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DIPLOMARBEIT

Analysis of pulmonary T cell responses to IC31®-adjuvanted model protective antigens

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Abbreviations

ab	Antibody
AF	Alexa Fluor
ag	antigen
APC	Antigen presenting cell, allophycocyanin
CFA/IFA	Complete Freund's adjuvant/Incomplete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
ConA	Concanavalin A
CopN	*Chlamydia pneumoniae* outer protein N
Cpn	*Chlamydia pneumoniae*
CT(B)	Cholera toxin (B subunit)
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN-γ ELISpot	Interferon-gamma enzyme-linked immunospot assay
IFU	Inclusion forming units
IM	Inclusion membrane
i.n.	intranasal
i.p.	intraperitoneal
LPS	Lipopolysaccharide
MACS	Magnetic cell separation
Ova	Chicken Ovalbumin
PMA	Phorbol-12-myristate-13-acetate
PVDF	Polyvinylidene difluoride
RCAS	Rat ConA supernatant
RT	Room temperature
s.c.	subcutaneous
SD	standard deviation
SEB	*Staphylococcus aureus* enterotoxin B
SFC	Spot forming cells
TCM	T cell medium

TLR	Toll-like receptor
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TTSS	Type III secretion system

1 Curriculum vitae

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2 Abstract

Chlamydia pneumoniae (*Cpn*) is a widely distributed human respiratory pathogen, that has also been implicated in COPD and atherosclerosis. There is strong evidence, that *Cpn* utilizes a Type 3 secretion system (TTSS) to export effectors out of its secluded, intracellular niche. A protein that has been shown to be associated with the TTSS is chlamydial outer protein N (CopN). Previous reports have shown the immunogenicity of CopN. Here, immunogenic epitopes of CopN were identified in BALB/c and C57BL/6 mice based on protein immunizations and IFN-γ ELISpots with a peptide library. Using these newly identified epitopes, it was possible to demonstrate the mucosal efficacy of Intercell's adjuvant IC31®. IFN-γ release of lung T cells from animals immunized intranasally with CopN and IC31® could be detected, as well as epitope-specific $CD8^+$ T cells in BALB/c mice using MHC class I pentamers. The relevance of CopN during a natural *Cpn* infection could also be demonstrated using a cultured ELISpot approach. Potential T cell factors, which might be differentially regulated during adjuvantation with IC31® were suggested by means of an initial microarray experiment using RNA from lung T cells.

The data shown here demonstrate efficient mucosal adjuvanticity of IC31® when applied intrasally to mice, suggesting a potential use in the human system.

3 Zusammenfassung

Chlamydia pneumoniae (*Cpn*) ist ein weit verbreitetes humanes Pathogen des Respirationstrakts, das auch mit COPD und Arteriosklerose in Zusammenhang gebracht wurde. Es wird vermutet, dass *Cpn* ein Type 3 secretion system (TTSS) verwendet, um Effektormoleküle zu exportieren. Chlamydial outer protein N (CopN) ist an das TTSS assoziiert. Frühere Publikationen berichteten über die Immunogenität von CopN. In dieser Arbeit wurden immunogene Epitope von CopN in BALB/c und C57BL/6 Mäusen identifiziert. Dies erfolgte mittels Immunisierung mit CopN und anschließenden IFN-γ ELISpots mit einer Peptidlibrary. Anhand dieser neu identifizierten Epitope konnte die Effizienz von IC31®, einem von Intercell entwickelten Adjuvans, in intranasal immunisierten Tieren gezeigt werden. IFN-γ Ausschüttung von T Zellen der Lunge aus Tieren, die mit CopN und IC31® intranasal immunisert wurden, konnte gemessen werden. Weiters wurden Epitop-spezifische $CD8^+$ T Zellen in BALB/c Mäusen anhand von MHC I Pentameren detektiert. Die Bedeutung von CopN während einer natürlichen *Cpn* Infektion konnte in einem cultured ELISpot gezeigt werden. RNA aus T Zellen der Lunge wurde für ein Microarray Trial-Experiment verwendet, um mögliche T Zellfaktoren, die im Zuge einer Immunreaktion nach Gabe von IC31® unterschiedlich reguliert werden zu identifizieren.

Die hier beschriebenen Daten zeigen eine erfolgreiche Adjuvantizität von IC31®, wenn es Mäusen intranasal dargeboten wird, was eine Applikation im humanen System versprechend erscheinen lässt.

4 Introduction

4.1 *Chlamydia pneumoniae*

Chlamydia pneumoniae (*Cpn*) is an obligate intracellular, Gram-negative eubacterium that is widely distributed. It is a small pathogen with a genome size of circa 1.2 Mbp [29]. *Cpn* is a causative agent of community-acquired pneumonia, bronchitis, sinusitis and other respiratory diseases [31,41]. Moreover, *Cpn* has been implicated in asthma, chronic obstructive pulmonary disease (COPD) [20] and atherosclerosis [9]. Throughout this thesis, the expression *"Chlamydia pneumoniae"* is used, despite recent suggestions of using *"Chlamydophila pneumoniae"* instead, which is not yet universally accepted.

Chlamydiae have no detectable amounts of peptidoglycan, but contain a dense array of disulfide cross-linked proteins in the outer membrane conferring structural integrity [41]. Despite their small genome, *Chlamydiae* have an intriguing biphasic life cycle, composed of a metabolically inert, infectious form termed elementary body (EB) and a metabolically active, replicating form termed reticulate body (RB). The EB of *Cpn* is typically pear-shaped (unlike the round EBs of *C. trachomitis*), with a size < 0.5 μm [9,31].

During the first few hours of infection, EBs differentiate into RBs capable of vegetative growth [14]. Throughout their developmental cycle, *Chlamydiae* are located within an intracellular, parasitophorous vacuole, also referred to as inclusion. The inclusion remains separated from endolysosomal pathways [25] and intercepts Golgi-derived vesicles to acquire sphingolipids and cholesterol [52]. After 18-20 hours after infection, a subset of RBs differentiates into EBs, which can cause another infectious cycle.

Chlamydia trachomatis can exit the infected host cell in two ways: cellular lysis and a packaged release mechanism referred to as extrusion [25]. Lysis implicates a sequential degradation of the inclusion membrane, followed by rupture of the host cell plasma membrane. In contrast, extrusion preserves the life of the host cell, and only a portion of the inclusion is released, leaving the host cell and the remaining inclusion intact.

In addition to the basic life cycle, *Cpn* is also able to endure suboptimal replicative conditions in a persistent form. Known inducers of persistence are deprivation of Fe and L-Trp [53]. The extrusion mechanism has also been

suggested to play a role in persistence [25].

Chlamydiae modulate cellular functions in a variety of ways. The pro-apoptotic effectors BAD and atypical PKCδ are inhibited. Chlamydial protease-like activity factor (CPAF) degrades BH-3 only domain pro-apoptotic proteins. Furthermore, CPAF degrades RFX-5 and USF-1 which are involved in MHC gene expression. Finally, *Chlamydiae* severely impact the cell cycle [24, 52, 54].

Several antibiotic agents were reported to be efficient against *Cpn* [31]. However, intensive long-term treatment is recommended in order to avoid re-infection with persistent bacteria.

Resistance against *Cpn* requires an intact T cell compartment [53]. In humans and mice, both $CD4^+$ and $CD8^+$ T cells were detected at the site of infection. In the mouse model, experimentally induced absence of the latter results in increased bacterial burdens as well as an increase in disease severity, indicating a major importance of $CD8^+$ T cells in the combat against this pathogen [51, 53]. This comes as no surprise, given the obligate intracellular nature of *Chlamydiae*. Particularly, $CD8^+$ T cells specific for known antigens of *Cpn* belong to the Tc1 subset, secreting IFN-γ, TNF-α and MIP-1α [53].

As mentioned before, *Cpn* might be a risk factor in atherosclerosis. Evidence comes from a multitude of seroepidemiological studies, which demonstrate, that there is a significant association between antibodies specific for *Cpn* and atherosclerosis. Furthermore, *Cpn* has been detected in foam cells in atherosclerotic lesions, but only rarely in arterial tissues that are histologically normal [9].

4.2 *Chlamydia* outer protein N (CopN)

In 1997, four putative type III secretion system (TTSS) genes were identified in *Chlamydia psittaci* [23]. Scc1, copN, cds1 and cds2 were suggested to be potential candidates of TTSS components based on their homology to *Yersinia* genes. After the genomes of *Ctr* and *Cpn* were sequenced in 1998 and 1999, respectively, it was found that these genes were conserved, further indicating a functional relevance of the TTSS [14, 29, 45].

The first experimental evidence for the expression of *Ctr* CopN came from Fields et al. in 2000 [14]. According to this study, CopN is present in EBs,

RBs and whole culture extracts harvested 20 hours after infection. Moreover, it was shown that CopN, which is homologous to YopN, could be secreted by the *Yersinia* TTSS. CopN is believed to act as a peripherally asociated "plug" of the injectisome, preventing unwanted passage.

CopN has been shown to interact with LcrH-2, which might function as a chaperone [44]. A major finding further strengthening the assumption that *Chlamydiae* possess a functional TTSS was the demonstration of ATPase activity of CdsN [48]. The same group demonstrated the interaction of CopN with CdsN, even at high salt concentrations.

Interestingly, heterologous expression of *Cpn* CopN in yeast and mammalian cells induced cell cycle arrest at the G2/M phase, presumably due to alteration in the microtubule cytoskeleton [24]. Moreover, functional knockout of CopN activity by means of inhibitors abrogated *Cpn* growth in infected cells, indicating an essential role of this protein.

Bearing these facts in mind, CopN is an attractive candidate for a future vaccine against *Cpn* and potentially other members of the family. Intranasal immunization of BALB/c mice with heat-aggregated CopN protein adjuvanted in E. coli heat-labile toxin resulted in antigen-specific production of antibodies and IFN-γ, lymphocyte proliferation and protection against *Cpn* challenge [50].

4.3 Adjuvants

IC31® is a dual component adjuvant, composed of the antimicrobial, cationic peptide $KLKL_5KLK$ (KLK) and an oligodeoxynucleotide composed of deoxy-inosine/deoxy-cytosine repeats (5'-ICICICICICICICICICICICIC-3').

IC31® is able to induce local depot formation and to elicit a specific type 1 and type 2 as well as a cytotoxic T cell response in mice [42]. According to extensive toxicological studies in rats, IC31® did not cause any toxic effects [42].

In addition to the adjuvant properties described in mice, IC31® induces antigen-specific T cell responses in monkeys and humans [40]. An application of IC31® in combination with a commercial, MF59-based subunit vaccine has been suggested, especially in immunosenescent mice: A better immune response was detected, when the vaccine was combined with IC31®, than

with the original vaccine [40].

IC31® signals via TLR9 and MyD88, as suggested by experiments using knock-out mice [42]. Recently, it was demonstrated that IC31® only activates a minor fraction of peripheral dendritic cells, without diffuse pro-inflammatory reactions. Only DCs, which directly interact with IC31® were shown to upregulate CD40, CD80 (B7.1), CD86 (B7.2) and IL12p40 [30].

KLK is thought to function as a delivery vector of ODN1a, and potentially also of antigen, acting through a series of conformational changes in its secondary structure to cross the plasma membrane of target cells (M. Aichinger, personal communication).

Given the promising results obtained so far, the question addressed in this thesis was, whether the adjuvant IC31® is also suitable in the mucosal context. Using the model antigen CopN of *Chlamydia pneumoniae*, it could be demonstrated that intranasal administration of protein antigen in combination with IC31® resulted in significant cellular responses.

Another adjuvant used here was cholera toxin (CT) or CT B subunit (CTB). CT is an enterotoxin produced by *Vibrio cholerae*, composed of an enzymatically active A subunit and a B subunit, that interacts with the GM1 ganglioside expressed on host cells [17]. Once inside a host cell, the A subunit ultimately leads to efflux of Cl^- and osmotic movement of water into the gut lumen, resulting in watery diarrhea. The B subunit, however, is nontoxic [22].

CT is considered to be the "gold standard" mucosal adjuvant, but causes high toxicity in humans [19]. On the other hand, CTB is used as an adjuvant in the Dukoral® (VBL Vaccin) cholera vaccine licensed for humans.

CpG1826 is a synthetic oligodeoxynucleotide containing unmethylated CpG motifs, which occur more often in bacteria than in mammals. The immunologic effects after administration of CpG ODNs were reported to be proliferation of B cells and secretion of ab, activation of monocytes, macrophages and DCs, as well as a Th1 like pattern of cytokine production [17]. Signaling depends on TLR9.

Freund's adjuvant is a water in mineral oil emulsion with a manide monooleate surfactant. Complete Freund's adjuvant (CFA), which was used for primary immunizations only, contains inactivated mycobacteria. Incom-

plete Freund's adjuvant (IFA), which was used for booster immunizations, does not contain mycobacteria. Due to its high reactogenicity, CFA cannot be used in humans [19].

4.4 Mucosal Immunity in the Lung and Mucosal Vaccines

80% of immunocytes are accumulated in, or in transit between various mucosa-associated lymphoid tissues (MALT) in the healthy adult [22], illustrating the large area of contact between the mucosal immune system and the outer world, as well as the resulting importance of an active, intact barrier.

Unlike the intestinal mucosa, the lung, particularly the lower respiratory tract, is sterile in healthy humans [26]. This may seem surprising, given the permanent exposure to environmental toxins, allergens and pathogens. The upper airways contain intraepithelial DCs and lamina propria DCs, whereas the lower respiratory tract contains lamina propria DCs and alveolar DCs.

The lungs are an example of type I mucosal surfaces, which are covered by a simple epithelium. Type I mucosal surfaces express polymeric Ig receptor (pIgR), which allows passage of secretory IgA into the lumen.

Mucosal DCs generally avoid secretion of proinflammatory cytokines, suppressing Th1 differentiation and preferably induce a Th2/Th3 type of response [19]. Furthermore, mucosal surfaces have a tendency towards induction of tolerance, when ag is administered without adjuvant.

The motivation to pursuit research in the field of mucosal vaccination reflects the fact, that most human pathogens gain entry through mucosal sites [13, 18]. Hence, the idea to prime an immune response using the same route as the pathogen only seems logical. A successful mucosal immune response abrogates initial entry of the pathogen beyond the defensive barriers of the host [17], whereas a systemic immunization approach could only act at a later stage.

