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EVALUATION OF ADJUVANTICITY OF GEL AND NIOSOMAL FORMULATIONS OF IC31®

Verfasser

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"There are no such things as applied sciences,
only applications of science."

Pasteur, Louis (1822-1892)

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I could always count on you.

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1 Abbreviations

Abbreviated terms	Non-abbreviated terms
7-AAD	7-amino-actinomycin D
Alum	Aluminium hydroxide
BD	Beckton Dickinson
CBA	Cytometric Beads Assay
CFSE	Carboxyfluorescein succinimidyl ester
Con A	Concanavalin A
CTB	*Vibrio Cholerae* Toxin Subunit B
CTL	Cytotoxic T lymphocytes
ELISpot	Enzyme-linked-immunosorbent spot assay
FBS	Fetal bovine serum
FCA	Freund´s complete adjuvant
FCS	Fetal calve serum
i.n.	Intranasal
i.p.	Intraperitoneal
ISCOMS	Immune stimulatory complexes
LPS	Lipopolysaccharide
M-cells	Microfold cells
MALT	Mucosal-associated lymphoid tissue
MDP	Muramyl peptides
MPL	Monophosphoryl lipid A
NALT	Nasopharyngeal-associated

	lymphoid tissue
oppAV (BAP006-2)	Periplasmatic oligopeptide-binding protein
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RT	Room temperature
s.c.	subcutaneous
SEB	*Staphylococcus aureus* enteroxin subunit B
SEM	Standard error of the mean
SFU	Spot forming unit
SOP	Standard operating procedure
STkP (SP1732-3)	Serine/Threonine-kinase Protein
T-stim	T-cell culture supernatant
Th1 cells	T helper subset 1 cells
Th17 cells	T helper subset 17 cells
Th2 cells	T helper subset 2 cells
TLR	Toll-like receptor
TRP-2	Tyrosinase-related Protein 2
FAE	follicle-associated epithelium
HEV	high endothelial venules
MADCAM1	mucosal vascular addressin cell adhesion molecule 1
VCAM1	vascular cell adhesion molecule 1

Table 1: List of abbrevations

2 Abstract

A look at recent media coverage implicates the constant need for more and better vaccines. Only effective, safe and easy-to-use vaccines can overcome unjustified fears and growing rejection of vaccine applications in the general public. This is especially true for adjuvant systems for human use, which have been the target of many of these concerns. Developing new adjuvant systems, which can be applied via non-invasive routes, is therefore pivotal for the success of a modern vaccine and it´s world-wide impact. The skin, an immunologically rich environment, as well as the respiratory tract have already frequently demonstrated to be able to induce potent systemic humoral and cellular immunity to various pathogens.

This study evaluates the adjuvanticity of Intercells´ novel adjuvant system IC31® and its individual components, applied topically on the skin or intranasal, as gel- or niosomal formulation. In total 22 different variants were tested in combination with a wide range of subcutaneous (s.c.) protein or peptide antigen injections, including StkP- (*Streptococcus pneumonie*) and oppAV- (*Borrelia burgdorferi*) analogues, as well as TRP-$2_{180\text{-}188}$ (murine melanoma). The immune response was profiled via various immunologic methods including ELISpot, Cytometric Beads Assay (CBA), CFSE proliferation staining and CD154 (CD40L) surface staining screening several systemic produced cytokines and T-lymphocyte populations in five to eight mice per group (C3H, C57BL/6). Data evaluation was performed with Graphpad Prism 5 and Microsoft Excel 2011 (OS X). Statistical significance was determined via student´s T-tests (95% confidence interval, $p<0.05$).

The results show that topical skin IC31® gel formulations, despite increased IC31® component concentrations, show only limited potential for further development. Niosomal formulation applied on the skin on the other hand, albeit delivering only suboptimal immune responses in contrast to the controls, have the potential to deliver a solid cellular and humoral immunity, once IC31® component concentrations are further increased. Intranasal administration of niosomal formulations proved to have the highest potential and delivered a very strong immune response in comparison to the controls. Alternative vesicular particle vectors, like Transferosomes, could also help increasing the potential humoral and cellular immune response in both a topical and intranasal use-case.

Further improved IC31® niosomal formulations have the potential to create a non-invasive, potent and easy-to-use topical/intranasal adjuvant system that can boost immune responses to future vaccines and ultimately foster world-wide application by improving the acceptance of vaccines in the population.

3 Zusammenfassung

Die fortwährende Diskussion in den Medien trägt dazu bei, dass die Nachfrage nach neuen und besseren Impfstoffen für die Weltbevölkerung auch in Zukunft nicht abreißt. Jedoch haben nur jene Impfstoffe eine Chance von der Bevölkerung akzeptiert zu werden, die sicher und leicht in der Anwendung sind. Ständige Verbesserung der verfügbaren Impfstoffe und ihrer Anwendungsformen tragen dazu bei, ungerechtfertigten Ängsten und genereller Ablehnung gegenüber Impfanwendungen in der Bevölkerung entgegen zu wirken. Dies ist umso wichtiger für Adjuvanssysteme, da diese speziell im Lichte derartiger Bedenken stehen. Es ist daher unabdingbar für den Erfolg und die Verbreitung eines modernen Impfstoffes, neue Adjuvanssysteme zu entwickeln die nicht-invasive Routen der Anwendung erschließen. Die Haut und der Atmungsapparat haben sich in früheren Studien als immunologisch vielversprechende Anwendungsumgebungen erwiesen denen ein hohes Potential zur Erzeugung starker systemischer, humoraler und zellulärer Immunität innewohnt.

