaluate constructs consisting of the remaining partial sequences as well as the whole recombinant proteins CP0177 and CP0020, which exceeded the aims of this study. Such investigations would be especially interesting for protein CP0177 as the in *Chlamydia* highly conserved domain is located in the C-terminal region of the protein. Therefore CP0177 represents a potential cross protective *Cpn* antigen. Furthermore such experiments have the potential for the identification of other immunogenic epitopes that could be part of a multi-epitope vaccine built in the way it was previously designed by Pinchuk et al. [97], or otherwise of a subunit vaccine consisting of several protective antigens and additionally containing the two component adjuvant IC31® as a potent inducer of T_H1-type immune responses in mucosal/nasal applications.

In spite of the encouraging results obtained for IC31® as a potential mucosal adjuvant during this study future experiments will have to show the applicability and efficacy in humans, thereby ultimately allowing the implication in the prevention of infectious human disease.

6 Materials and Methods

6.1 Antigen purification

6.1.1 Growth of *E.coli* BL21 Codon Plus and over expression of 6x His-tag *Chlamydia pneumoniae* proteins

Glycerol stocks (10% Glycerol, Sigma) of *E.coli* BL21 Codon Plus containing the vector pET28B, carrying an insert of one of the *Chlamydia pneumoniae* proteins CP0529 (hypothetical protein, [*Chlamydia pneumoniae* AR39]), CP0177-1 (amino acid 1-380 of 755, hypothetical protein, [*Chlamydia pneumoniae* AR39]), CP0020-1 (amino acid 2-418 of 812, hypothetical protein, [*Chlamydia pneumoniae* AR39]), CP0504 (hypothetical protein, [*Chlamydia pneumoniae* AR39]), CP0879 (hypothetical protein, [*Chlamydia pneumoniae* AR39]) and CP0409 (hypothetical protein, [*Chlamydia pneumoniae* AR39]) with a C-terminal 6x His tag were used throughout this study.

For the production of biomass, 100 ml of LB medium (PAA) were inoculated in a 500 ml flask by adding a bit of material scratched from the respective *E.coli* BL21 Codon Plus glycerol stock and incubated over night (o/n) at 37 °C and 180 rpm in a shaker, in the presence of 50mg/ml Kanamycin (Serva) and 50 mg/ml Chloramphenicol (Calbiochem).

On the following day the 100 ml o/n culture was added to 2000 ml LB medium containing 50 mg/ml Kanamycin and incubation at 37 °C, 180 rpm was continued for 2 hours (OD_{600} = 0.3 – 0.5). At this point, 0.1 mM IPTG (Serva) was added for the induction of recombinant protein expression. The culture was further incubated for 4 hours at 37 °C, 180 rpm. Subsequent to this incubation period cells were harvested by centrifugation. The pellet was resuspended in 30 ml PBS (Gibco), transferred into a 50ml falcon and stored at -20 °C.

6.1.2 Lysis

Lysis of recombinant *E.coli* biomass was performed using the high pressure homogenizer (HPH) “Panda 2K”, manufactured by Niro-Soavi, in the presence of protease inhibitors (1 µg/ml Leupeptin (Serva), 1 µg/ml Pepstatin A

(Sigma), 1 µg/ml Aprotinin (Sigma), 40 µM Bestatin (Sigma), 10 µM E-64 (Sigma) and 1000 µM AEBSF (Serva).
Prior to lysis, the biomass was resuspended in 50 mM TrisHCl (Serva), pH 8.0, 500 mM NaCl (Sigma), 1% TritonX-100 (Fluka) (final volume: 250 ml). Additionally 1 mM DTT (Serva) was added to those suspensions of *E.coli* biomass which expressed proteins containing > 2 cysteines.

For the subsequent degradation of DNA, DNase I grade 2 (100 µg/ml, Sigma) and $CaCl_2$ (1 mM, Fluka) were added to the crude lysate and incubated for 45 min at RT and 180 rpm. The soluble fraction of the lysate was separated from the insoluble fraction by centrifugation (10000 g, 10 min).