In addition to the the effect mentioned above, this list summarizes more advantages of mucosal - and particularly intranasal - vaccines. [17, 18, 22]:

- Both mucosal and systemic immunity can be attained.

- Increased stability and shelf-life.

- No need for needles and specifically trained personnel.
- Improved safety for the vaccinator and vaccinee.
- Decreased or eliminated injection site pain.
- Easier and faster vaccine delivery.
- Mucosal administration frequently requires less ag and fewer doses than does parenteral immunization. This could be of particular relevance, when the demand for a vaccine exceeds manufacturing capabilities, as in a pandemic.
- Immunity through intranasal immunization is intimately associated with vaginal immunity. This might be a feasible way of immunizing against sexually transmitted diseases.

An example of a vaccine that is applied intranasally is FluMist® (MedImmune). FluMist® is a live, cold-adapted, trivalent influenza vaccine, administered via a prefilled single-use device that sprays the vaccine into the nostrils [18].

4.5 A brief note on the mouse model

Mice, rabbits and monkeys were tested for susceptibility against *Cpn* [31]. It was found that mice are most susceptible to infection.

Specifically, primary challenge of BALB/c mice resulted in a self-restricted infection [37]. No culturable bacteria could be detected by day 27 after infection. Interestingly, a $CD4^+$ $CD8^+$ double-positive population of T cells appeared after primary challenge, which was more apparent after rechallenge.

The two mouse strains used here were BALB/c and C57BL/6. It has been reported, that BALB/c mice preferentially develop a Th2 response and are more susceptible to infection of intracellular pathogens, compared to C57BL/6 mice [11]. Experimental infection with the intracellular parasite *Leishmania major* caused a protective, Th1-driven response in C57BL76 mice, whereas BALB/c mice developed a non-protective Th2 response. This might be due to a greater population of $CD4^+CD25^+$ regulatory T cells found in BALB/c mice.

5 Results

5.1 CopN peptide library screening

The library used here covered the entire sequence of CopN. The individual peptides were 15-mers with an overlap of 10 amino acids. Thus, for a 399 amino acid protein, this gave rise to 78 peptides. According to the position in the synthesis plate, the peptides were systematically designated as A1, A2, A3 ... A12, B1, B2, ... until the last peptide G5.

Groups of mice were immunized three times with CopN whole protein + IC31®(100/4), protein alone, adjuvant alone or left naïve. Initially, the strong adjuvant CFA/IFA was also used.

The IC31®-containing vaccine formulation was analyzed in terms of protein-adjuvant binding behavior using a micro-bicinchoninic acid assay as well as a HPLC-analysis. Both methods yielded similar results, indicating that 60% of CopN were bound to IC31®, while 40% remained unbound.

Screening was based on an IFN-γ ELISpot with the individual peptides as recall stimuli. Data for BALB/c mice was obtained from 3 independent sets of animals. Screening with the entire peptide library was performed 5 times. For the first two sets of animals, the immunization route was intraperitoneally, whereas for the last set of animals it was subcutaneously.

A representative example of a single round of screening is given in Figure 1 for BALB/c and in Figure 2 for C57BL/6 mice. For BALB/c mice, signals from peptides B8 and E8 appeared in each screening round for IC31® adjuvanted protein administrations, while absent in control groups. For C57BL/6 mice, D10 and E11 gave the most apparent signal. At this point, no irrelevant peptide controls were used, because the library itself contained irrelevant peptides.

CFA/IFA-adjuvanted protein administrations (not shown) resulted in a general increase of average peptide-signal. The main rationale for including this treatment was to have another adjuvant in case there is no effect in IC31®-adjuvanted groups. Interestingly, adjuvantation of CopN with IC31® gave dominant signals for e.g. peptides B8 and E8, while corresponding signals were significantly lower or even not detectable in the case of CFA/IFA. However, this might as well be due to a trend towards over-

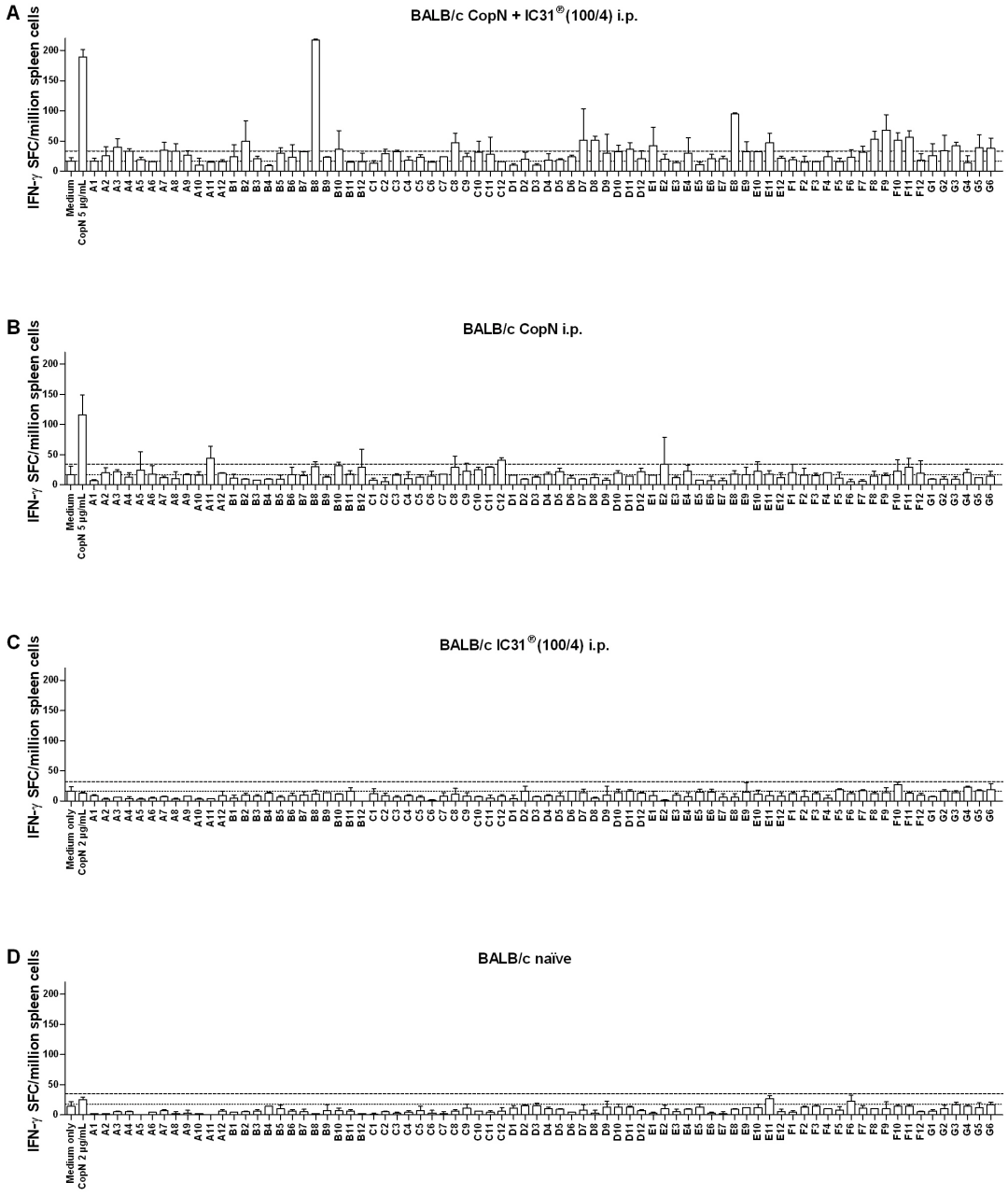

Figure 1: IFN-γ ELISpot screening in BALB/c mice. **A**, **B**, **C**: Animals were immunized 3 times at day 0, 14 and 28. **D**: PBS-mock-immunized. On day 42, mice were sacrificed. Concentration of peptides was 10 μg/mL. SFC extrapolated to 1x10^6 total spleen cells. Mean $\pm$ SD. Similar results were obtained in 2 independent experiments

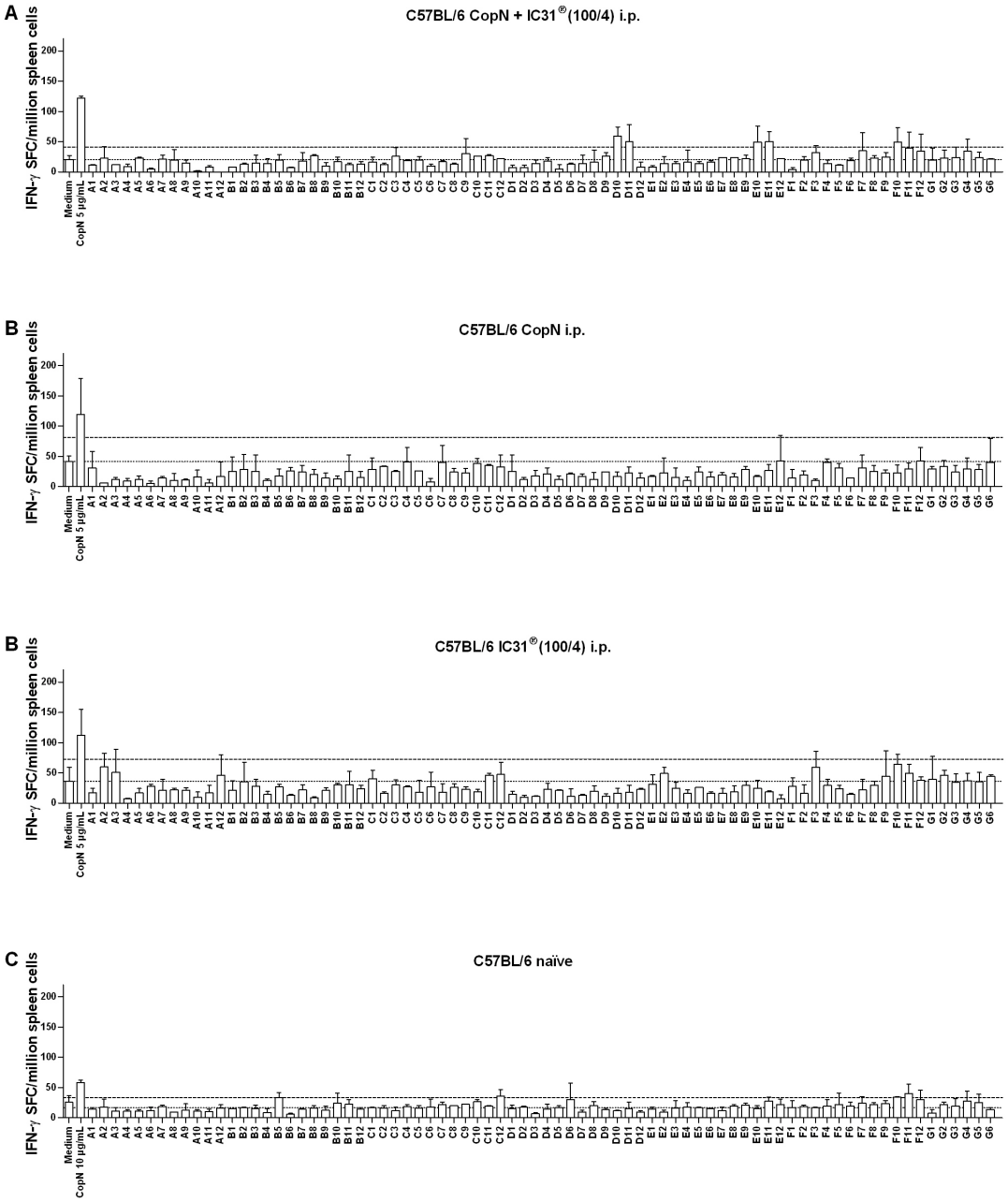

Figure 2: IFN-γ ELISpot screening in C57BL/6 mice. **A**, **B**, **C**: Animals were immunized 3 times at day 0, 14 and 28. **D**: untreated. On day 42, mice were sacrificed. Concentration of peptides was 10 μg/mL. SFC extrapolated to $1x10^6$ total spleen cells. Mean $\pm$ SD. Similar results were obtained in 2 independent experiments

Name	Sequence	Candidate in
A3	GGTQGVNLAAVEAAA	BALB/c
B6	ESTEEKPDTDLADKY	BALB/c
B7	KPDTDLADKYASGNS	BALB/c
B8	LADKYASGNSEISGQ	BALB/c
C8	SQGKLKEALIQARNT	BALB/c
D8	TCDQLLSMLQDRYTY	BALB/c
E8	SYDYFESRVPILLDS	BALB/c
F3	YHKIINDKFPTASKV	BALB/c
F8	DVDSVTGVLNLFFSA	BALB/c
F9	TGVLNLFFSALRQTS	BALB/c
F11	LRQTSSRLFSSADKR	BALB/c
G3	MIANALDAVNINNED	BALB/c
A5	VEAAAAKADAAEVVA	C57BL/6
D10	DRYTYQDMAIVSSFL	C57BL/6
E10	ILLDSLKAEGIQTPS	C57BL/6
E11	LKAEGIQTPSDLNFV	C57BL/6
D11	QDMAIVSSFLMKGMA	BALB/c + C57BL/6
F10	LFFSALRQTSSRLFS	BALB/c + C57BL/6

Table 2: Summary of candidate epitopes for both BALB/c and C57BL/6.

stimulation in the latter case.

Since the purpose of this screening approach was to get a quick yes/no answer as to whether a certain peptide is an immunogenic epitope or not, a simple criterion was established: If the mean of the peptide-specific signal obtained in the ELISpot was greater or equal than the double mean from the medium-specific signal, the counter of this peptide was incremented by one. In addition to the automated read-out, a visual inspection was also performed. For example, B8 is a clear candidate in terms of quantitative IFN-γ response, whereas E8 gave rise to significantly larger spots than all other peptides, indicating a difference in the quality of response.

After successive rounds of screening, it was apparent which peptides could be considered as candidates and which peptides could be regarded as non-immunogenic under these conditions. Table 2 summarizes the findings of the screening experiments. There were 12 candidate epitopes in BALB/c mice, 4 in C57BL/6 and 2 shared epitopes.