Diese Studie untersucht die Effektivität von Intercells´ neuem Adjuvanssystem IC31® sowie dessen Einzelkomponenten, als topisch aufgetragene oder intranasal applizierte Gel- oder niosomale Formulierung, in Kombination mit subkutanen Protein- bzw. Peptidantigeninjektionen von einer weiten Palette von Pathogenen. Die Immunantwort selbst, im Besonderen deren Zytokine und T-Lymphozyten Populationen, wurde mit der Hilfe von diversen

immunologischen Methoden wie ELISpot, Cytometric Beads Assay (CBA), CFSE Proliferations-Färbung und CD154 (CD40L) Oberflächenfärbung in Mäusen(C3H, C57BL/6) dargestellt.

Die Ergebnisse zeigen, dass topisch Haut-applizierte IC31® Gelformulationen, trotz bereits erhöhter IC31® Komponentenkonzentrationen, nur ein stark eingeschränktes Potential für die weitere Entwicklung bieten. Niosomale Formulierungen auf der anderen Seite, trotz ihrer bis dato suboptimalen Immunantwort im Vergleich mit den Kontrollen, könnten das Potential für eine solide zelluläre und humorale Immunität haben, sofern die IC31® Komponentenkonzentrationen weiter erhöht werden. Intranasale Anwendung von niosomalen Formulierungen zeigte das größte Potential in der Studie und lieferte eine signifikant stärkere Immunantwort im Vergleich zu den Kontrollen. Alternative vesikuläre Partikelvektoren, wie zum Beispiel Transferosomen, könnten ebenfalls dazu beitragen die potentielle humorale und zelluläre Immunantwort im Falle einer topischen oder intranasalen Anwendung weiter zu erhöhen.

Weiter entwickelte IC31® niosomale Formulierungen haben nicht nur dass Potential als nicht-invasive, einfach anzuwendende und topische /intranasale Adjuvanssysteme zukünftige Impfstoffe immunologisch zu verstärken, sondern auch deren weltweite Anwendung und Akzeptanz zu fördern.

4 Introduction

The following section of the diploma thesis gives a short overview of currently used vaccine types and adjuvants, as well as some historical facts. Moreover the immune systems of the skin and mucosa are explained. Further, it introduces to Intercells´ novel adjuvant system IC31®, its delivery methods and the antigens used in this study.

4.1 Vaccines

The development of vaccines represents one of the greatest scientific achievements in medical history, yet they remain our greatest challenge. Hardly any other treatment has changed our world to a greater extent and gave us better means to control diseases that used to plague humanity for centuries.

The first official vaccine ever used, was developed by Edward Jenner in 1796 to provide protection against smallpox. He administrated cowpox to children via inoculation to achieve a cross protection against the human pathogen. The general idea of inoculation already existed in local customs, but it was Jenner who finally put the pieces together. In the early 19th century, Louis Pasteur experimented with vaccinations as well. He managed to immunize chicken against chicken cholera with, using what today would be called a live attenuated vaccine. Later, his focus switched to rabies and anthrax, where he was as successful as Jenner. This early pioneer work was the start of a long series of successful of vaccines, starting in the late 18th century continuing until

today. The scientific and medical community managed, also thanks to the great acceptance of the general public, to eradicate smallpox in 1980 More vaccines were introduced to target diphtheria, mumps, and measles, among many others. Most of them showed a tremendous effect on the distribution of the disease, once they were used in large parts of the population. [1-5]

Todays vaccines can be classified in 5 different groups:

- Killed pathogen vaccine:

Pathogens are treated chemically or physically with antibiotics, heat or similar actions to achieve a "killing effect". A solution containing dead, non-infectious pathogen agents is injected to induce an immune response against (e.g. Rabies vaccine) [6].

- Live attenuated vaccine:

The pathogens are biologically changed to lose their virulence. The big advantage of these vaccines is their effectiveness. The pathogen is still alive and can still replicate in the body thus guaranteeing a strong immune response. Often a single shot is enough to achieve live-time immunity. There are, however, downsides: the pathogen could revert in the host and infect the unvaccinated population (e.g. Measles vaccine).[7]

- Toxoid vaccines:

Involves toxic agents derived from the pathogens, which can provoke an immune response. They are typically able to provoke a very strong immune response up to a systemic cytokine storm in some cases. This group also includes very successful vaccines (e.g. Tetanus vaccine). [8]

- Subunit vaccines:

Subunit vaccines resemble one of the safest groups of vaccines, due to their low but very defined immunogenicity. These vaccines are composed of retrieved fragments of the pathogen and injected to induce an immune response (e.g. Hepatitis B vaccine). [9]

- Recombinant vaccines:

The latest group of vaccines consists of specially designed and purified fragments, toxin subunits or similar agents, which are recombinant produced in cultures to generate a very specific vaccine. Such vaccines can include entire structures as well as proteins or only certain peptides, which showed a potent immunogenicity in prior studies (e.g. Hepatitis B vaccine) [10]. DNA vaccines resemble a special subtype of these vaccines. They are specially designed DNA fragments, which are transported to antigen presenting cells via a vector (e.g. Immune Stimulatory Complexes or Virus-Like Particles). They integrate into the host cell genome and produce the antigen target, which is then expressed directly in the cell and presented on the cell surface to the immune system [11].