For the assessment of protein expression a sample was taken from the crude lysate, the soluble fraction and the insoluble fraction followed by SDS-PAGE analysis.

Insoluble Proteins were further washed in 1 M Urea (Sigma), 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 1 mM EDTA (Sigma) and 10 mM DTT. For the estimation of the optimal washing condition a small portion was further washed with increasing concentrations of Urea (1 mM, 2 mM and 3 mM) followed by analysis through SDS-PAGE.

After washing the insoluble pellet twice in 10 x volume of the previously determined washing buffer the purified inclusion bodies were one time washed and subsequently resuspended in the final wash buffer, 50 mM TrisHCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT.

6.1.3 SDS-PAGE

For SDS-PAGE gold precast gradient gels containing 4-20% Polyacrylamid (Lonza) were used throughout this study. The Marker used for the estimation of molecular weights was the prestain protein ladder (Fermentas). For staining of protein bands the staining solution “SimplyBlue Safe Stain” (Invitrogen) was used.

6.1.4 Soluble gravity column affinity purification

Binding to Ni-sepahrose 6 fast flow (GE Healthcare) of soluble 6x His-tag recombinant protein was performed at 4 °C over night while rotating.
1 ml of Ni-sepharose 6 fast flow was used for binding 10 mg of recombinant protein.

Washing occurred with: 15 column volumes (CV) of buffer containing 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100 and 1 mM DTT followed by 10 CV 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 1 mM DTT

followed by 5 CV 20 mM Imidazol (Sigma), 50 mM TrisHCl, pH 8.0, 500 mM NaCl and 1 mM DTT followed by 5 CV 40 mM Imidazol (Sigma), 50 mM TrisHCl, pH 8.0, 500 mM NaCl and 1 mM DTT

Soluble Protein was eluted by washing with: 3 CV 250 mM Imidazol, 50 mM TrisHCl, pH 8.0, 150 mM NaCl and 1 mM DTT

Remaining insoluble protein was eluted by washing with: 3 CV 250 mM Imidazol, 50 mM TrisHCl, pH 8.0, 6 M GuaHCl (Sigma), 500 mM NaCl and 1 mM DTT (note: for the isolation of CP0529 no DTT was added in any step)

6.1.5 Insoluble gravity column affinity purification

Inclusion bodies were denaturated by suspending the biomass in buffer containing 6 M GuaHCl, 50 mM Tris, pH 8.0, 500 mM NaCl, 0.1 % Triton X-100 and 1 mM DTT.

Binding to Ni-sepahrose 6 fast flow (GE Healthcare) of soluble 6x His-tag recombinant protein was performed at 4 °C over night while rotating. 1 ml of Ni-sepharose 6 fast flow was used for binding 10 mg of recombinant protein.

Washing occurred with 10 CV 6 M GuaHCl, 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100 and 1 mM DTT followed by 10 CV 6 M GuaHCl, 50 mM TrisHCl, pH 8.0, 500 mM NaCl and 1 mM DTT followed by 5 CV 20 mM Imidazol, 6 M GuaHCl, 50 mM TrisHCl, pH 8.0, 500 mM NaCl and 1 mM DTT.

Insoluble protein was eluted by washing with: 3 CV 250 mM Imidazol, 6 M GuaHCl, 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 1 mM DTT, 0.1 % Tween20, 100 mM L-Arg (Serva) and 200 mM Sorbitol (Serva).

6.1.6 Refolding

Refolding conditions were tested for those insoluble proteins which showed precipitation during dialysis after gravity column purification. The optimal refolding buffer was assessed by diluting the eluted and denaturated protein 1:50 in 10 different buffers varying in pH, L-Arg concentration and/or Urea concentration. This dilution was put on 4 °C o/n while shaking.

On the subseqent day an aliquot of the soluble fraction of each buffer was analyzed by SDS-PAGE. The different protein bands were photographed using a CCD camera (Alpha Innotech) and compared by using the software “alpha view” (Alpha Innotech). The buffer which showed to have the largest amount of protein present in the soluble fraction was chosen for refolding and isolation.