Name	Sequence
B8	LADKYASGNSEISGQ
B8.1	KYASGNSEI
B8.2	YASGNSEIS
B8.3	DKYASGNSE
E8	SYDYFESRVPILLDS
E8.1	DYFESRVPI
E8.2	YFESRVPIL
E8.3	YDYFESRVP
E8.4	SYDYFESRV

Table 3: Potential minimal epitopes of peptides B8 and E8.

5.2 Identification of minimal epitopes

The screening was performed without any *in silico* predictions done *a priori*. However, at a certain time point the entire sequence of CopN was entered into an online MHC-prediction server [39]. The top-scoring peptides for BALB/c mice were also those found experimentally, i.e. B8 and E8. According to this prediction, both 15-mers contain H2-K^d nonamers.

With these findings in mind, several potential minimal epitopes were synthesized for B8 and E8. These 9-mers are depicted in Table 3, along with the original 15-mers. B8.1 and E8.1 were the highest-scoring nonamers *in silico*.

As can be seen in Figure 3, B8.1 and B8.2 gave a significantly higher response than B8.3. Nevertheless, there was still a considerable amount of IFN-γ release for B8.3 compared to medium or irrelevant peptides A5-A7, which might indicate the presence of only one anchor residue and no significant sterical hindrance of the remaining peptide with the MHCI-binding pocket.

The situation was less clear for the E8-derived nonamers. According to the data shown in Figure 3, E8.1 might be the principal nonamer. 14 days after the third immunization, similar results were obtained (not shown). Further evidence for the relevance of peptides B8.1 and E8.1 is provided in Section 5.7 on page 20.

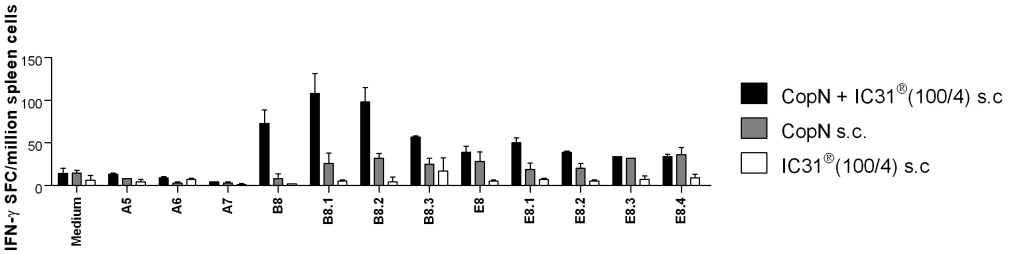

Figure 3: Identification of minimal epitopes in BALB/c mice through an IFN-γ ELISpot. Animals were immunized two times at day 0 and 14 s.c. On day 28 mice were sacrificed. Concentration of peptides was 10 μg/mL. A5, A6 and A7 were irrelevant peptides. SFC extrapolated to 1x10^6 total spleen cells. Mean $\pm$ SD. Similar results were obtained 14 days post third immunization

5.3 Titration of minimal epitopes B8.1 and E8.1

To demonstrate the potency of these newly found minimal epitopes, as well as to show specificity, titrations at semi-logarithmic steps were performed. As Figure 4 demonstrates, there was a broad range of concentrations, which caused significant IFN-γ release. Interestingly, this applied to subcutaneously and intranasally immunized mice (Figure 4A and B, respectively), when spleens were assessed.

Peptides 19 and 27 are irrelevant peptides, which are also H2-K^d-restricted. In the data shown before, concentration of peptides was given in μg/mL. Here, concentration is expressed in terms of molarity, thereby allowing direct comparison between the two epitopes. Thus, there is a higher precursor frequency of T cells specific for B8.1 than for E8.1, in agreement with the *in silico* prediction score.

5.4 CD4/CD8 restriction of candidate peptides

To find out, whether the candidate peptides defined in BALB/c and C57BL/6 mice are CD4 or CD8 epitopes, the following experiment was made: Spleen cells from immunized animals were split into three aliquots. The first was used as is (termed whole suspension), the second aliquot was depleted of $CD8^+$ T cells and the third was depleted of $CD4^+$ T cells. Depletion was chosen to avoid the possibility of unspecific stimulation, which could be an effect of positive selection after direct labeling of CD4 or CD8.

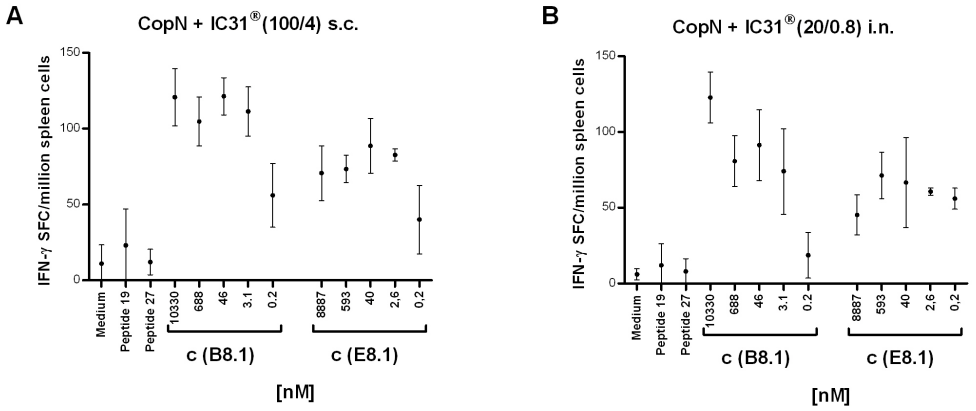

Figure 4: IFN-γ ELISpot: Titration of minimal epitopes B8.1 and E8.1 in BALB/c mice. Animals were immunized three times at day 0, 14 and 28 s.c. or i.n. On day 42 mice were sacrificed. Concentration of irrelevant peptides 19 and 27 was 10 μg/mL. SFC extrapolated to 1x10^6 total spleen cells. Mean $\pm$ SD. Comparable results were obtained in an independent experiment.

The results of this isolation approach are depicted in Figure 5. According to these data, B8 and B8.1 as well as E8 and E8.1 are CD8-restricted epitopes (Figure 5A). In the second experiment performed with BALB/c mice there were minor signals originating from $CD4^+CD8^-$ cells for A3, B6, B7 and F3 (not shown).

In C57BL/6 mice, A5, D10 and D11 might be CD4-restricted epitopes (Figure 5B).

The purity of the isolated cells was assessed via a FACS analysis as depicted in Figure 5C.

5.5 Analysis of isolated lung T cells

Data shown so far originated from spleen cells. Already it was seen, that intranasal administration could elicit a splenic response (Figure 4B). In this section, also lung T cells will be discussed.

In analogy to the isolation approach mentioned in section 5.4, the principle of untouched isolation was also followed here. This time on lung T cells, however. Non-T cells (i.e. B cells, NK cells, dendritic cells, macrophages, granulocytes and erythroid cells [1]) were labeled and retained, enabling further analysis of the unlabeled T cells.

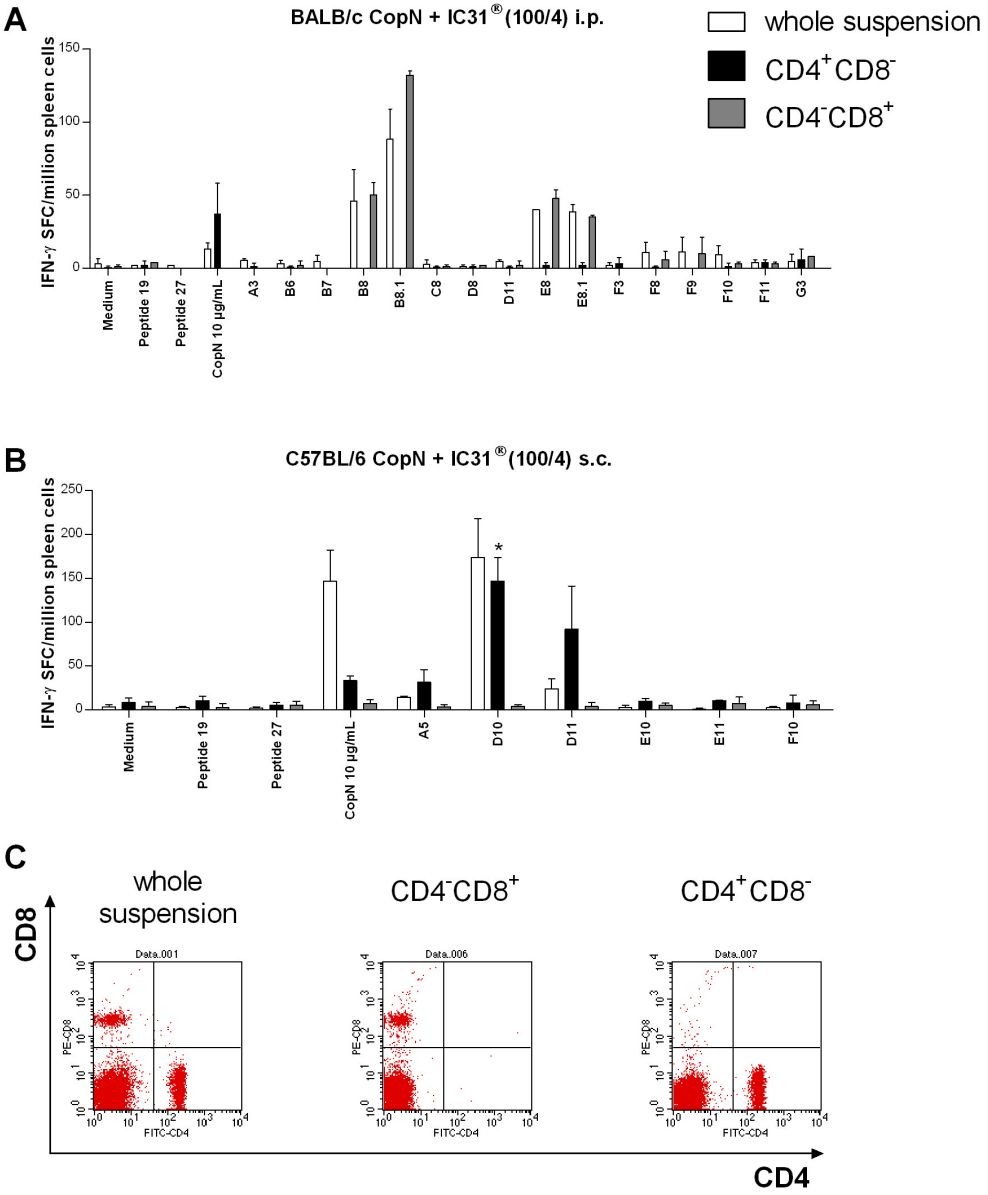

Figure 5: IFN-γ ELISpot: CD4/CD8 restriction of candidate peptides in BALB/c and C57BL/6 mice. Animals were immunized three times at day 0, 14 and 28 as shown. On day 42 mice were sacrificed. Concentration of peptides was 10 μg/mL. Peptides 19 and 27 are irrelevant peptides. **B**: *indicates, that the signal exceeded the upper detection limit. SFC extrapolated to $1x10^6$ total spleen cells. Mean $\pm$ SD. Similar results were obtained in an independent experiment for BALB/c mice. **C**: Flow cytometric analysis demonstrating cell purity of cells after isolation, which were used in **A**

Consequently, a source of antigen presenting cells must be supplied. Spleen cells from naïve mice were used, either without any treatment or after an irradiation step.

Figure 6A and B shows the lung T cell response of BALB/c and C57BL/6 mice. Most candidate peptides identified via the screening of spleen cells from mice immunized with CopN parenterally were also recognized by lung T cells from mice immunized with CopN by mucosal immunization. No signals were obtained in control groups which were immunized with CopN alone, IC31® alone or left naïve (not shown).

In particular, the IFN-γ signal in response to the minimal epitopes B8.1 and E8.1 was significantly higher here than in spleen cells from parenterally immunized mice (Figure 6A). Interestingly, the signal for peptide E8, which was always lower than B8 when spleen cells were assessed, reached a comparable value in this context (reproduced in an independent experiment).

To have a means of comparison for the mucosal administration of IC31®, BALB/c mice were also immunized with a CopN formulation adjuvanted with CTB or CpG1826. The response to selected epitopes is depicted in Figure 6C. In the case of IFN-γ response to peptides, IC31® outperformed CpG1826. A significant difference between CTB and IC31® was only apparent for peptide B8.

The mucosal response for C57BL/6 mice was of lower intensity (6B. Still, peptide D10 was readily detectable, while other peptide candidates showed a lower response.

The purity of lung T cells is depicted in Figure 6D. Before lung T cell isolation there were circa 40% $CD4^{-}CD8^{-}$ events in the $FSC/SSC^{lymphocyte}$ gate. These events dropped to circa 3% after isolation.

5.6 Comparison of subcutaneous and intranasal immunization

The data shown so far comprised analysis of spleen cells from parenterally or intranasally immunized mice, as well as lung T cells from intranasally immunized mice. A remaining question is, whether parenteral immunization could also elicit a lung T cell response.