Future vaccines against global pandemics like HIV or Hepatitis C need to be easy to administrate (ideally topically over the skin, inhalation or via ingestion), while remaining highly effective and without major side effects at the same time. Ultimately, they also have to be affordable to be distributed among a critical mass of our population to induce a herd immunity. In order to meet these criteria, a wide political and social acceptance of vaccination is as critical as scientific progress and further clinical studies. [12]

4.2 Adjuvants

Especially the safer vaccine types like recombinant or subunit vaccines, which are less likely to cause unwanted side effects, often face the problem of being poorly immunogenic. Many vaccines tend to merely induce a humoral immune response, which is not too effective against intracellular pathogens [13]. For this purpose special agents are mixed with vaccines to manipulate the immunogenicity of the antigen used. These so-called adjuvants boost a broad immune response to develop not only a humoral but also a cellular response. Despite the positive effects of adjuvants there have been public concerns about their safety lately, which in extreme cases lead to refusal of vaccination. Since most of these concerns originate from misunderstandings and misleading media coverage, improved public communication is of outmost importance. [14-17]

Vogel et al. [18] classified adjuvants into five main groups:

- Gel-type adjuvants:

This group includes substances like the frequently used aluminium hydroxide (alum). Alum was one of the first adjuvants in wide use and already discovered in 1926. It is able to boost a potent Th2-type humoral antibody response against an antigen by inducing the release of uric acid in the body, but proved poorly effective in inducing a cellular type reaction. [19, 20]

- Bacterial adjuvants:

This group is among the most potent immune stimulatory. Bacterial derived peptides or lipids, like Muramyl peptides (eg. MDP), are included in this group. MDP can be used, depending on the administration conditions, to induce either a humoral or cellular immune response [21]. Lipopolysaccharides (LPS) are also included in this group since they originate from the cell wall of Gram-negative bacteria and also directly activate TLR 4 via lipid A for example [22]. Monophosphoryl lipid A (MPL) is also part of this group [23].

- Particle adjuvants:

A common example of this group are Liposomes. They are vesicular membrane structures, composed of lipid layers that can activate antigen-presenting cells and transport antigens, incorporated inside them, into the cell via endocytosis. They proofed effective in inducing both a humoral and cellular immune response as well as prolonging the

half-life of the transported agent [24]. They further increase the transdermal transport of material through the skin [25]. ISCOMS, or immune stimulatory complexes, are open cage like structures composed of phospholipids, cholesterol and Saponins that can also act as transportation vector and provoke an immune response but avoid the toxicity of the Saponin-only formulations. [26, 27]

- Oil-emulsions and emulsifier-based adjuvants:

One of the most famous adjuvants in this group is Freund´s incomplete adjuvant, an emulsion of water in mineral oil based on Freund´s complete adjuvant (FCA) discovered in 1936. Freund´s complete adjuvant also contains a bacterial component originating from killed mycobacteria. FCA is one of the most potent adjuvants available, but at the same time known to cause severe side effects, which render it unsuitable for most human vaccines. Saponins (e.g. QS21), originally derived from plants and marine organisms, also belong to this group. They are known for their ability to induce a strong Cytotoxic T lymphocyte (CTL) response [28]. Saponin solutions can show toxic side effects. Squalene (e.g. MF59) was also originally derived from plants and shark liver oil. The literature describes it as strong immune stimulator, able to elicit both a Th1- and Th2-type response. [29]

- Synthetic adjuvants:

Synthetic adjuvants are the most modern group of adjuvants, where natural agents and particles are mimicked in order to achieve a clear immune stimulatory effect with fewer side effects than other adjuvants.

Typical examples for this class of adjuvants are synthetic lipid A, as toll-like receptor 4 (TLR 4) agonists, or synthetic CpG motifs, as TLR 9 agonists [30, 31]. Poly L-arginin (pR) has also shown to be an effective adjuvant in studies with the murine melanoma protein TRP-2. Here pR was able to induce a type 1 and type 2 immune response against this antigen [32]. By carefully selecting the synthetic components of the adjuvant mixture a cleaner immune response without systemic side effects can be achieved in contrast to classical adjuvants like FCA.

Just a fraction of the adjuvants mentioned above - and even a more negligible part of all immune stimulatory substances known - are being used in vaccines for humans. In fact, alum is still the predominant adjuvant on the market. Hence, there is a tremendous demand for new adjuvant systems that are able to elicit a solid cellular immune response in addition to a humoral one, and are safe enough to be used in humans. Ideally such an adjuvant system would forgo injections, and thus facilitate its global acceptance and ease of administration. [33]

4.3 IC31®

IC31® is Intercells'' next generation adjuvant system that was designed to meet the requirements mentioned in Chapter 3.1. It is a fully synthetic mixture composed of two defined components in a characteristic ratio:

- KLK (H-KLKL$_5$KLK-OH):

Positively charged KLK is an antimicrobial peptide, which enhances the uptake of an antigen and ODN1a (Oligodeoxynucleotide) into antigen presenting cells (APCs) by attaching itself to the negatively charged membrane [34]. There it first forms a regular β-sheet structure and mediates the uptake of ODN1a, via a subsequent α-helical structural change, into the cell. It has been shown that KLK leads to ultrastructural changes in the membrane like a decrease of membrane fluidity and an increase of microviscosity, without actual pore formation.[35] Additionally, KLK possesses a protective structural trait when in solution with ODN1a as it randomly coils around it. Once administrated, KLK leads to a depot formation at the injection site, which can be tracked up to 58 days[36]. Beside all these passive effects KLK itself also induces a type 2 immune response when injected in combination with proteins and characterized by IL-4, IL-5 and IgG1 [37].