Refolding was performed by first changing the buffer to 8 M Urea, 50 mM TrisHCl, pH 8.0, 500 mM NaCl, 1 mM DTT and 0.1 % Tween20 by dialysis.
This protein solution was then drop-wise diluted to 100 µg/ml at 4 °C while stirring, using the respective refolding buffer.

After 12 hours Ni-Sepharose 6 fast flow beads were added to the soluble fraction of the solution (for the isolation of CP0409 the pH was changed from 5.5 to 7.0 by adding NaOH prior to the addition of Ni-sepharose 6 fast flow).
After letting the protein bind to the Ni-sepharose 6 fast flow beads for 12 hours, isolation of the recombinant protein was performed by soluble gravity column purification.
(note: prior to the elution of remaining insoluble protein a washing step with 3 CV 250 mM Imidazol, 50 mM TrisHCl, respective pH, 150 mM NaCl, 1 mM DTT and 500 mM L-Arg was included).

6.1.7 Dialysis

Dialysis was performed over night at 4 °C on a magnetic stirrer. The volume of buffer was 100 x the sample volume. The dialysis buffer was changed two times in order to achieve full buffer change. Large sample volumes were dialyzed in dialysis soft tubes (Spectrum Labs), smaller volumes (< 10 ml) were dialyzed in dialysis cassettes (Slide-A-Lyzer, Thermo Scientific).

6.1.8 BCA assay

Protein concentration was determined using the BCA Protein Assay (Pierce)

6.1.9 Western Blot “semidry”

The marker used for Western Blot was the Magic marker XP (Invitrogen, “Western Standard”). The Tranfer Buffer concited of 39 mM Glycine, 48 mM Tris, 0.037% SDS (Serva), 20% Methanol (Merck).
The Gelsandwich consisted of 3 pieces Whatman 3 mm-filter (Schleicher-Schuell), followed by the Gel (gold precast gradient gel with 4-20% Polyacrylamid (Lonza)) and followed by 3 pieces Whatman 3 mm-filter (Schleicher-Schuell). Western Blot conditions were 15 V, 80 mA per gel, 1 hour.

After transfer, the NC-membrane (Immobilon-P, Millipore) was washed with MilliQ followed by staining with Ponceau S (Sigma) for 1-2 minutes. The membrane was destained by washing with MilliQ and subsequently blocked for 1 hour with PBS containing 5% milk powder (Fluka). Incubation with primary

antibody (penta-his, BSA-free, mouse monoclonal IgG, 1:5000 in PBS, 1% Tween20, 5% milk powder) was performed at 4 °C o/n with gentle agitation. Subsequently the membrane was washed and incubated for 1 hour with the secondary antibody (Duko, polyclonal, rabbit anti-mouse HRP, 1:5000 in PBS, 1% Tween20, 5% milk powder).

After a washing step the developer solution Chemiglow substrate (Apha Innotech; "ChemiGlow West") was added for 5 minutes. Pictures were acquired using a CCD camera (Alpha Innotech).

6.1.10 LPS assay

The amount of LPS-contamination was measured using the "PyroGene Recombinant Factor C endotoxin Detection System" (Lonza).

6.1.11 LPS removal by filtration

Mustang E Membrane filter (PALL)

6.1.12 SEC analysis

Size exclusion chromatography was performed by HPLC using a Superdex 200 column with a runtime of 60 minutes and an injection volume of 50 µl measuring at the wavelength of 214nm.

6.1.13 Concentration of isolated protein in final buffer

Amicon Ultra 10K centrifugal filter unit (Millipore)

6.1.14 Sterile filtration

Prior to the final assessment of protein concentration, LPS contamination and the percentage of oligomerization the protein solution was 0.2 µm sterile filtered.

6.1.16 Storage

Protein solutions ready for immunization studies were stored at –80 °C.