The data shown in Figures 7 and 8 shows all four different combinations

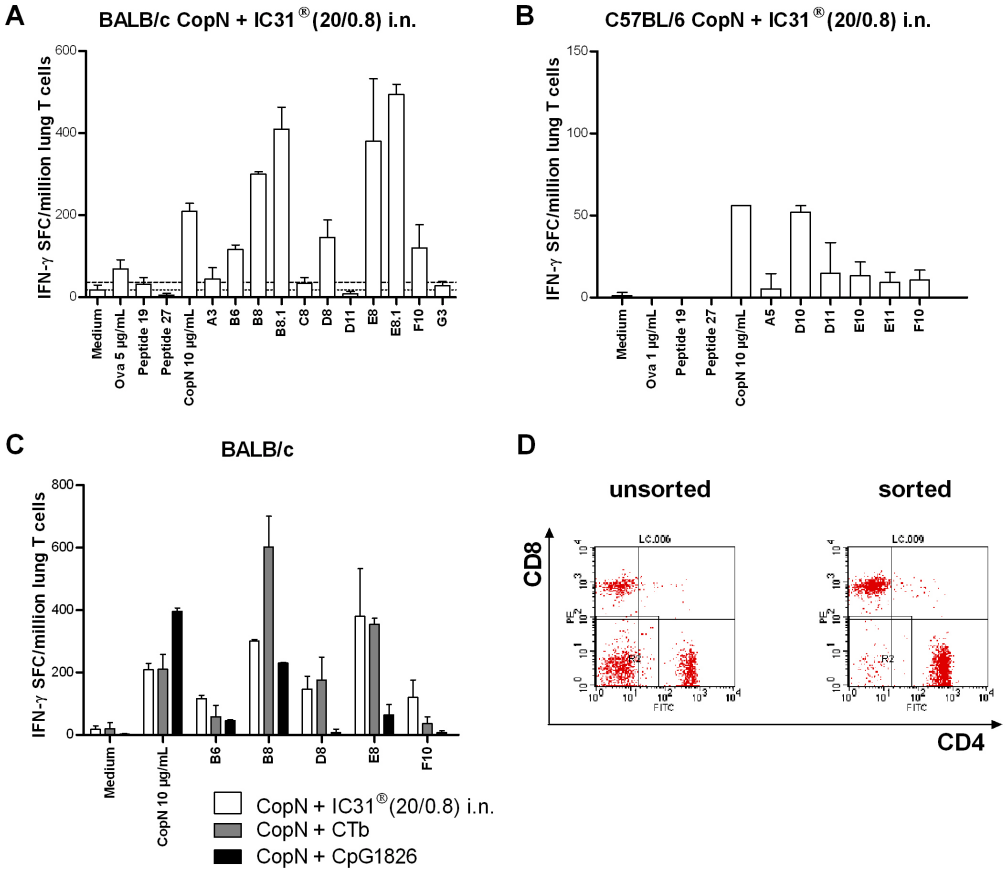

Figure 6: IFN-γ ELISpot using isolated lung T cells. Animals were immunized 3 times at day 0, 14 and 28 i.n. On day 42, mice were sacrificed. Concentration of peptides was 10 μg/mL. Ovalbumin was chosen as an irrelevant protein. Peptides 19 and 27 are irrelevant peptides. **C**: Comparison between IC31®, cholera toxin B and CpG1826. **D**: Demonstration of increased purity of lung T cells after isolation. Refer to text for details. SFC extrapolated to $1x10^6$ total lung T cells. Mean $\pm$ SD. Similar results were obtained in one independent experiment

Administration of CopN	Epitope	pentamer$^+$ cells [%]	
		Spleen cells	Lung cells
IC31®(100/4) s.c.	B8.1	0.23	0.03
IC31®(20/0,8) i.n.	B8.1	0.13	0.47
IC31®(100/4) s.c.	E8.1	0.14	0.05
IC31®(20/0,8) i.n.	E8.1	0.13	0.24

Table 4: CD8$^+$ T cell-specific probes for BALB/c mice. Animals were immunized 3 times at day 0, 14 and 28. On day 44, mice were sacrificed. Events shown here were gated from a FSC/SSC$^{\text{lymphocytes}}$ and a CD8$^{\text{high}}$ population. A background subtraction using signal obtained from a CopN-immunized control group was performed. A similar trend was observed in an independent experiment for spleen cells.

of administration route and organ assessed in BALB/c and C57BL/6 mice, respectively. It entails the lung T cell data shown in Figure 6.

There is some evidence, that subcutaneous immunization causes almost no priming of lung T cells in BALB/c mice, although a response could be detected in C57BL/6 mice. The observation, that intranasal immunization caused a splenic response was already discussed for BALB/c mice. Figure 8 suggests that it might also be true for C57BL/6 mice. Finally, it is also noteworthy that the magnitude of the lung T cell response after intranasal immunization was significantly greater in BALB/c than in C57BL/6 mice.

5.7 CD8$^+$ T cell-specific probes

The use of CD8$^+$ T cell-specific probes has become an accepted method for accurate quantification of precursor frequencies [6, 35, 47]. The probes used here consisted of a pentameric complex of H2-K$^{\text{d}}$ MHC class I molecules, connected though a coiled-coil domain to a fluorescent compound [2], which was allophycocyanin (APC). Due to the high costs of these reagents, only two pentamers were purchased for the most promising epitopes of BALB/c mice.

The data shown in Figure 9 shows the precursor frequencies of B8.1- and E8.1-specific lung T cells. It is apparent that lung T cells from mice immunized with CopN adjuvanted with IC31® had higher levels of epitope-specific cells than those from control groups.

Among the control groups, the number of specific CD8$^+$ T cells was higher in CopN immunized animals than the other two. Exact percentages

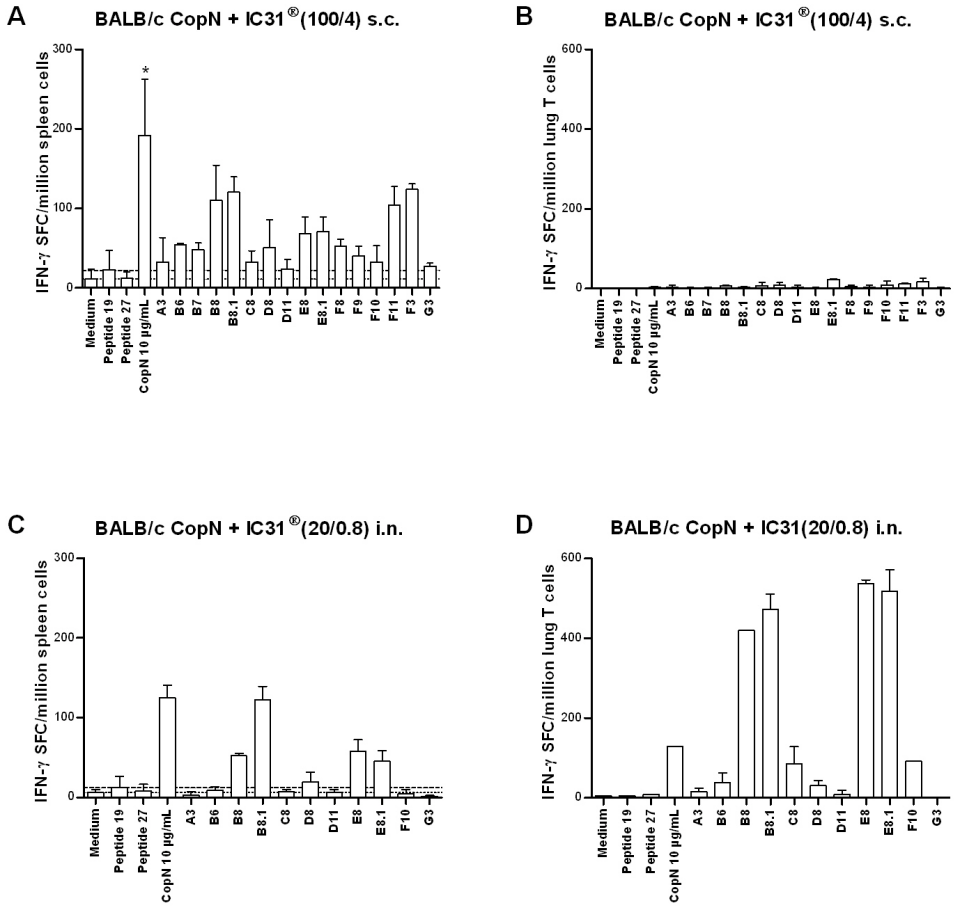

Figure 7: IFN-γ ELISpot: Combinations of administration route/organ assessed in BALB/c mice. Animals were immunized at day 0, 17 and 31. On day 46, mice were sacrificed. Concentration of peptides was 10 μg/mL. Peptides 19 and 27 are irrelevant peptides. SFC extrapolated to $1x10^6$ spleen cells or total lung T cells. Mean $\pm$ SD. **A**: *indicates, that the signal exceeded the upper detection limit. Similar results were obtained in at least one independent experiment, despite for the combination i.n./spleen cells

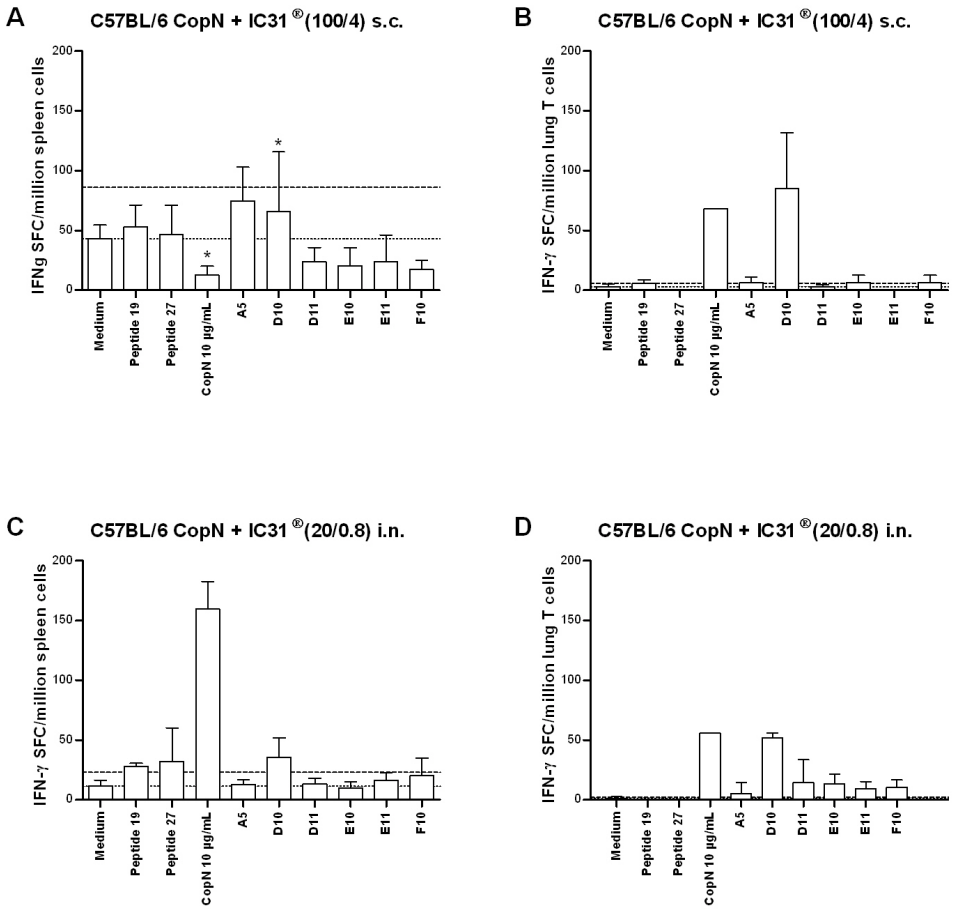

Figure 8: IFN-γ ELISpot: Combinations of administration route/organ assessed in C57BL/6 mice. Animals were immunized at day 0, 17 and 31. On day 46, mice were sacrificed. Concentration of peptides was 10 μg/mL. Peptides 19 and 27 are irrelevant peptides. SFC extrapolated to $1x10^6$ spleen cells or total lung T cells. Mean $\pm$ SD. **A**: *indicates, that the signal exceeded the upper detection limit. Similar results were obtained in one independent experiment, despite for the combination i.n./spleen cells

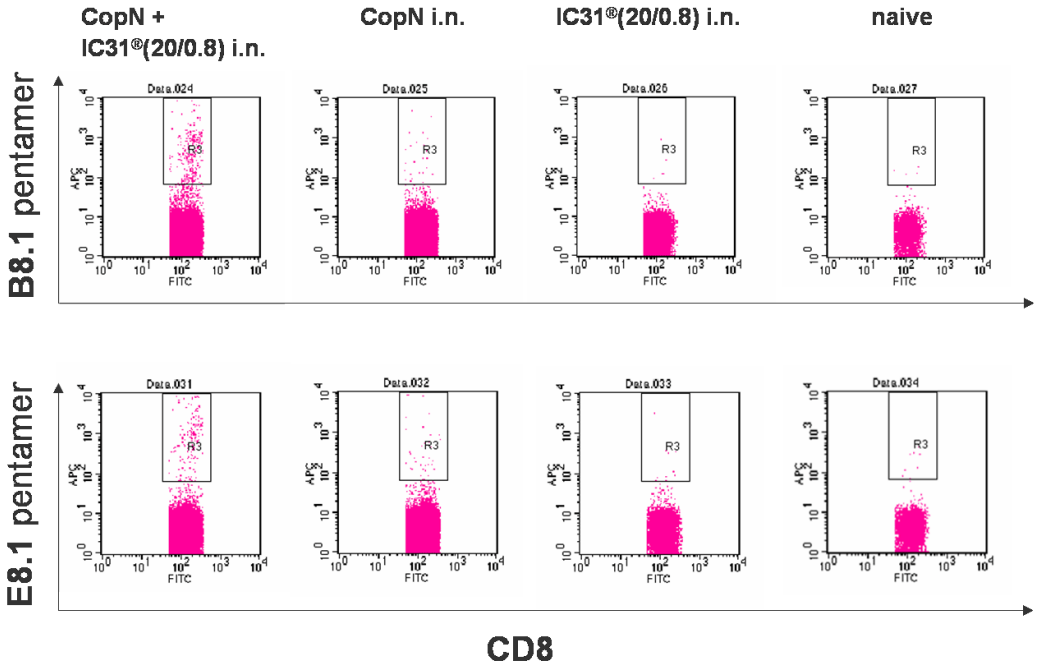

Figure 9: CD8$^+$ T cell-specific probes for BALB/c mice. Animals were immunized 3 times at day 0, 14 and 28. On day 44, mice were sacrificed. Events shown here were gated from a FSC/SSC$^{\text{lymphocytes}}$ and a CD8$^{\text{high}}$ population. A similar trend was observed in an independent experiment for spleen cells.

of background-corrected precursor frequencies are given in Table 4. Interestingly, subcutaneous application resulted in a considerable signal from spleen cells, but a negligible signal from lung T cells. On the other hand, intranasal application resulted in a considerable signal in lung T cells as well as in spleen cells.

In order to compare the percentages of specific T cells shown in Table 4 with ELISpot data, it is necessary to refer to the percentages to T cells instead of CD8$^+$ T cells. Previous experiments showed that there are ca. 25% of CD8$^+$ and 75% of CD4$^+$ T cells in BALB/c mice (not shown). Bearing this factor in mind, there were more pentamer$^+$ cells than IFN-γ releasing cells, as anticipated.