- ODN1a (5'ICICICICICICICICICICICIC-3'):

ODN1a is an oligodeoxynucletoide of a repeating sequence. It acts as a direct agonist of the toll-like receptor 9 (TLR9) and the downstream acting MyD88 pathway. Thus ODN1a upregulates the transcription factor NfκB and induces a potent immune response of mainly cellular but also humoral characteristics[36].

Mixed in Tris buffer, IC31® builds up nanometer-sized particles and can be formulated directly, without the need for conjugation or coupling, with the antigen. IC31® has shown to be effective with a variety of antigens in different use cases [38-40]. Developing this system further to

be effective not only via injections but also via topical application is the logical next step in making tomorrows vaccines easier to use and safer without systemic proinflammatory side effects.

4.4 Cholera Toxin Subunit B

Cholera Toxin Subunit B (CTB) is a typical bacterial adjuvant originating from *Vibrio cholerae*. The whole exotoxin is composed of six subunits and is the active agent causing severe diarrhea in cholera infections. It binds to lipid rafts (GM1 gangliosides) and is taken up by the host cells, where it induces the transport of water and ions into the lumen [41-43]. The B subunit, which is less toxic than the A subunit, has been successfully used to boost mucosal immune responses in combination with various antigens during intranasal immunizations. Thus CTB is an ideal candidate as positive control for the intranasal experiments in this study [44, 45].

4.5 Imiquimod

Imiquimod is a synthetic TLR7 agonist, which is already in use to treat basal cell carcinomas and genital warts. For example, it is commercially available as Adlara 5% cream, which was also used in this study as positive control for transdermal immunizations [46-50]. Despite the real mode of action is not fully understood, Imiquimod frequently proved its strong potential as transdermal adjuvant. Via TLR 7 it has the capacity to modulate and potentiate the immune response to an administrated protein. Only rare negative systemic effects are known

[51]. Therefore Imiquimod resembles the ideal positive control for this study to demonstrate the possible effectiveness of transdermal immunizations [46, 52].

4.6 Topical Skin Immunity and transdermal vaccination

There are different routes of vaccination besides the frequently used classical muscular injection. Finding easier-to-use, safe and non-invasive administration methods is pivotal for the global success and distribution of modern vaccines. Among these are transdermal vaccinations. The skin itself resembles the first barrier pathogens face when interacting with our body. As a first-level lymphoid organ the skin shows a complex layered structure (see Figure 1).

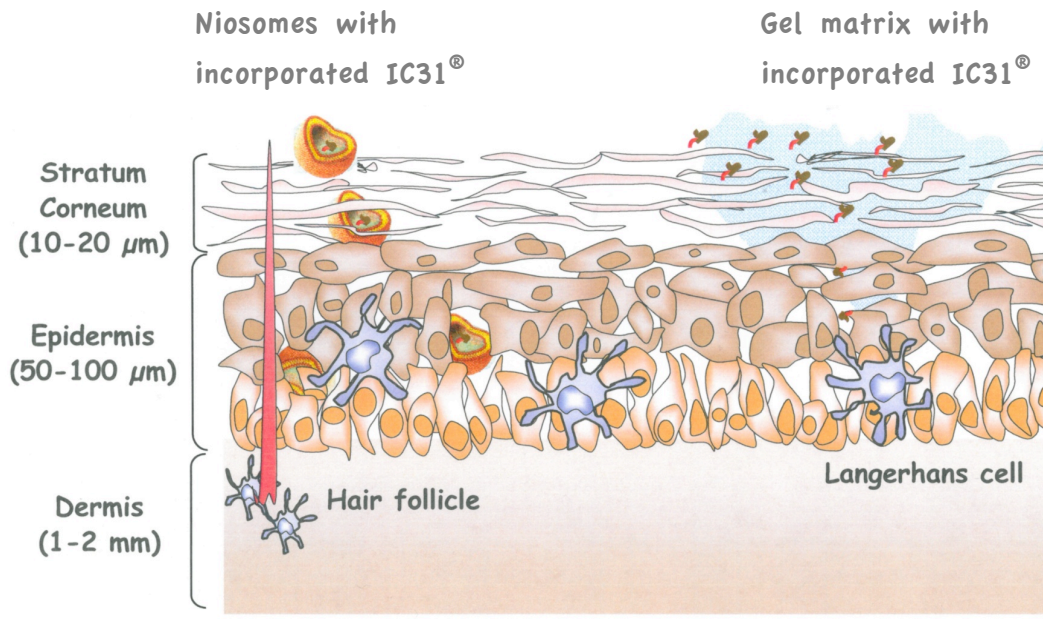


Figure 1: **Layer-model of the skin** showing two possible ways of transdermal adjuvanting of vaccinations with IC31® (red/brown particles) via gel or niosomal formulations. Langerhans cells in the Epidermis act, once activated, as immunologic mediator and transport antigens to

the next draining lymph node to promote a systemic and local immune response. Picture kindly provided by Benjamin Wizel, Intercell AG

The top layer of the skin is the *Stratum Corneum,* which mainly consists of dead Corneocytes. In the process of transdermal vaccination this layer is sometimes either removed partly (e.g. 20% of thickness) by pre-vaccination treatment at the application site or made more permeable by additional application of gel formulations like shown in Figure 1. The next layer is the *Epidermis,* a squamous and proliferating area of Keratinocytes. Underneath lies the *Dermis,* built up mainly of Fibroblasts, Collagen, Elastin, Adipocytes, which also contains a lot of macrophages. [53-55]