6.2 Additional Antigens

Chlamydia outer protein N (CopN, Low Calcium Response E [*Chlamydophila pneumoniae* CWL029]) with an N-terminal 6x His tag and *Chlamydia* major outer membrane protein (MOMP, ompA major Outer membrane protein [*Chlmydophila pneumoniae* CWL029]) with a C-terminal 6x His tag were used as control antigens during this study. In cultured ELIspots with splenocytes from *Cpn* K6 infected animals the in house identified *Borrelia* protein antigen BAP038 was used as irrelevant antigen for stimulation.

The recombinant proteins were isolated in house by gravity column affinity purification from purified inclusion bodies after over expression in *E. coli* BL21 codon plus strain. The final buffer was 50 mM TrisHCl, pH 8.0, 150 mM NaCl.

6.3 Peptides for ex vivo restimulation

The CP0177-1 and CP0020-1 peptide libraries used in this study consisted of 15mers overlapping by ten residues covering the entire sequence of the recombinant protein. The peptides were synthesized in house by solid phase synthesis on preloaded 2-chlorotrityl resins (Pepchem) on a multiple peptide synthesizer (Syro, Multisyntech). Following synthesis, the peptides were cleaved from the resin and purified by RPHPLC. The identity and purity was confirmed by mass spectroscopy using a MALDI-TOF mass spectrometer Voyager-DE-STR (Applied Biosystems). All peptides were solubilized in DMSO (Sigma) to a concentration of 20 mg/mL. Peptides were stored at -20 °C. Peptides B8.1 (KYASGNSEI, CopN) and E8.1 (DYFESRVPI, CopN) were synthesized in house, solubilized and stored as described before.

6.4 Mice

Naïve female BALB/c ($H2^d$) and C57BL/6 ($H2^b$) mice were purchased from Charles River laboratories Germany at an age of 6-8 weeks. Mice were kept under specified pathogen free conditions with free access to water and food. Experiments were initiated before the mice reached the age of 12 weeks. All animal experiments were conducted according to Austrian law and approved by the Austrian Federal Ministry of Science and Research.

6.5 Adjuvants

The classical preparation of IC31® in PBS (Gibco) was used throughout this study. $KLKL_5KLK$ (NH_2-KLKLLLLLKLK-COOH) and ODN1a (phosphor-diester backbone ODN, oligo-$(dIdC)_{13}$) were purchased from Bachem (KLK) and Transgenomic (ODN1a). The single components of IC31® were mixed in PBS (pH = 7) at a molar ratio of 2000 nmol KLK / 80 nmol ODN1a and stored at 4 °C.

Before preparing the vaccine the IC31® preparation was vigorously mixed at room temperature. For intra nasal immunizations the amount of IC31® in the vaccine was 20 nmol KLK and 0.8 nmol ODN1a per mouse (IC31® (20/0.8)). For intra peritoneal, subcutaneous and intra dermal immunizations the amount of IC31® in the vaccine was 100 nmol KLK and 4 nmol ODN1a per mouse (IC31® (100/4)).

In some experiments Cholera toxin b subunit (Sigma) was used as an adjuvant for intra nasal vaccination of control groups. The amount given to each mouse of those groups was 10 µg.

6.6 Vaccination of mice

The amount of antigen delivered to each mouse irrespective of the route was 40 µg. The volume for intra peritoneal injection was 200 µl. Subcutaneous injection combined with intra dermal injection was given at the tail base delivering each 100 µl.

Intra nasal vaccination was performed using a pipette while keeping the mice under light anesthesia with Isofluorane (Baxter). The volume for intra nasal vaccination was 40 µl per mouse. Control groups either received adjuvant alone or were left naïve. .Vaccination occurred on day 0, 14 and 28 of each experiment.

6.7 Infection of mice with *Chlamydia pneumoniae* K6

The stock solution of *Chlamydia pneumoniae* Kajaani 6 (K6) purified elementary bodies was provided by Dr. Benjamin Wizel. The concentration in IFU/ ml of this stock was $3*10^8$. *Cpn* K6 elementary body stocks were stored at -80 °C. Prior to intra nasal delivery the stock solution was diluted in ice cold PBS (Gibco). Each mouse received $1*10^6$ IFU in a volume of 40 µl using a pipette, while keeping the mice under light anesthesia with Isoflurane (Baxter).