Specifically, the ratio of pentamer$^+$ cells and IFN-γ-releasing cells was higher in subcutaneously immunized animals than in intranasally immunized animals.

5.8 Single dose immunization with CopN

The results shown so far originated from animals immunized three times. This section discusses single-dose administration on CopN whole protein adjuvanted with IC31® or CpG1826.

Figure 10 suggests that IFN-γ release was detected after a single immunization of CopN at the base of the tail in BALB/c and C57BL/6 mice. As anticipated, the T cell precursor frequency observed here was lower than in animals which received three immunizations. Remarkably, peptide E8 was readily detectable when adjuvanted with IC31®, but not with CpG1826. This has also been observed in the intranasal administration of CopN.

A similar principle might apply for peptide D10 in C57BL/6 mice: It could be detected when IC31® was used as adjuvant, but not when CpG1826 was used.

5.9 Immunizations with candidate peptides

A relevant question is, whether peptide immunizations could also prime a detectable amount of specific T cells. Whereas up to now, immunizations were performed with 40 μg of CopN per dose, here 50 nmol of peptide were used. Due to the large amount of peptide required, only the top two peptides per mouse strain were used.

Results are depicted in Figure 11. Peptide E8 in BALB/c and D10 in C57BL/6 mice were able to prime enough T cells in order to detect them by means of an IFN-γ ELISpot. The apparent lack of signal in the case of B8 and E11, however, might suggest the need of a CD4 helper epitope [28, 38]. Another possible explanation might be, that impurities in the peptide synthesis acted as altered peptide ligands, promoting anergy instead of IFN-γ-releasing T cells [43].

5.10 IFN-γ response at later time points

A crucial aspect in vaccine development is the induction of memory T cells, which can exert protective functions long after vaccination. As a first step in this direction, IFN-γ response at time points later than 14 days after the third immunization were assessed.

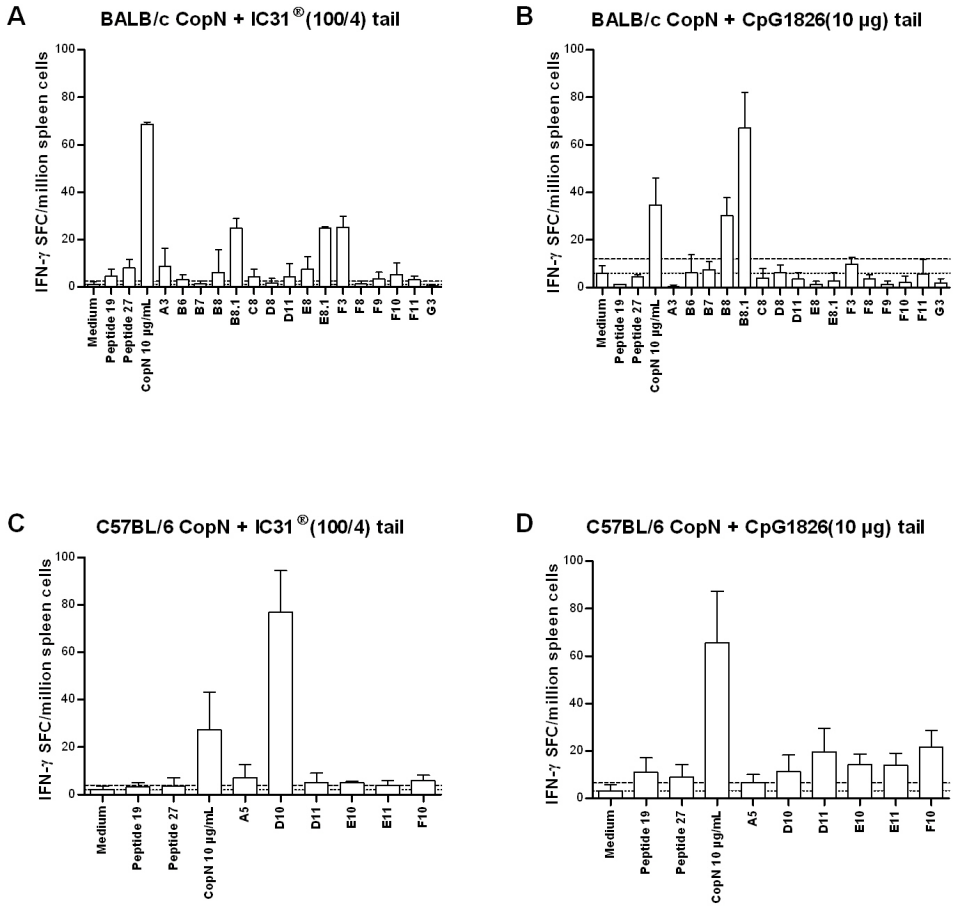

Figure 10: IFN-γ ELISpot after single dose immunization with CopN in BALB/c and C57BL/6 mice. Animals were immunized at day 0 at the base of the tail. On day 8, mice were sacrificed. Concentration of peptides was 10 μg/mL. Peptides 19 and 27 are irrelevant peptides. SFC extrapolated to 1x10^6 spleen cells. Mean $\pm$ SD. This experiment was only performed once

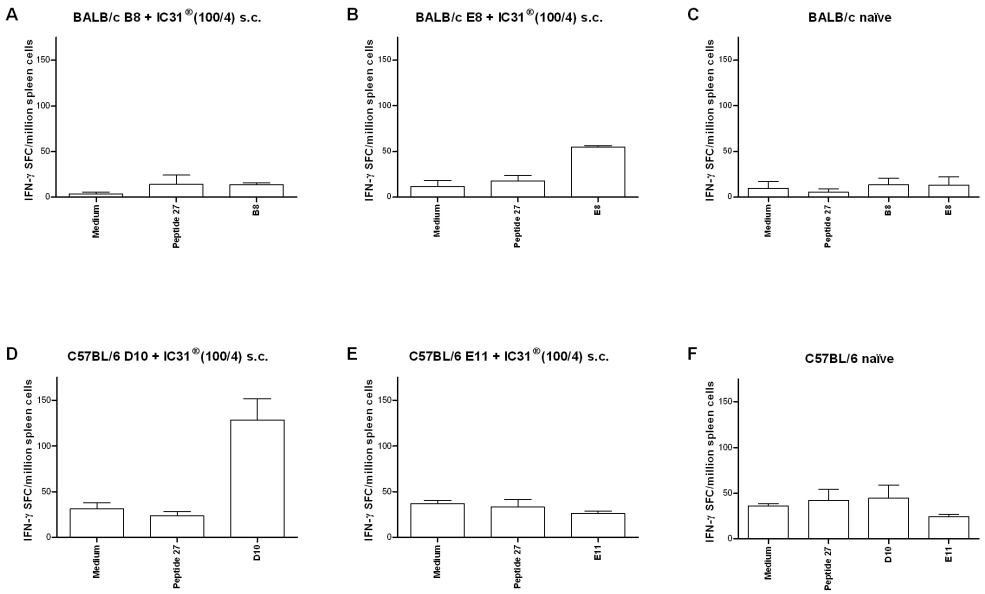

Figure 11: IFN-γ ELISpot after two immunizations with peptide in BALB/c and C57BL/6 mice. Animals were immunized at day 0 and 6 at the base of the tail with 50 nmol of peptide. On day 13, mice were sacrificed. Concentration of peptides was 10 μg/mL. Peptide 27 is an irrelevant peptide. SFC extrapolated to 1x10^6 spleen cells. Mean $\pm$ SD. This experiment was only performed once

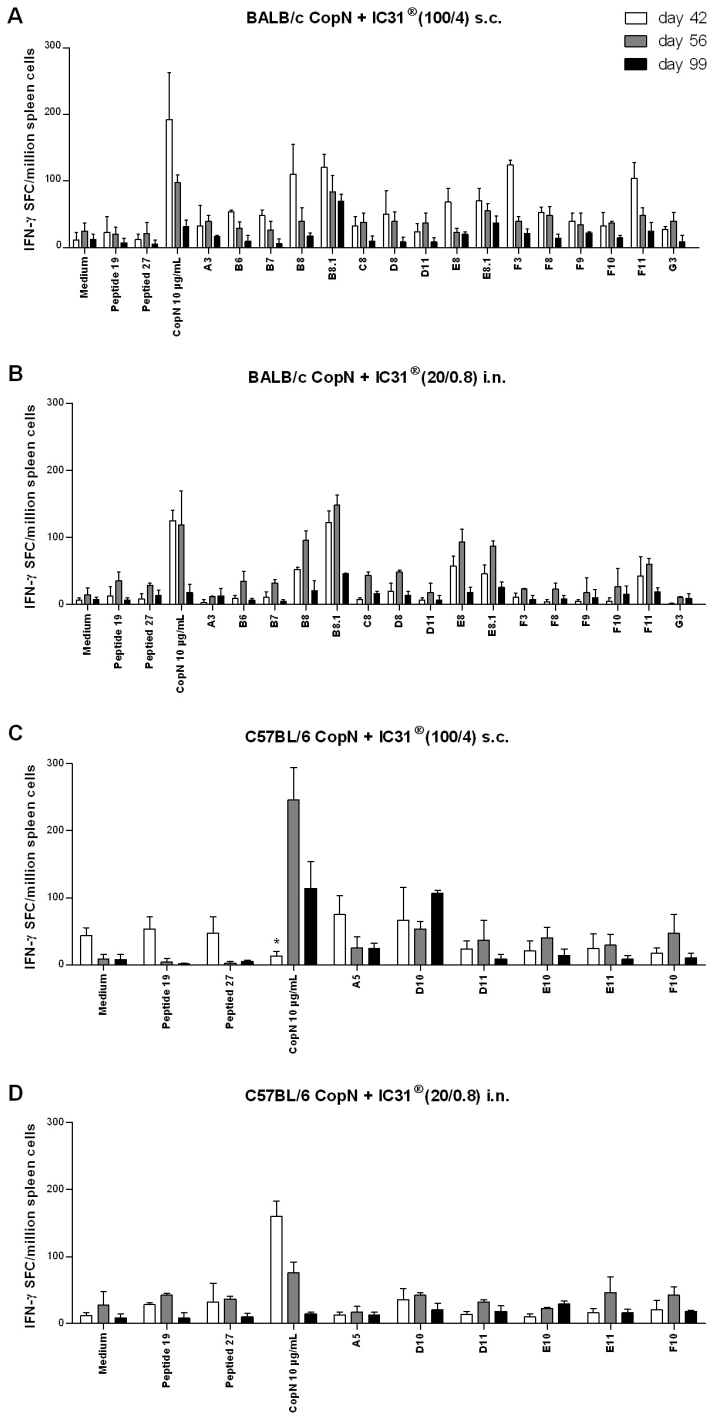

Figure 12: IFN-γ ELISpot at later time points in BALB/c and C57BL/6 mice. Animals were immunized at day 0, 14 and 28. Mice were sacrificed at three time points as indicated. Concentration of peptides was 10 μg/mL. Peptides 19 and 27 are irrelevant peptides. **C**: *indicates, that the signal exceeded the upper detection limit. SFC extrapolated to $1x10^6$ spleen cells. Mean $\pm$ SD. Data from day 42 and 99 was only obtained in one experiment.

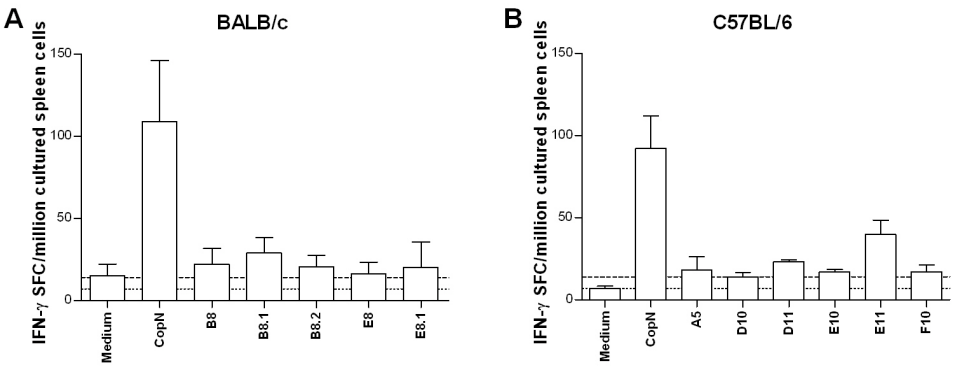

Figure 13: cultured IFN-γ ELISpot after *Chlamydia pneumoniae* infection in BALB/c and C57BL/6 mice. Animals were infected intranasally at day 0 and 27 (**A**) or 29 (**B**). Mice were sacrificed 12 days later. Concentration of stimuli was 10 μg/mL during the 48 hours culture, and half of this concentration during the ELISpot. SFC extrapolated to $1x10^6$ cultured spleen cells. Mean $\pm$ SD. Data for BALB/c was only obtained in one experiment. A similar trend was observed for C57BL/6 in one independent experiment

In BALB/c mice, the minimal epitopes B8.1 and E8.1 could still be easily detected at the latest time point, irrespective of subcutaneous or intranasal application (Figure 12A and B). Once again this elegantly demonstrates the experimental advantage of minimal epitopes.

In BALB/c mice, intranasal immunization caused a greater response of spleen cells on day 56 than on the other time points. This might reflect the migration of locally primed T cells to central organs such as the spleen. On the other hand, data at this time point was only assessed once.

It was also tried to obtain data from lung T cells at these later time points. This was not possible due to overstimulation of cells.

5.11 Priming of T cells during a *Chlamydia pneumoniae* infection

The data shown so far was obtained by immunizing mice with CopN whole protein or candidate peptide, mostly adjuvanted with IC31®. The evident question is whether reactivity towards CopN is of relevance during a *Chlamydia pneumoniae* infection. In other words: can a natural infection prime T

cells specific for CopN?

To solve this question, a cultured IFN-γ ELISpot was performed. After obtaining spleen cells from infected mice, these cells were incubated for circa 48 hours prior to the IFN-γ ELISpot. During this incubation period, the same stimuli were used as in the following ELISpot. This has the effect of amplifying small T cell populations, which could not be detected in an *ex vivo* ELISpot as performed before.