Beside other defense mechanisms on the surface of the skin like nucleases or proteases, the skin in general is an immunologic rich environment [56]. This environment can be defined as skin immune system (SIS) and is constantly challenged with pathogens. Helper and suppressor T-cells are abundant throughout all inner layers of the skin, as well as around hair follicles and sweat glands [57]. Beside T-cells the skin also houses a large number of specialized dendritic cells. These so-called Langerhans cells are dendritic cells situated in the skin and originate from monocytes [58]. They are present in their immature form in the epidermal layer, where they form a dense web of dendrites constantly sampling the surrounding area for potential pathogens. Once a presentable antigen is found the cells mature and travel to the next draining lymph node where they initialize an immune response of the T-cells. TLR triggering also plays a pivotal role in immune stimulatory processes along the skin [59]. Langerhans cells-triggering and boosting such a local immune response to a systemic immunity

against potential pathogens is a great challenge for tomorrow´s vaccines and adjuvants.

Transdermal vaccination in general can happen via a variety of methods ranging from high-pressurized jet injectors that penetrate the upper layer of the skin with a liquid, gas or even solid particle stream, over electroporation, to patch technology featuring a constant release of agents into the skin [60-66]. In this study two different methods of transdermal adjuvanting of a subcutaneous (s.c.) injections were tested:

Gel formulations of IC31®:

The gel formulations consist of a combination mixture of RO water, Glycerin monostearate, Plant oils, Carbomer 940, AloeVera gel, Lanoline, Allantoin, Paraffin oil, Triethanolamine and Emulgator: Tego betaine. This forms a matrix in which IC31® or its single components can be incorporated as shown in Figure 1. The gel has the capability to mediate agent transport across the outer layers of the skin to directly reach the underlying Langerhans cells.

Niosomal formulations of IC31®:

Niosomes are bilayered liposome-like vesicles formed by non-ionic surface-active agent in aqueous media like Span 40 or Span 60. They are stabilized by cholesterol integration and show high deformability and the potential to protect the encapsulated agent [67]. Additionally they enhance the delivery through the skin [68]. Niosomes have frequently shown to be able to promote the uptake of incorporated material, like

antigens or agents, into the host cell, by fusing with the cell membrane and subsequently activating the immune system [69-71]. It is also possible that Niosomes by themselves have an immune stimulatory and depot effect, where they constantly release material over time [24]. In contrast to Liposomes, Niosomes are cheaper to produce, since no phospho-lipids are needed and they are more stable when stored at 4°C [72]. Figure 2 shows the IC31® niosomal construct. In contrast to the usual IC31® nano-sized particle, where KLK directly contacts ODN1a, the components are separated here. While ODN1a is protected inside the vesicle KLK is adsorbed to its surface. Figure 2 also illustrates the double layer of vesicle where hydrophobic parts face inside the layer and hydrophilic face outside of it.

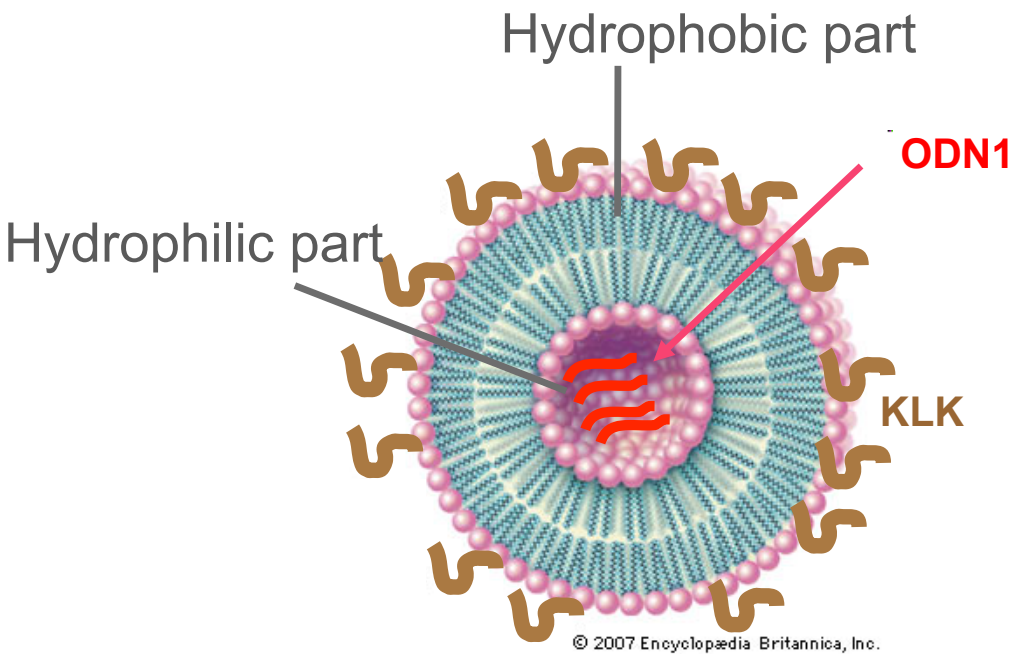


Figure 2: Schematic illustration of IC31® Niosomes. In contrast to the IC31 particle, IC31 Niosomes incorporate ODN1a and adsorb KLK to the vesicle surface. Picture altered from Encyclopedia Britannica, ©2007