Intra nasal infection of mice in immunization-challenge studies was performed 10 days following the 3rd vaccination. Infection priming of an immune response to *Cpn* K6 occurred by a primary intra nasal infection on day 0 of the experiment followed by a secondary intra nasal reinfection on day 30.

6.8 Media

For the isolation and in vitro incubation of splenocytes or total lung cells, defined T cell medium (TCM) was used throughout this study. TCM consisted of RPMI 1640 + L-glutamine (Gibco), 10% FCS (PAA), 20 mM HEPES (Gibco), 1 mM sodium pyruvate (PAA), 0.1 mM non essential amino acids (PAA), additional 2 mM L-glutamine (Gibco), 50 µM β-mercaptoethanol and 100 units/ml penicillin and 100 µg/ml streptomycin (PAA).

For the work with HEp-2 cells no β-mercaptoethanol was added to the medium. For the subsequent work with *Chlamydia pneumoniae* 100 µg/ml gentamycin was used instead of penicillin and streptomycin. This medium was defined as RPMI complete. For the incubation of *Cpn* K6 infected HEp-2 cells defined IMDM complete was used. This medium consisted of Iscove's Modified Dulbecco's Medium (Gibco), 10% FCS (PAA), 20 mM HEPES (Gibco), 1 mM sodium pyruvate (PAA), 0.1 mM non-essential amino acids (PAA), additional 2 mM L-glutamine (Gibco), 0.26 mg/mL sodium-bicarbonate (PAA), 0.5 mg/mL glucose (Gibco) and 100 µg/mL gentamycin (PAA).

6.9 Assessment of inclusion forming units (IFUs) in murine lungs

Mice were sacrificed by cervical dislocation on day 10 after intra nasal challenge with *Cpn* K6. Lungs were removed and individually kept in each 2 ml ice cold sucrose-phosphate-glutamic acid (SPG) buffer. Subsequently homogenates were prepared using an electrical homogenizer. Fat and tissue was separated from the fluid by centrifugation (1000 rpm, 10 min). The Supernatant was transferred into cryotubes and stored at -80°C.

For the assessment of IFUs, HEp-2 cells were seeded on 13 mm diameter round glass slides (Thermo-Scientific) inside 24-well plates (Nunc) and incubated at 37 °C and 5% CO_2 until a confluent monolayer was formed. Dilutions (1:50, 1:100 and 1:250) were prepared from individual lung homogenates in RPMI complete. For the infection of HEp-2 cells the medium was removed and 0.5 ml of the different dilutions were added in duplicates to

the HEp-2 cells, followed by centrifugation at 550 g and 35 °C for 1 hour. Subsequent the medium was removed and 1.5 ml IMDM complete containing 0.5 µg cycloheximide (Sigma) was added to each well. Infected HEp-2 cells were incubated for approximately 60 hours prior to immunofluorescent staining of *C. pneumoniae* inclusions.

After washing with PBS (Gibco), cells were fixed by adding -20 °C cold methanol (Merck) fro 10 min. After another washing step, a blocking step with 1% BSA in PBS was performed for 1 hour at RT. Following this blocking step, the primary antibody RR402 Mouse Mab anti-MOMP (B. Wizel), was added to each well. Incubation was performed at RT for 1 hour in a dark humid chamber.

After washing with PBS (Gibco) the secondary antibody, AF488 Goat anti-mouse Ig (Invitrogen) was added. Incubation occurred at RT for 1 hour in a dark humid chamber. After another washing step the glass slides were carefully removed from the wells and put onto cover slips covered with Mowiol 4-88 (Sigma). Counting of fluorescently labeled inclusions was performed using an Axioplan 2 microscope (Zeiss).