Figure 13 shows the outcome of this approach. A strong signal was obtained for CopN in both strains. Interestingly, signal for candidate peptide E11, which did not produce a strong signal in previous experiments, was slightly elevated compared to other peptides in C57BL/6 mice, indicating a particular role of E11 during an infection.

The signal for CopN may seem promising, however at this point it must be stated that a necessary control is missing: An aliquot of cells that was treated with CopN during the 48 hours culture should have been stimulated with an irrelevant protein control like Ovalbumin in the IFN-γ ELISpot. A similar control was performed with Ovalbumin also being present during the culture. The obtained signal was lower than for CopN (not shown), but this approach might not be a true irrelevant control.

It was also tried use the pentamers specific for B8.1 and E8.1 on a short term line from reinfected animals. No significant signals could be obtained (not shown), in agreement with the data from the cultured IFN-γ ELISpot.

5.12 *In vivo* cytotoxicity assay

The major functional response assessed so far has been the release of IFN-γ. However, the ultimate ability of $CD8^+$ T cells is to lyse target cells, which display a non-self epitope.

In an *in vivo* cytotoxicity assay, this ability can be measured. Naive spleen cells were incubated with a particular epitope and labeled with a fluorescent dye. These cells were then mixed with reference cells which were incubated with buffer only and labeled with a lower concentration of fluorescent dye.

In the experiment performed here, the cells were administered intranasally to immunized and infected animals, along with control groups. Due to the complexity of the experiment, only the top two peptide candidates for

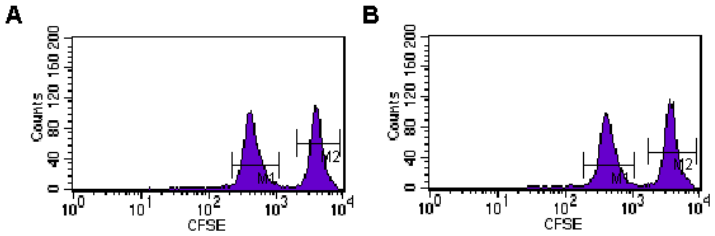

Figure 14: *In vivo* cytotoxicity assay: target cells for BALB/c mice before they were administered. Events shown here were gated from a FSC/SSC$^{\text{lymphocytes}}$ and a CFSE$^{\text{high}}$ population.

BALB/c mice were used. The fluorescent signal from these target cells before administration is depicted in Figure 14.

18 hours after administration, lungs were obtained from individual mice and measured as before. It was anticipated, that the number of cells which were pulsed with peptide decreases relative to the medium-control cells. No significant difference could be detected between infected or immunized and control groups (not shown). Possible explanations are discussed on page 38.

5.13 CD154 assay: Activation of helper T cells

CD154, also known as CD40L, is expressed by helper T cells activated by antigen and costimulators. CD40-CD40L interaction is involved in direct cell contact between helper T cells and B cells or macrophages, providing costimulatory signals [5]. Because CD154 is specifically upregulated on recently activated cells, it can be used as an activation marker [10, 16].

Data in Figure 5 suggested the presence of CD4-restricted epitopes in C57BL/6 mice. As can be seen in Figure 15, there was a CopN-dependent upregulation of CD154 compared to medium alone in mice that were immunized with CopN + IC31® subcutaneously. Furthermore, increase in background-corrected CD154 expression could not be detected in control groups, which received CopN alone or IC31® alone.

A specific upregulation of CD154 was not observed in reinfected mice compared to naive mice. However, it was not attempted to perform a preculture prior to the assay, which might amplify an existing helper T cell population specific for CopN, in a similar fashion as in Figure 13.

Increase in CD154 could not be observed in BALB/c mice, in agreement

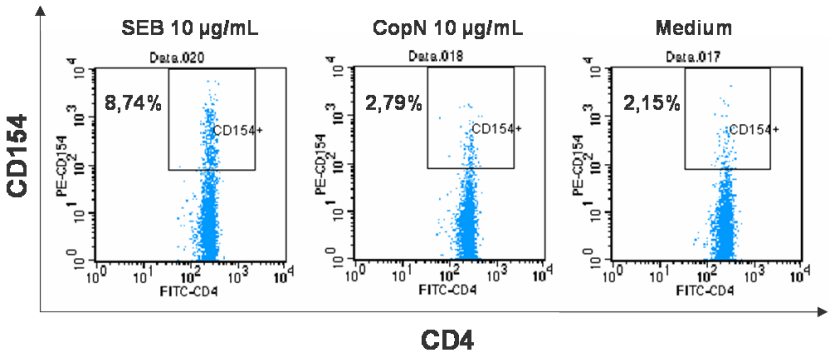

Figure 15: CD154 assay: Activation of helper T cells in C57BL/6 mice. Animals were immunized 3 times at day 0, 14 and 28 with CopN + IC31®(100/4) subcutaneously. On day 42, mice were sacrificed. The stimuli during the incubation are shown on top of each dot blot. SEB served as a positive control. 6x10^4 events in the FSC/$SSC^{lymphocytes}$ gate were collected. Events shown here were gated from a FSC/$SSC^{lymphocytes}$ and a $CD4^{high}$ population. This experiment was only performed once.

with the preponderant CD8 restriction of epitopes.

5.14 CD107 mobilization assay: Activation of $CD8^+$ cells

CD107 is present in the membrane of pre-formed cytotoxic granules inside $CD8^+$ T cells. Soon after recognition of antigen, activation-induced degranulation occurs, releasing cytotoxic compounds like perforin and granzyme. The exposure of the CD107 marker provides an indication of the cytotoxic potential of the responding $CD8^+$ T cells [7, 34].

The CD107 mobilization assay was attempted in five independent experiments, but no positive signal was obtained. Cells used originated from peptide-immunized mice, infected mice and cells from a short-term line. Signals for the following positive controls were obtained, in decreasing magnitude: concanavalin A, *Staphylococcus aureus* enterotoxin B and PMA/ionomycin (not shown). A detailed discussion of possible reasons is given on page 39.

5.15 Linear B cell epitope screening

As a side project, the peptide library was also used to perform a linear B cell epitope screening. Individual peptides were spotted on a membrane,

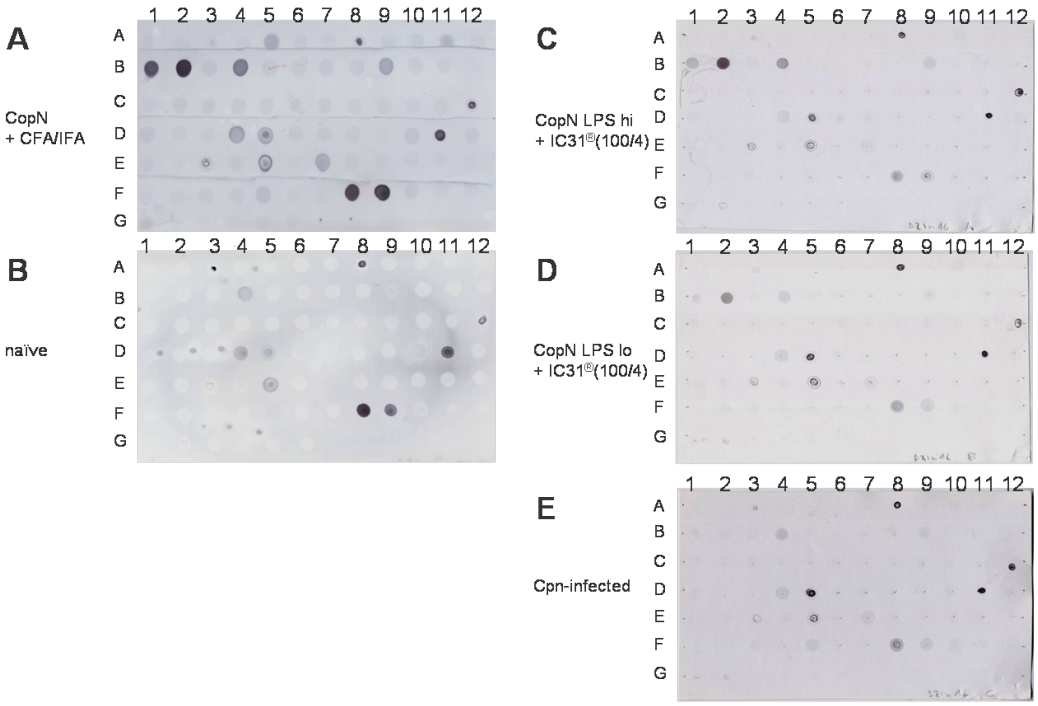

Figure 16: Linear B cell epitope screening. Serum was obtained from terminal bleedings. Circa 20 μg of peptide were spotted on a PVDF membrane. **C**: LPS content was ca. 14500 EU/mg during the first immunization and ca. 4500 during the subsequent immunizations. **D**: LPS content was 21 EU/mg at all three immunization. Similar results were obtained for **B** in one independent experiment.

which was then incubated with serum obtained from terminally bled animals followed by a colorimetric reaction.

As can be seen in Figure 16, there were several spots that differed between immunized, reinfected and the naïve group. There was a faint signal for peptide E7 that appeared in immunized and reinfected groups, but not in naïve. Peptide F5 only appeared in the CFA/IFA-immunized group, not in naïve or IC31®-adjuvanted groups.

A pronounced signal was peptide B2. It appeared in immunized groups, but not in naïve or infected groups. Although antibodies against B2 might not be generated in a natural infection, they might still be involved in recognizing live bacteria.

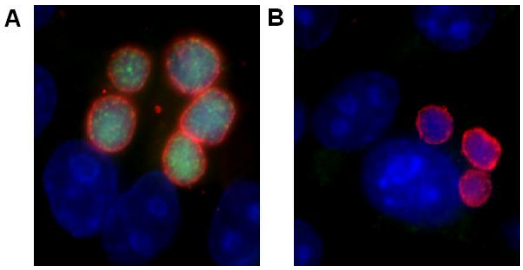

Figure 17: Immunofluorescence microscopy of cells infected with *Chlamydia pneumonia*: HEp-2 cells were grown on coverslips in 24-well plates. They were infected with *Chlamydia pneumoniae* K6 and incubated for 72 hours. Then, they were incubated with mouse serum and a rabbit antibody specific for the inclusion membrane. Serum in **A** and **B** was from BALB/c mice immunized with CopN + IC31®(100/4) i.p. **A**: LPS content was ca. 14500 EU/mg during the first immunization and ca. 4500 EU/mg during subsequent immunizations. **B**: LPS content was ca. 4500 EU/mg during the first immunization and 21 EU/mg during subsequent immunizations. Green signal: AF488-conjugated goat anti-mouse Ig. Red signal: AF594-conjugated goat anti rabbit IgG. Nuclei were stained with DAPI (blue).

5.16 Immunofluorescence microscopy of cells infected with *Chlamydia pneumoniae*

The serum obtained from immunized animals was also used to stain infected cells. Thereafter, a fluorochrome-labeled anti-mouse secondary antibody was used.

Interestingly, detection of *Chlamydia pneumonia* was only possible with serum from animals immunized with CopN + IC31®, where CopN had a high LPS content (Figure 17A). Animals immunized under similar conditions, where only the batch of CopN differed, did not produce serum capable of staining bacterial structures (Figure 17B). Moreover, animals which were immunized with CopN high LPS without adjuvant produced serum which stained *Chlamydia pneumonia*, if a high exposure time was set. Serum from animals immunized with IC31® only or naïve did not stain infected cells (not shown).

5.17 ELISA of serum from immunized animals

Figure 18 depicts the results of an ELISA against whole IgG. Reminiscent of the Immunofluorescence microscopy experiments, there was a LPS-dependent behavior (Figure 18A and B).

Serum from BALB/c mice immunized with CopN + IC31®(100/4) i.p.

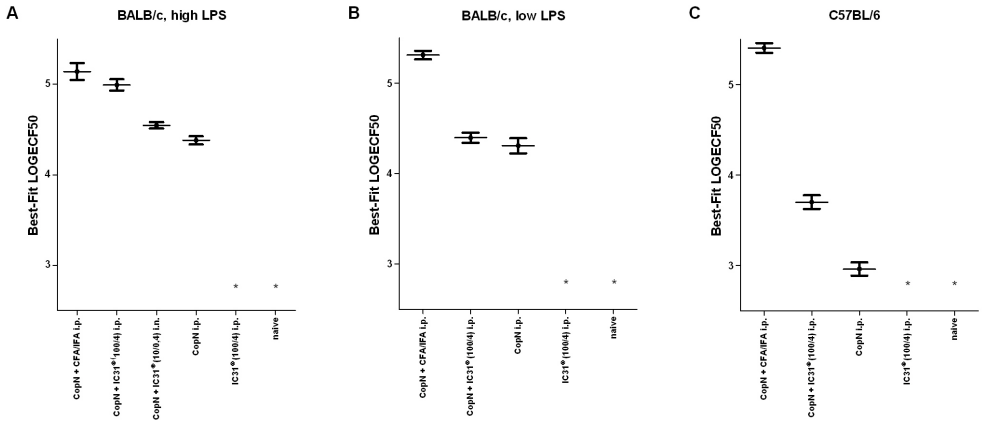

Figure 18: ELISA of serum from immunized animals. Mice were immunized three times at day 0, 14 and 28 as depicted. Mice were terminally bled at day 42. **A**: LPS content was ca. 14500 EU/mg during the first immunization and ca. 4500 EU/mg during subsequent immunizations. **B** and **C**: LPS content was ca. 4500 EU/mg during the first immunization and 21 EU/mg during subsequent immunizations. * below limit of detection. Data obtained from non-linear regression with constrained bottom of blank-subtracted means from photometric readouts. Mean ± 95% CI.

showed a comparable IgG titer as mice immunized with CFA/IFA only when CopN with high amounts of LPS was used. The titer dropped by one order of magnitude when CopN with low LPS contamination was used. This effect was even more apparent in the more Th1-tilted C57BL/6 strain.

Because the focus of this thesis was more on the T cell response to administrations of IC31®, this issue was not followed up.

5.18 Microarray analysis of total RNA from lung T cells

In order to identify factors, which are differentially regulated during intranasal administration of CopN + IC31® in lung T cells, a microarray experiment was performed. Freshly isolated lung T cells from immunized and control mice were homogenized and total RNA was isolated.