4.7 Mucosal Immunity in the Lung and nasal Vaccination

Mucosal tissue includes not only the intestines, but also the urinary tract and respiratory parts of our body. The mucosal immune system is among the most crucial of all defense barriers available in our body. After all, mucosal tissue resembles the largest body/outer world connective of all surfaces of an individual. Specialized compartments or mucosal-associated lymphoid tissue (MALT) are situated throughout the mucosal system and house all lymphoid T-cells, Macrophages, Dendritic cells, high endothelial venules (HEVs) and properties necessary to screen the interface of the endothelial cells and the outer world and induce an immune response if necessary [73]. In case of the intestine, Peyer´s patches resemble such MALT [74]. These structures have antigen-sampling M-cells (microfold cells) as gateway to the lumen. Antigens enter through these cells and are taken up by APCs, which mediate an immune response or in most cases suppress such. Nasopharynx-associated lymphoid tissue (NALT), like the Waldeyer´s ring (in humans), resembles a similar structure like the Peyer´s patch but is located in the respiratory tract [75]. NALT itself consists of follicle-associated epithelium (FAE), high endothelial venules (HEV) as well as B- and T-cell areas [76, 77]. One of the major differences between Peyer´s patches and NALT is the organogenesis. Whereas Peyer´s patches are already developed during embryogenesis, NALT has to build up postnatal in mice [78]. Another major difference is seen in the expression of adhesion molecules in NALT and Peyer´s patch HEVs. Whereas NALT HEVs express the T-cell homing receptor vascular addressin cell-adhesion molecule 1 (VCAM1), Peyer´s patch HEVs

express mucosal vascular cell-ahesion molecule 1 (MADCAM1), which is also an indicator for differentiated development [79].

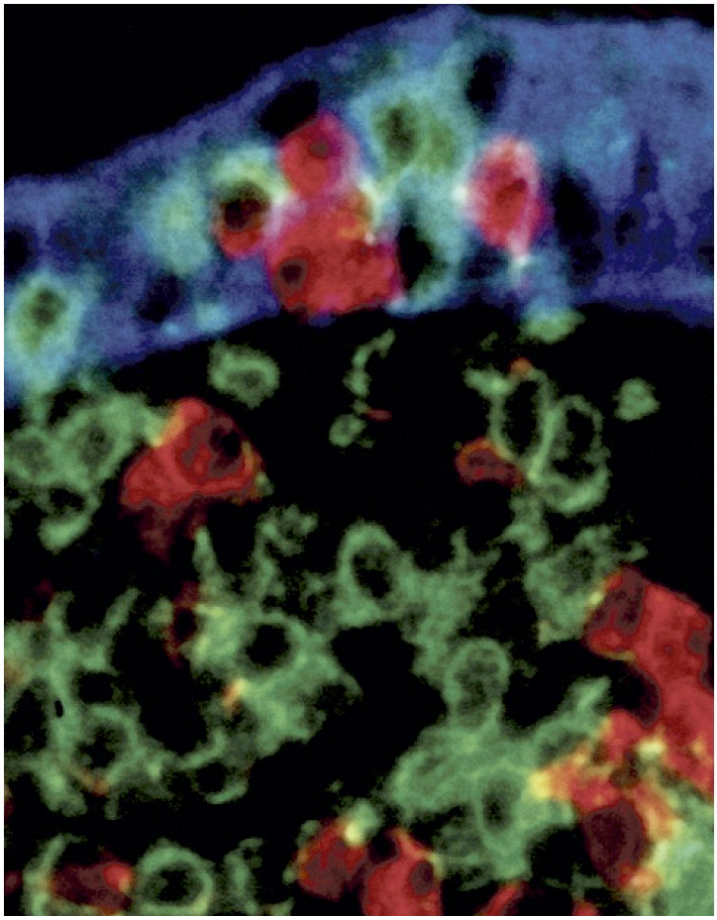


Figure 3: Immune fluorescence staining of nasopharynx-associated lymphoid tissue (NALT). Mucosal cells are shown in blue. B-cells are shown in green and T-cells in red. Antigens are sampled through specialized mucosal cells and presented to the lymphocytes by APCs. Picture from Janeway´s Immunobiology 7th edition, ©2008 by Garland Science.

Figure 3 shows the typical T- and B-cell rich environment of NALT tissues, which are the target of intranasal (i.n.) vaccinations. It has been shown that intranasal vaccination leads to the development of germinal centers, local IgA secretion and furthermore even a systemic immune response towards antigens [80, 81]. Such immune responses could either be of humoral or cellular type. Potent adjuvants administrated

intranasal, like IC31®, in combination with recombinant antigens might be able to trigger a systemic immunity against certain kinds of pathogens. This, with the obvious benefits of an easy-to-use intranasal vaccine, could be the key factor to develop a widely successful new vaccine.

To protect the IC31® components from degradation, especially the oligonucleotide ODN1a, vesicle structures like Niosomes resemble a great possibility. Niosomes have already been frequently used for intranasal vaccinations and proved to be a successful vector for antigens[82-84]. For this study the same niosomal formulations as mentioned in Chapter 3.6 are used for intranasal vaccinations and are directly formulated with the antigen mixture, thus testing a potential alternative application route of IC31® Niosomes.