6.10 Cell preparation and purification

10 days post 2^{nd} and 3^{rd} immunization as well as 10 days post reinfection, mice were sacrificed by cervical dislocation. Spleens and/or lungs were collected in each 8 ml of ice cold RPMI 1640 + L-glutamine (Gibco). Spleens and/or lungs of the same experimental groups were pooled prior to mechanical preparation of the single cell suspension in RPMI 1640 + L-glutamine (Gibco). Reed blood cell lysis was performed in commercially available red blood cell lysis buffer (Sigma). Subsequently cells were washed in RPMI 1640 + L-glutamine (Gibco) and resuspended in TCM. Viable cell count was determined by trypan blue exclusion staining.

6.11 Cultured IFN-γ ELIspot assay

For culturing total spleen cells, quadruplicates of each experimental group containing $5*10^6$ cells/well were incubated at 37 °C and 5% CO_2 in sterile 24-well plates (Nunc) in a volume of each 2 ml TCM for 48 hours in the presence of 5 µg/ml stimulus (relevant antigen, irrelevant antigen, medium). After this incubation period, cells of each experimental group were harvested by extensive washing with TCM and pooled. Subsequently viable cell count was

determined by trypan blue exclusion staining. The following procedure was performed as described in section 6.10 IFN-γ ELIspot assay.

6.12 IFN-γ ELIspot assay

For IFN-γ ELIspot assay sterile 96-well filtration plates were purchased from Millipore (Catalog number: MAHAS4510). Wells were pre-rinsed with PBS (Gibco) prior to the coating with the rat anti-mouse IFN-γ antibody (BD Pharmigen). The antibody was diluted to a concentration of 3 µg/ml in coating buffer. Coating buffer consisted of 0.1 M NaHCO3 in bidistilled water (Mayrhofer Pharmaceuticals) pH 9.2-9.4. Coating was performed o/n at 4 °C in a humid chamber. In advance of the assay plates were blocked with 1% BSA (PAA) in PBS (Gibco) at 37 °C for 1 hour.

Subsequent to the blocking step 0.25-1.0*10^6 cells in TCM were added in duplicates to each well. Additionally RACS (Becton Dickinson) was added to a final concentration of 1%. Finally stimuli were added. ConA (Sigma) was added as a positive control. TCM was used as negative control. Incubation occurred for approximately 20 hours at 37 °C and 5% CO_2.

Following incubation, plates were washed with washing buffer which consisted of 0.1% Tween20 (Sigma) in PBS (Gibco) for 15 minutes at RT. Secondary biotin anti-mouse IFN-γ antibody (BD Phrmigen) was diluted in PBS (Gibco) to a final concentration of 1 µg/ml and added to each well. Incubation occurred at 37 °C for 2 hours. After washing Streptavidin-labeled horseradish peroxidase (Roche) was added and incubated at 37 °C for 30 minutes. After another washing-step, substrate was added, which consisted of 0.8 mg/ml 3,3'diaminobenzidine, 0.4 mg/ml $NiCl_2$ and 0.015% H_2O_2 in 100 mM Tris.

The reaction was performed at RT and was usually terminated after approximately 4-6 minutes, when visible spots had formed, by flushing the plates under cold tap water. Dry plates were analyzed with the Bioreader 5000 (BioSys, Germany).

6.13 IL-4 ELIspot assay

IL-4 ELIspot was performed as described for IFN-γ ELIspot in section 6.10 with the exception of not adding RACS after plating the cells. Multiscreen HTS filter plates were used for IL-4 ELIspot assays (Catalog number: MSIP S4510, Millipore). The rat anti-mouse IL-4 antibody (BD Pharmigen) was used as primary antibody at a final concentration of 2 µg/ml in coating buffer. The

secondary antibody, biotin anti-mouse IL-4 (BD Pharmigen), was diluted in blocking buffer to a final concentration of 1 µg/ml.

6.14 Serum preparation and antigen-specific ELISA

Terminal bleedings were collected 10 days post 2nd and 10 days post 3rd immunization or 10 days post reinfection, if not stated otherwise in the text. For the collection of blood Vacutainer SST tubes (Becton Dickinson) were used. Serum was prepared as described in the manufacturers' manual and stored at -80 °C until needed.