In this trial experiment, only BALB/c mice were chosen. Mice received CopN + IC31®(20/0.8), CopN only or were left naïve. Purity of the isolated RNA was assessed photometrically. Values for A_{260}/A_{280} were above 2 and values for A_{260}/A_{230} were above 1.7, indicating negligible protein and salt

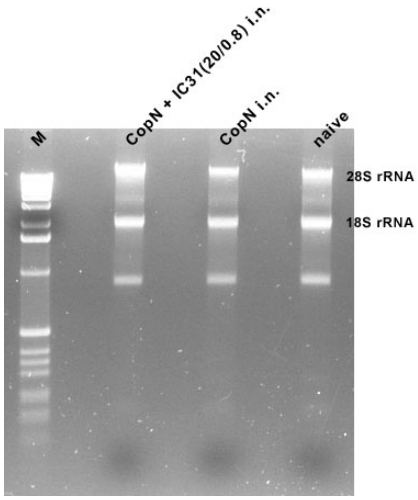

Figure 19: Non-denaturing agarose gel of total RNA from freshly isolated lung T cells from BALB/c mice. A RNeasy Midi isolation kit (Qiagen) was used to isolate RNA after MACS. 1 μg of RNA was loaded onto a 1.2% agarose gel. The marker (M) used here was only of internal use.

contaminations, respectively.

Furthermore, a non-denaturing gel was run to detect potential degradation events. According to Figure 19, no degradation occurred. The upper two bands might correspond to 28 and 18S rRNA. The lowest band might be 5S rRNA.

Microarray analysis of RNA was performed by SABiosciences™. The array covered mouse inflammatory cytokines and receptors. A list of probes which were sampled is given in the appendix on page A.

Candidate genes which were significantly up- or downregulated in the sample group CopN + IC31®(20/0.8 i.n.) compared to both control groups (CopN i.n. or naïve) were further analyzed. The cellular source and main function of a given gene were assessed individually in the Cytokines & Cells Online Pathfinder Encyclopaedia [3], Online Mendelian Inheritance in Man [4] or in a textbook [5]. Table 5 shows differentially regulated mRNA known to be expressed in T cells. A discussion is given on page 39.

Upregulated	
Name	**GeneBank**
Interferon alpha 2	NM_010503
Interleukin 12B	NM_008352
Downregulated	
Name	**GeneBank**
Chemokine (C-C motif) ligand 5	NM_013653
Interferon gamma	NM_008337
Interleukin 10	NM_010548
Interleukin 10 receptor, beta	NM_008349
Interleukin 18	NM_008360
Interleukin 1 beta	NM_008361
Interleukin 3	NM_010556
Integrin beta 2	NM_008404
Secreted phosphoprotein 1	NM_009263
Tumor necrosis factor	NM_013693

Table 5: Microarray analysis of total RNA from lung T cells: Genes up- or downregulated in sample group compared to control groups. Refer to text for more information.

6 Discussion

The data shown here demonstrate encouraging effects of the adjuvant IC31® on mucosal immunity in the murine system. Specifically, intranasal administration of the whole protein CopN in combination with IC31® resulted in local and systemic immune responses, as determined via IFN-γ ELISpot of lung T cells and spleen cells, respectively.

Preceding these findings, immunogenic epitopes of CopN were identified. Using a purely experimental approach avoids the risk of missing peptides, which could be identified as false negatives *in silico*. However, the design of the peptide library had a bias towards CD8-restricted epitopes: Peptides presented on MHC class I have a relatively narrow size distribution of 8-11 amino acids (rare exceptions exist, though), whereas peptides displayed on MHC class II can be between 10-30 amino acids or even longer [5, 33]. The peptides used for the screening were 15-mers. Furthermore, only IFN-γ and no Th2 cytokine was used during the screening.

Despite these obvious limitations, attractive candidate epitopes were suggested here, among which B8.1 and E8.1 were the most promising in BALB/c mice. Here, further evidence was also provided by using MHCI pentamers. Both B8.1- and E8.1-specific $CD8^+$ T cells could be detected in lungs and spleens from animals immunized i.n.

Titration experiments suggested, that more T cells are primed for B8.1 than for E8.1. *In silico* predictions, which base their calculations on the affinity of individual amino acids to the MHC, yielded the same ranking. Interestingly, it has been described that peptide affinity towards MHC directly correlates with immunogenicity [43].

A follow-up experiment would be to test the potential minimal epitopes B8.2-3 and E8.2-4 also at lower concentrations, because they gave responses significantly above medium background. If other sites are reproducibly shown to be immunogenic, this immunogenic "hot spot" might be interesting from a sequence conservation point of view: Should a population be immune due to exquisite recognition of a single epitope, a pathogen could rapidly mutate and evade immune recognition. However, should a population be able to recognize multiple neighboring epitopes, the probability for the pathogen to mutate both of these epitopes in one generation, while maintaining a functional gene

product, is very low. The necessity of *Cpn* to maintain a functional CopN was already demonstrated by Huang et al. using a functional knock-out of CopN [24].

Direct comparison of IFN-γ SFCs among lung T cells from animals immunized with CopN and IC31®, CTB or CpG1826 gave interesting results: Signals from IC31® were, with the exception of B8, stronger than signals from the "gold standard" CTB. With the exception of CopN whole protein, IC31® always performed equal or better than CpG1826.

A potentially interesting observation was, that the ratio of pentamer$^+$ cells and IFN-γ-releasing cells was higher in subcutaneously immunized animals than in intranasally immunized animals. An intriguing FACS-based follow-up experiment might be to take multiple cytokines into account. I.n. administration might preferably induce effector T cells compared to parenteral administration, which might induce a broader repertoire of cytokine secretion.

According to preliminary results, s.c. immunization caused almost no priming of lung T cells in BALB/c mice, while IFN-γ release was detectable in C57BL/6 mice. As mentioned in the introduction, this might be an effect of the preference of C57BL/6 mice to mount a Th1-type of reaction. On the other hand, i.n. administration in BALB/c mice caused a significantly stronger priming of lung T cell than in C57BL/6 mice.

An encouraging result was the detection of CopN-specific cells during a *Cpn* infection. This is in agreement with the data shown by Tammiruusu et al. [50]. The logical follow-up would be to detect a reduction in bacterial load in immunized vs. control groups. Furthermore, an optimized protocol for obtaining short-term lines might also detect candidate epitope-specific *Cpn*-primed T cells.

A functional assay that was attempted here was the in vivo cytotoxicity assay. Unfortunately, no specific killing was observed, which is likely due to wrong assay conditions. The two most critical parameters in this context might be the choice of peptide and the incubation period.

Here, 15-mers (B8 and E8) were used. However, other papers reporting this method used minimal epitopes, mostly nonamers [8]. Thus, a repetition with B8.1 and E8.1 might yield a better result.

Furthermore, the incubation period used here was 18 hours, as reported previously [51]. However, it has been shown that killing can occur as early as 15 minutes after administration of labeled cells [8], in agreement with the observation that target cell death occurs within 5 minutes of CTL-target cell contact [46]. Thus, assessment of killing at multiple earlier time points might also be feasible.

The fact that significant IFN-γ release was detected for B8 and E8, but all attempts of the CD107 mobilization assay being unsuccessful raises the question if the experimental conditions have been optimal.

First of all, only anti-CD107a has been used. Betts et al. performed trial experiments with CD107a and CD107b and used a mixture of both throughout the rest of their publication [7].

Second, a brighter fluorochrome like allophycocyanin might be superior over AF488 which was used here. APC-CD107 is very likely to produce an easily discernible $CD107^{high}$ population.

Third, a minor optimization might be to add the secretion inhibitor monensin one hour after incubation instead of adding it already at the beginning of the incubation.

Finally, it might also be worth considering to lower the amount of FCS present in the culture medium, because high levels of background-stimulation were observed. Another optimization in this direction would be to define a dump channel for B cells and monocytes, which were reported to contribute significantly to non-specific background [7]. Then, all confounding CD107-signals from these cells could easily be excluded.

Concerning the peptide-immunization experiments, no signal was obtained for B8 and E11. It is possible that B8 requires the presence of $CD4^+$ helper epitopes in order to be immunogenic [38]. Before any conclusions are drawn, this experiment would have to be repeated, preferably with a very pure synthesis in order to avoid tolerance induction by altered peptide ligands [43].

Despite the initial trial character of the microarray experiment, which awaits optimization and reproduction of results, a brief description of the biological activities of differentially regulated factors are given here. Information was collected from sources mentioned before [3–5].

All known subtypes of IFN-α, including IFN-α2 shown to be upregulated here, display the same antiviral, antiparasitic, antiproliferative activities, which might indicate a general effect mediated by IC31®.

IL-12B is part of the heterodimer IL-12, together with IL-12A. IL-12B was shown to be upregulated, but not IL-12A. Murine IL-12 has been shown to alter $CD4^+$ subset differentiation. It is also involved in protective immunity against intracellular parasitic infections such as Leishmania major. However, IL-12B may also form homodimers, which antagonize the aforementioned effects. Interestingly, IL-10, shown to be downregulated, is an inhibitor of IL-12 production.

The following factors were shown to be downregulated:

Chemokine (C-C motif) ligand 5 (RANTES) is induced by TNF-α and IL-1α, but repressed following the stimulation of T cells. RANTES is involved in recruiting lymphocytes to inflammatory sites.

IFN-γ is mainly produced by T an NK cells in response to ag. The main function of IFN-γ is to activate macrophages. It is also involved in promoting a Th1-type response and repressing a Th2-type response. Secreted phosphoprotein 1, also known as osteopontin, which was also shown to be downregulated, is secreted by a plethora of cells, including activated T and NK cells. It was also shown to be involved in promotion of robust Th1 responses. In close analogy, IL-18, also downregulated, functions as a growth and differentiation factor for Th1 cells.

IFN-γ also inhibits the growth of B-cells induced by IL-4, as well the production of IgG1 and IgE. Interestingly, also IL-12 has been shown to inhibit production of IgE.

In monocytes and macrophages IFN-γ induces the secretion of TNF-α, which was also shown to be downregulated here. TNF-α is intimately associated with inflammatory responses.

Furthermore, TNF-α, along with type I and II interferons and microbial products, induces the synthesis of IL-1. The main biological activity of IL-1 is the promotion of Th1-type responses. Low concentrations of IL-1 are associated with local inflammation, whereas high concentrations are associated with systemic inflammation.

In summary, these initial microarray results point towards an anti-in-

flammatory, anti-IgE state with a repression of factors that favor a Th1-type response. It must be stressed that this is only a "snapshot" of the developing immune response, hence interpretation must be performed carefully.

Although the findings related to mucosal administration of IC31® described here might indicate significant adjuvanticity in the mouse model, extrapolation to man must be treated with caution. It has been reported that bronchus associated lymphoid tissue (BALT) is significantly lower or absent in man compared to mice [21, 36]. Furthermore, TLR9, which was reported to be a target of IC31® [42] is expressed in different DC subsets in mice and humans, and CpG sequences resulting in optimal immune response are also species-specific [12, 19, 27].

Concerning the comparison between BALB/c and C57BL/6 mice in response to IC31®, it has been reported that DCs isolated from the spleens of naive C57BL/6 mice preferentially expressed TLR9 mRNA. [32]. Also, IL-12 production was much higher in C57BL/6 DC in response to phosphorothioate-stabilized CpG ODN.

In addition to differences in the mucosal immune system, one must not forget that man has 20-40% lymphocytes among white blood cells, whereas mice have 50-70% [21].

Finally, the CTL epitopes identified here might be incorporated in a multi-epitope vaccine, as demonstrated in a paper by Pinchuk et al. [38]. In this DNA-based vaccine, 7 $CD8^+$ T cell epitopes and a $CD4^+$ epitope are combined, resulting in protective immunity against *Cpn*. Mucosal administration of a similar construct, in combination with IC31®, might also prove to be protective in mice.

7 Materials and methods

7.1 Mice

Female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice were obtained from Charles River laboratories, Germany, or Harlan Winkelmann, Germany, at an age of 6-8 weeks. Experiments were initiated before mice reached an age of 12 weeks. Mice were kept in our animal facility under specific pathogen-free conditions with free access to food and water. All animals experiments were conducted according to Austrian Law.

7.2 Antigens

Chlamydia outer protein N (CopN, Low Calcium Response E [*Chlamydophila pneumoniae* CWL029] Accession AAD18473) with an N-terminal 6xHis tag was used throughout this study. CopN was overexpressed in E. coli BL21 Codon Plus under standard conditions. Lysis was performed via sonication in 50 mM Tris pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100 (Fluka), 1 mM DTT (Serva) in the presence of protease inhibitors: 1 μg/ml pepstatin A (Sigma), 1 μg/ml aprotinin (Sigma), 1 μg/ml leupeptin (Serva), 40 μM bestatin (Sigma), 10 μM E-64 (Sigma), 1000 μM AEBSF(Serva). DNA was digested using DNase 1 grade II (Roche).

Isolation was performed using gravity flow-columns with Ni-sepharose 6 fast flow (GE Healthcare). Washing occurred in 20 mM imidazole (Sigma), 50 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol and 0.5 mM DTT. Elution was performed with 500 mM imidazole and 0.5 mM DTT in TNG buffer. TNG buffer consisted of 50 mM Tris pH 8.0, 120 mM NaCl, 5 mM KCl and 10% glycerol. The eluate was dialyzed against TNG buffer, followed by sterile filtration.

Protein concentration was determined using the BCA Protein Assay (Pierce). The amount of LPS-contamination was measured using the QCL-1000 Endpoint Chromogenic Limulus Amoebocyte Lysate Assay (Lonza).

The peptide library which was used here consisted of 15-mers overlapping by 10 amino acids, covering the entire sequence of CopN. Peptides were synthesized in-house by solid phase synthesis on preloaded 2-chlorotrityl resins (Pepchem) on a multiple peptide synthesizer (Syro, Multisyntech). After syn-

thesis, 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). Candidate peptide epitopes were either synthesized in-house or purchsed from ProImmune. All peptides were solubilized in DMSO (Sigma) to a concentration of 20 mg/mL. Peptides were stored at -20°C until needed.