4.8 Antigens

4.8.1 TRP-2$_{180-188}$

Tyrosinase-related protein 2 peptide 180-188 (TRP-2$_{180-188}$) is a murine melanoma peptide. It was shown in the literature that subcutaneous vaccination with this peptide in combination with CpG motifs leads to a potent CD8+ T-cell response [85]. Internal studies at Intercell AG also showed the potential of IC31® in provoking a similar effect. Having proven effective in s.c. immunizations and having shown to be able to elicit a restricted CD8+ T-cell immune response makes this melanoma peptide an ideal model antigen for this study.

4.8.2 SP1732-3

SP1732-3 is an in-house purified protein of *Streptococcus pneumonia* closely related to Serine/Threonine-kinase Protein (STkP). Studies have shown that STkP is a potent target for the bodies humoral and potentially also cellular immune response [13]. It could be shown that a Th17-type immune response is typical for this antigen, in addition to Th1- or Th2-types, to reduce the carriage time clear the infection [86, 87]. Further vaccination studies in-house confirmed immune stimulatory effect, thus suggesting SP1732-3 as a high potential protein antigen model for topical vaccination studies.

4.8.3 BAP006-2

BAP006-2, which was purified in-house is originally named Periplasmatic Oligopeptide-binding protein (oppAV) and is part of the ABC transporter in *Borrelia burgdorferi* [88]. It was shown in the literature that oppAV belongs to a limited set of immunogens, which could be used as potential protein antigen targets for a vaccine against in *Borrelia burgdorferi.* A potent Th2-type immune response could already be provoked by intraperitoneal (i.p.) injections and coadministration with FCA [89]. Intercells´ novel adjuvant system IC31® is able to boost a variety of antigens and thus could potentially also be used with this protein. Since IC31® can promote both a Th1-type and Th2-type immune response BAP006-2 is an ideal alternative protein antigen model beside SP1732-3.

4.9 Study objectives

The aims of this study were to assess the immunomodulatory activity of Intercells'' next generation adjuvant system IC31®, as well as of its single components KLK and ODN1a, when applied topically as gel or niosomal formulations on the skin in combination with s.c. injected antigens.

This was done by profiling the immune response in mice in terms of the elicited cytokine-producing T-cells via a wide variety of state of the art immunologic assays (enzyme-linked immunosorbent spot assay, cytometric beads assay, CFSE proliferation staining and CD154 (CD40L) staining). To address the wide spectrum of IC31® use-cases, different peptide and protein models were used as antigens.

In addition, an alternative route of administration, namely intranasal immunization, was tested with niosomal formulations in combination with IC31® or its single components and the selected peptide antigen model to evaluate the immune stimulatory potential for further i.n. studies.

5 Material & Methods

All experiments were performed at the Intercell AG laboratory according to the Austrian law and the corresponding standard operating procedures (SOPs) of the company. The experiments were carried out between August 2010 and April 2011.

5.1 Materials

This section gives an overview of the used laboratory equipment, reagents and antibodies. It also includes information about the antigen and adjuvant formulations used.

5.1.1 Laboratory Equipment

Material	Company/	Cat. No.
Micro Well Plates (sterile):		
24 well flat bottom	NUNC	142475
96 well U bottom	NUNC	143982
37°C / 5% CO_2 Incubator	Kendro	99100061
Cell strainer 70µm, (sterile)	Falcon	352350
Centrifuges:		
Megafuge 2.0R	HERAEUS	286331
Megafuge 2.0	HERAEUS, No.;	271629

Biofuge fresco	HERAEUS	270208
ELISpot plates:		
IFN-γ	Millipore	MSHAS4510
IL4, IL17	Millipore	MSIPS4510
ELISpot Reader:		
Bioreader 2000 Gen 6	BIOSYS	O3M105
FACS tubes (sterile), 5ml	Falcon	352054
FACS:		
FACSCalibur	Becton Dickinson (BD)	401/009
Flow Cytomix 10-plex (CBA)	Bender MedSystems	BMS820FF
Hemocytometer:	VWR	6311111
Neubauer Improved	VWR	6319440
Cover glass 20x26mm		
Laminar Flow Hood	HERA Safe	99100632
Petri dishes (sterile):		
60x15mm	Falcon	351016
pH-Meter	InoLab	02020005
Pipette boy	Integra Biosciences	848422
Pipettes (sterile):		
5ml	Falcon	7543
10ml	Falcon	7551
25ml	Falcon	7525
Pipettes & tips	Eppendorf	n.a.
Polypropylene tubes (sterile):		
15ml	Greiner	188271

50ml	Greiner	226271
Reagent reservoirs (sterile)	Costar	4870
Syringes (sterile): Omnifix-F 1ml	Braun	6120110
Transfer pipettes (sterile) 3ml	Falcon	357575

Table 2: List of Laboratory Equipment used at Intercell – Smart Vaccines

5.1.2 Reagents

Reagent	Company	Cat.No.
10xPBS	Invitrogen	14190
3.3`-Diaminobenzidine (DAB)	Sigma-Aldrich	D-5637
Aldara 5% Crème (Imiquimod)	Meda	
Anasthesia (intranasal application): Isofluran	Baxter	HDG9623A
Anasthesia (topical application): 0,9% saline solution (phosphate buffered)	Braun	190/1260605 4/0608
2% (Xylazin) Rompun 20mg/ml	Bayer Healthcare	8-14840
10% (Ketamine) Ketamidor 100mg/ml	Richter Pharma Ag	8-00158