For the estimation of the optimal coating concentration with antigen, each recombinant protein was titrated using serum from mice immunized with the relevant antigen and serum from mice immunized with an irrelevant antigen as well as sera from naïve mice as controls. The coating concentration which allowed the best differentiation between sera from mice immunized with the relevant antigen in comparison to sera from control groups was chosen for the subsequent antigen-specific ELISA. The selected coating concentrations were 1.25 µg/ml for CP0529, 0.63 µg/ml for CP0177-1, 2.5 µg/ml for CP0020-1, 0.63 µg/ml for CP0504, 2,5 µg/ml for CP0879, 1.25 µg/ml for CP0409, 0.31 µg/ml for CopN and 0,63 µg/ml for MOMP.

ELISA plates (Nunc Immuno plate F96 Maxisorp) were coated with 50 µl of antigen diluted in PBS (Gibco) at 4 °C over night. Blocking was performed after flicking the liquid, by adding 100 µl/well 2% BSA (PAA) in PBS (Gibco) for 1.5 hours at 37 °C.

The liquid was removed by flicking onto paper towels. Sera were serially diluted (as indicated in the text) in dilution buffer which consisted of 0.1% BSA (PAA) in PBS (Gibco). 50 µl of diluted serum was added to each well and incubation occurred for 1.5 hours at 37°C. Following plates were washed 3 times with washing buffer which consisted of 0.1% Tween20 (Sigma) in PBS (Gibco). For the measurement of total serum IgG titers, polyclonal rabbit anti-mouse immunoglobulin/HRP antibody (Dako Cytomation) was used. For the estimation of serum IgA titers, goat anti-mouse IgA/HRP antibody (Southern Biotech) was used. Secondary antibodies were diluted in dilution buffer and 50 µl were added to each well. Incubation occurred for 1 hour at 37°C.

Wells were washed 3 times with washing buffer before 50 µl of ABTS (Sigma) was added to each well. Plates were incubated for approximately 20 minutes in the dark before measuring the color intensity at 405 nm on a Tecon Sunrise microplate reader. Using Graph Pad Prism 5 software

background subtraction and non linear regression was performed across all data points.

6.15 Flow cytometry – surface staining

Surface staining of cells isolated from murine spleens or lungs was performed by washing in FACS buffer followed by resuspension in 100 µl of FACS buffer containing 1 µg/ml rat IgG2a anti-mouse CD16/32 (eBioscience). Incubation occurred at RT for 20 min in the dark. After washing the cells in FACS buffer, cells were resuspended in 100 µl FACS buffer containing the appropriate antibody and incubated at RT, for 20 min in the dark. Antibodies used for surface staining in this study were 1 µg/ml rat IgG2a anti-mouse CD4 FITC-conjugated antibody (eBioscience), 2 µg/ml Armenian hamster IgG anti-mouse CD3 APC-conjugated antibody (eBioscience), 2µg/ml rat IgG2b anti-mouse CD4 PE-Cy7-conjugated antibody (eBioscience) and respective isotype controls. Before measurement, cells were extensively washed and finally resuspended in FACS buffer.

6.16 Flow cytometry - CD154 assay

For the CD154 assay $1*10^6$ cells derived from lung or spleen were plated in 96-well U-bottom plates (Nunc), in TCM and a volume of 100 µl. Subsequently stimuli at a concentration of 10 µg/ml were added. As negative control, only medium was added. As positive control 1 µg/ml SEB (Sigma) was added to the respective well. Following stimulation, 1µg/ml GoligiStop (Becton Dickinson), 1.25 µg/ml Armenian hamster anti-mouse CD154 PE-conjugated antibody (eBioscience) as well as respective isotype controls were added and cells were incubated for 6 hours at 37 °C and 5% CO_2.

Anti-CD4 surface staining was performed as described in section 6.14. Cells were measured on a FACSCalibur equipped with CellQuest software. The data was analyzed using FCS Express 3 software (De Novo software).