Irrelevant peptides 19 and 27 were also synthesized in-house. Peptide 19 has the sequence KIYNRNIVNRLLGD and encodes an H-2^d-restricted cytotoxic T lymphocyte epitope [15]. Peptide 27 has the sequence CYHASVTDI and encodes an H-2^d-restricted cytotoxic T lymphocyte epitope [49].

Crystallized chicken Ovalbumin (Sigma) dissolved in PBS (Gibco) was used as an irrelevant antigen for *ex vivo* re-stimulation of cells in ELISpot assays or activation-based assays using flow cytometry.

7.3 Adjuvants

$KLKL_5KLK$ (NH_2-KLKLLLLLKLK-COOH) and ODN1a (phosphodiester backbone ODN, oligo-$(dIdC)_{13}$) were purchased from Bachem (KLK) and Transgenomic (ODN1a). The individual components of IC31® were dissolved in 10 mM Tris, 135 mM NaCl (pH 7.5–8) at a stock concentration of 2000 nmol KLK/80 nmol ODN1a. Before preparing the vaccine formulation, this stock solution was mixed vigorously at RT. The final amount of IC31® in the vaccine formulation is given in brackets throughout the text: the first number designates the amount of KLK in nmol and the second the amount of ODN1a in nmol.

Initially, also CFA and IFA (Difco) were used intraperitoneally. The vaccine formulation consisted of 50% v/v of CFA for the first immunization and IFA for subsequent immunitaions.

Cholera toxin or Cholera toxin b subunit (Sigma) and CpG1826 (Purimex) were used at 10 μg/dose intranasally.

7.4 Vaccination of mice

Irrespective of route, 40 μg of CopN were administered. In the peptide-immunization experiments, 50 nmol of peptide were administered. Intraperi-

toneal vaccination of mice occurred in a volume of 200 μL per dose. Subcutaneous vaccination of mice occurred in a volume of 100 μL per dose at the left flank. Intranasal vaccination was performed under light anesthesia of Isoflurane (Baxter) in a volume of 40 μL per dose, administered with a pipettor. Vaccinations at the base of the tail were performed in 100 μL per dose. Naïve mice or mice which received antigen or adjuvant alone served as controls.

7.5 Infection of mice with *Chlamydia pneumoniae*

Mice received a hypothetical bacterial load of 1x10^6 *Chlamydia pneumoniae* K6 IFUs/dose. This was performed intranasally, under light anesthesia.

7.6 Media

Unless stated otherwise, the medium used here 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), an additional 2 mM L-glutamine (Gibco), 50 μM β-mercaptoathanol (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin (PAA). This was defined as T cell medium (TCM).

For experiments with HEp-2 cells, no β-mercaptoathanol was added.

For subsequent experiments with *Cpn*, 100 μg/mL gentamycin (PAA) were used instead of penicillin and streptomycin.

For Immunofluorescence microscopy experiments, the 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), an 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).

7.7 Cell preparation and purification

At different time-points after single and booster immunization or infection, mice were sacrificed by cervical dislocation and spleens and/or lungs were collected. All organs from animals in the same experimental groups were pooled and single cell suspensions were prepared mechanically in TCM followed by the lysis of erythrocytes in a solution containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 10 μM EDTA buffer pH 7.2-7.4. or in a commercially available erythrocyte lysis buffer (Sigma).

Whenever possible, cells were stored on 4°C. After washing, viable cell count was determined by trypan blue exclusion staining.

If an isolation step was performed, cells were first washed in MACS-buffer (0.5% BSA, 2 mM EDTA in PBS). For CD4 and CD8 depletion experiments, CD4 (L3T4) and CD8a (Ly-2) microbeads were used in combination with LD columns. For isolation of lung T cells, the Pan T Cell Isolation Kit with pre-separation filters and LS columns were used, according to the manufacturer's instructions (MACS, Miltenyi Biotec). Purity was determined by fluorescent staining of cells followed by flow cytometry as outlined in section 7.9.

7.8 IFN-γ ELISpot assay

Sterile MS 96-well filtration plates for the IFN-γ ELISpot were purchased from Millipore (Cat. MAHAS4510). Wells were pre-wetted with PBS (Gibco) and coated with anti-mouse IFN-γ antibody (BD Pharmingen) diluted in coating buffer to a concentration of 3 μg/mL. Coating buffer was made of 8.4 g/L $NaHCO_3$ in aqua bidestillata (Mayrhofer Pharmaceuticals) pH 9.2-9.4. Coating was performed on 37°C for at least one hour or on 4°C in a humid chamber for at least one day. Before the assay, plates were blocked with 1% BSA in PBS on 37°C for one hour or on RT for at least two hours.

After the blocking step, $2.5 - 10\text{x}10^5$ cells in TCM were added per well. In the case of APC-derived cells, a roughly equimolar amount of naïve spleen cells was added. For the majority of experiments, these cells were irradiated with circa 20 Gy. Subsequently, stimuli were added. ConA (Sigma) was added as a positive control. Usually, Ova, peptides 19 and 27 were added as irrelevant controls. TCM was added as a negative control. Finally, RCAS (Becton Dickinson) was added to a final concentration of 1%.

Incubation at 37°C/5% CO_2 usually lasted around 20 hours. For cultured ELISpots, conditions were similar: cells were not placed in the ELISpot-plates, but in standard round-bottom 96-well plates at a density of $1\text{x}10^6$ cells/well. No RCAS was added. After 48 hours of incubation, old medium was removed and cells were transferred into ELISpot plates. Stimuli were added at half the concentration as during the pre-culture. RCAS was added to a final concentration of 1%. Then it was preceeded as in a standard IFN-γ ELISpot.

Afterwards, plates were washed with washing buffer, which consisted of 0.1% Tween20 (Sigma) in PBS, for circa 15 min at RT. Biotin-labeled detection antibody (BD Pharmingen) was added to a final concentration of 1 μg/mL in PBS.

Incubation on 37°C lasted two hours or o/n, if incubated on 4°C in a humid chamber. After washing, Streptavidin-labeled horseraddish peroxidase (Roche) was added and incubated on 37°C for 30 min. After a last round of washing, substrate was added, consisting 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 on RT and was usually terminated after circa 10 min by flushing the plates under cold tap water. The dry plates were analyzed on a Bioreader 5000 (BioSys, Germany).

7.9 Flow cytometry

Antibodies were obtained from eBioscience and used at 1 μg/mL unless stated otherwise. Antibodies used here were anti-CD4-FITC, anti-CD8a-PE, anti-CD8a-FITC, anti-CD3e-APC, anti-CD107a-AF488 (circa 2 μg/mL) and anti-CD154-PE as well as appropriate isotype controls. H2-K^d MHC class I pentamers against B8.1 (KYASGNSEI) and E8.1 (DYFESRVPI) were APC-conjugated and purchased from ProImmune. Pentamers were cross-titrated with anti-CD8 and used at 8 μL per stain.

$1 - 2\text{x}10^6$ cells were stained in a total volume of 100 μL for 20-30 min at 4°C. For pentamer experiments, incubation was exactly 10 minutes at RT. After excessive washing cells were measured immediately on a FACSCalibur equipped with CellQuest software (Becton Dickinson)

7.10 CD154 and CD107 assay

For the CD154 assay, $2\text{x}10^6$ cells were plated in a 96-well U-bottom plate. Subsequently, stimuli were added. SEB was used as a positive control. Finally, anti-CD154, GolgiStop (Becton Dickinson, concentration as recommended) and RCAS (final concentration: 1%) were added and cells were incubated at 37°C/5% CO_2 for 8 hours or overnight. Thereafter, the staining protocol outlined in the previous section was performed.

For the CD107 assay, $1 - 2\text{x}10^6$ cells were plated in a 96-well U-bottom plate. Subsequently, stimuli were added. ConA, PMA/Ionomycin and SEB were used as a positive control. Finally, anti-CD107a, GolgiStop (Becton Dickinson, concentration as recommended) and RCAS (final concentration: 1%) were added and cells were incubated at 37°C/5% CO_2 for 6 hours. Thereafter, the staining protocol outlined in the previous section was performed.

7.11 *In vivo* cytotoxicity assay

A single cell suspension from spleen cells from naïve mice was prepared and adjusted to 1x10^7 cells/mL in TCM. Aliquots were pulsed with stimuli at 10 μg/mL or medium only at 37°C/5% CO_2 for 1 hour. Cells were gently washed with PBS and adjusted to 2x10^7 cells/mL. Pulsed cells were incubated with 2.5 μM CFSE and mock-pulsed cells were incubated with 0.25 μM CFSE for 3 minutes at 37°C. The labeling reaction was stopped by adding FCS to a final concentration of 10%. After gently washing, cells were counted and adjusted to 2x10^8 cells/mL. Equal parts of pulsed and mock-pulsed cells were mixed to yield 2x10^7 cells/100 μL. Each mouse received 100 μL intranasally, under light anesthesia of isoflurane.

Lungs were harvested 18 hours later and cell suspensions were prepared for each individual mouse. Flow cytometric analysis was performed immediately afterwards.

7.12 Serum preparation, antigen-specific ELISA

Terminal bleedings were collected with Vacutainer SST tubes (Becton Dickinson). Serum was prepared as recommended in the manufacturer's instructions and stored at -37°C until needed.

ELISA-plates (NUNC low binding Maxisorp) were coated with 70 μL of purified CopN at 1 μg/mL in PBS at 4°C over night. After washing the wells with PBS, blocking was performed with 0.1% BSA in PBS at 37°C for 30 min. Wells were briefly washed with 0.05% Tween20. A dilution series of each serum sample was performed in PBS-BSA-Tween (concentrations as before) and incubated at RT for 90 min. After washing with PBS-Tween, goat anti-mouse IgG (H+L) horseraddish-peroxidase (Southern Biotech) detection antibody was added in PBS-BSA-Tween and incubated at RT for 90 min. Wells were washed with PBS-Tween before ABTS substrate solution (Sigma) was applied. After 10-20 min the colour intensity was measured at 405 nm on a Tecan Sunrise microplate reader.

Using Prism 5 (GraphPad Software), a nonlinear regression was performed across all data points and the titer that gave an optical density 50% between Top and constrained Bottom was reported as the Best-Fit LOGECF50.

7.13 Immunofluorescence microscopy

HEp-2 cells were grown to confluence on coverslips placed inside 24-well plates. After addition of *Chlamydia pneumoniae* K6, plates were centrifuged at 550xg on

35°C for 1 hour. New medium containing 0.5 μg of cycloheximide (Sigma) was added. Incubation at 35°C/5% CO_2 lasted for circa 48 hours.

After washing with PBS (Gibco), -20°C cold methanol (Merck) was carefully added into the wells. After another washing step, a blocking step with 1% BSA in PBS was performed for 1 hour. Primary antibodies were sera from immunized mice (according to text) and rabbit anti Cpn0585 (B. Wizel). Incubation was peformed in a dark humid chamber at RT for 1 hour. Coverslips were washed in PBS and stained with secondary antibodies, which were AF488-conjugated goat anti-mouse and AF594-conjugated goat anti-rabbit (Molecular Probes). Incubation was performed as before, only for 30 minutes. Coverslips were washed and stained with a 1 μg/mL solution of DAPI (Sigma) at RT for 10 minutes. After a final washing step, coverslips were placed on a glass slide covered with Moviol. Analysis was performed on an Axio Observer Z1 (Zeiss).

7.14 Linear B cell epitope screening

Circa 20 μg of peptide were spotted onto a PVDF Immobilon-P transfer membrane (Millipore). The membrane was air-dried and placed into blocking buffer consisting of 1% BSA in PBS for 30 minutes. After a brief washing step in PBS, serum from immunized mice diluted in ab-buffer consisting of 0.1% BSA, 0.1% Tween20 in PBS was added and incubated for 90 minutes at RT. Afterwards, the membrane was washed in PBS. Goat anti-mouse IgG coupled to horseraddish peroxidase (Southern Biotech) was used as secondary ab. diluted in ab-buffer. Incubation was performed as before followed by a final washing step. The substrate solution used here was the same as described in section 7.8. The reaction was stopped after 90 seconds and scanning was performed immediately.

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Oligo GEArray® Mouse Inflammatory Cytokines & Receptors Microarray

Functional Gene Grouping Chemokines and Cytokines: Ccl1, Ccl11, Ccl12, Ccl17, Ccl19, Ccl2, Ccl20, Ccl21a, Ccl21b, Ccl22, Ccl24, Ccl25, Ccl3, Ccl4, Ccl5, Ccl6, Ccl7, Ccl8, Ccl9, Cx3cl1, Cxcl1, Cxcl10, Cxcl11, Cxcl12, Cxcl13, Cxcl14, Cxcl15, Cxcl2, Cxcl4, Cxcl5, Cxcl9, Ifna2, Ifng, Il10, Il11, Il12a, Il12b, Il13, Il15, Il16, Il17, Il17b, Il17e, Il18, Il1a, Il1b, Il2, Il20, Il22, Il3, Il4, Il5, Il6, Il9, Lta, Ltb, Mif, Scye1, Spp1, Tnf, Tnfsf5 (CD40 Ligand), Xcl1.

Chemokine Receptors and Cytokine Receptors: Blr1, Ccr1, Ccr2, Ccr3, Ccr4, Ccr5, Ccr6, Ccr7, Ccr8, Ccr9, Cx3cr1, Cxcr3, Gpr2, Il10ra, Il10rb, Il12b, Il12rb, Il13ra1, Il1r1, Il2rb, Il2rg, Il5ra, Il6ra, Il6st, Il9r, Xcr1.

Interleukins and Receptors: Il1r2, Il1rn, Il8ra, Il8rb, Il10, Il10rb, Il12a, Il12b, Il13, Il18, Il18, Il1a, Il1b, Il1r1, Il1r2, Il2, Il20, Il3, Il4, Il5, Il6, Il9, Tollip.

TNF Ligands and Receptors: Tnfrsf1a, Tnfrsf1b, Lta, Ltb, Tnf (TNF-α), Tnfsf5 (CD40 Ligand).

Other Factors Involved in the Inflammatory Response: C3, Fcer1g, Fcgr1, Igh-4, Itgam, Itgb2, Nos2, Prkca, Rac1, Tgfb1, Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, Tlr6, Tlr7, Tlr8, Tlr9.

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