6.17 Flow cytometry - CFSE staining of splenocytes

$1*10^6$ cells were plated in 96-well U-bottom plates (Nunc), in TCM in a volume of 100 µl. Cells were washed in PBS (Gibco) containing 5% FCS (PAA). CFSE staining was performed by carefully resuspending the cells in 100 µl PBS (Gibco) containing 5% FCS (PAA) and 10 µM CFSE (Invitrogen) followed by incubation at 37 °C, 5% CO2 for 5 min. The reaction was stopped

by washing the cells with 5% FCS (PAA) in PBS (Gibco). Following another washing step cells were resuspended in 200 µl TCM containing the appropriate stimuli. Peptides and protein antigens were used at a concentration of 10 µg/ml. TCM was used as negative control. 0.5 µg/ml ConA (Sigma) were used as positive control. Incubation occurred for 5 days at 37 °C and 5% CO2.

Subsequent anti-CD3 combined with anti-CD4 surface staining was performed as described in section 6.15. Measurement was performed using the Cytomics FC 500 (Beckman Coulter). The data was analyzed using FCS Express 3 software (De Novo software)

6.18 Immunofluorescence microscopy

Infection of HEp-2 cells with *Chlamydia pneumoniae* K6 was performed as described in section 6.9 with the deviations in the incubation period prior to immunofluorescent staining (indicated in the text). *Cpn* K6 elementary bodies were added at a multiplicity of infection of 2. After removing the supernatatnt cells were washed with PBS (Gibco). Fixation was performed by the addition of 300 µl Cytofix/Cytoperm solution (BD Biosciences) and incubation for 20 min at 4 °C. Cells were washed and subsequently blocking was performed by adding Perm/Wash solution (BD Biosciences) containing 1% BSA (PAA) followed by incubation for 1 hour at RT.

Primary antibodies were sera from immunized animals, as mentioned in the text, and rabbit anti-Cpn0585 (B. Wizel). Antibodies were diluted in Perm/Wash solution (BD Biosciences) followed by incubation for 1 hour at RT in a dark, humid chamber. After washing, the secondary antibodies were diluted in Perm/Wash solution (BD Biosciences) and added. Secondary antibodies were AF488-conjugated goat anti-mouse IgG (Invitrogen) and AF594-conjugated goat anti-rabbit IgG (Invitrogen). Incubation was performed as before. Cover slips were subsequently washed and stained with 1 µg/ml DAPI for 10 minutes. After another washing step, cover slips were placed on glass slides and covered with Slowfade Gold (Invitrogen). Pictures were taken with an upright Laser Scanning Microscope LSM-510 Meta (Zeiss).

7 Acknowledgements

I gratefully acknowledge Benjamin Wizel for guidance, supervision, discussion and support throughout the process of this study as well as for critical review of the manuscript.

I wish to thank all members of the MAT/IDA team who supported my work by providing a helping hand and being open for inspiring discussions: Petra Schlick, Olga Lorenz, Sandra Jost, Gregory Bouchard, Manuel Zerbs, Gabor Nagy, Paer Comstedt, Karin Riedl, Zoltan Magyarics and my diploma college Christian Schenk.

Thankfully I acknowledge all people who supported me during the process of protein purification: Markus Hanner, Verena Salletmeyer, Jan Sinzinger and especially Christina Satke.

Furthermore I wish to thank all people at Intercell who helped me at one or another time point and who supported my work, as well as Michael Aichinger and Christine Marizzi of the Max F. Perutz Laboratories for excellent help with the LSM.

Finally I want to thank Eszter Nagy and Alexander von Gabain for discussion and guidance as well as for giving me the opportunity to fulfill my diploma thesis at Intercell.

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Mother tongue	German
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Computer skills and competences	Operating systems: Windows 2000, Windows XP, Windows Vista

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-cell culture (general)

-ELIspot

-T cell culture

-Handling of mice and removal of organs (infectious (lvl. 2) and non-infectious work)

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-Quantification of gene expression in single cells by real-time qPCR

-High resolution melting curve analysis

-LAMP PCR