Blocking buffer:	PAA Lab GmbH	H15-002
1x PBS	PAA Lab GmbH	K41-001-500
1%BSA (Bovine Serum Albumin)		
Calcium Ionophore	Sigma Aldrich	P-8139
Carboxyfluorescein succinimidyl ester (CFSE) (5mM)	Invitrogen	C1157
Cholera Toxin B	Sigma Aldrich	C-7771
Coating buffer:		
0,1M NaHCO3	Sigma-Aldrich	S-6014
ph 9,2-9,4		
Complete Medium -FCS:		
DMEM (500ml)	PAA Lab GmbH	E15-009
2mM L-Glutamine	PAA Lab GmbH	M11-004
1mM Sodium Pyruvate	PAA Lab GmbH	S11-003
1mM Non-essential amino-acids	PAA Lab GmbH	M11-003
100µg/ml Gentamicin	PAA Lab GmbH	P11-004
50µM 2-Mercaptoethanol	GIBCO	31350-010
Complete Medium:		
DMEM (500ml)	PAA Lab GmbH	E15-009
5% (v/v) heat-inactivated fetal bovine serum (FBS)	PAA Lab GmbH	A15-041
2mM L-Glutamine	PAA Lab GmbH	M11-004
1mM Sodium Pyruvate	PAA Lab GmbH	S11-003
1mM Non-essential amino-acids	PAA Lab GmbH	M11-003

100µg/ml Gentamicin	PAA Lab GmbH	P11-004
50µM 2-Mercaptoethanol	GIBCO	31350-010
Concanavalin A (ConA) (10mg/ml)	Sigma Aldrich	C5275
Endotoxin-free water	Fresenius Kabi	7151-4
GolgiStop (Monensin) (1500x)	Becton Dickinson	554724
H2O2 30%	Sigma-Aldrich	H-1009
IC31®nano (100nM KLK/ 4nM ODN1a)	Intercell	
MACS/FACS buffer:		
1x PBS	PAA Lab GmbH	H15-002
0,5% BSA (Bovine Serum Albumin)	PAA Lab GmbH	K41-001-500
2mM EDTA (Ethylendiamintetraacetic Acid)	Sigma-Aldrich	E-5134
NiCl2	Sigma-Aldrich	N-5756
Paraformaldehyde 4% (PFA)	Sigma	P6148
PMA	Sigma	P8139
Rat T-stim without Con A	Becton Dickinson	354116
Red blood cell lysis buffer	Sigma	R7757
Staphylococcus enterotoxin B	Sigma Aldrich	S4881
Streptavidin-Horseradish Peroxidase (SA-HPO)	Roche	1089153

500U/ml		
Trypan Blue Stain 0.4%	GIBCO	15250-061
Tween 20	Sigma-Aldrich	P-7949
Washing buffer:		
1x PBS	PAA Lab GmbH	H15-002
0,1% Tween 20	Sigma-Aldrich	P-7949
T-cell culture supernatant without ConA, rat (T-stim)	Becton Dickinson	354116

Table 3: **List of Reagents** used at Intercell – Smart Vaccines

5.1.3 Antibodies

Antibodies for ELISpot Assays	Company/	Cat. No.
Anti mouse IFN-γ antibody coating antibody (1mg/ml)	Becton Dickinson	551216
Biotinylated rat anti mouse IFN-γ detection antibody (1mg/ml)	Becton Dickinson	551506
Anti mouse IL 4 antibody coating antibody	Becton Dickinson	554434
Biotinylated anti mouse IL4 detection antibody	Becton Dickinson	554390
Anti mouse IL17 antibody coating antibody	Becton Dickinson	555068
Biotinylated anti mouse IL17 detection antibody	Becton Dickinson	555067

Table 4: **List of Antibodies used for ELIspot assays** at Intercell-Smart Vaccines.

Antibodies (FACS assays)	Isotype	Fluochrome	Stock conc.	Company	Cat. No.
CD 154 (CD40L)	Hamster IgG1	APC	0,2mg/ml	eBioscience	17-1541
Hamster IgG1	n.a.	APC	0,2mg/ml	eBioscience	17-4888
CFSE	n.a.	n.a.	50mM	Invitrogen	C1157
FcγR	Rat IgG2b	n.a.	0,5mg/ml	Beckton Dickinson	553142
CD4	Rat IgG2a	FITC	0,2mg/ml	eBioscience	11-0042
Rat IgG2a	n.a.	FITC	0,2mg/ml	eBioscience	11-4321
CD8a	Rat IgG2a	APC	0,2mg/ml	eBiosicience	17-0081
7-AAD (7amino-actinomycin D)	n.a.	PercP	0,5mg/ml	eBioscience	00-6993-50

Table 5: **List of Antibodies used for flow cytometric** assays at Intercell-Smart Vaccines.

5.1.4 Antigens

TRP-$2_{180\text{-}188}$:

A murine melanoma peptide of the tyrosinase-related protein-2 (TRP2) synthesized by Proimmune (Batch CPD4396). This peptide was used for subcutaneous and intranasal immunizations, as well as for ex-vivo re-stimulations.

SVN$_{19\text{-}28}$:

A murine lung cancer peptide of the apoptosis-related Survivin protein synthesized by Think Peptide (p2093). It is commonly expressed in malignant gliomas. This peptide was used as an irrelevant stimulus in

various ex-vivo assays when immunizations with TRP-$2_{180-188}$ were performed in the experiment.

SP1732-3:

An in-house purified protein of *Streptococcus pneumonia* closely related to Serine/Threonine-kinase Protein (STkP). This protein was used for subcutaneous immunizations, as well as for ex-vivo re-stimulations.

BAP006-2:

Part of the ABC transporter in *Borrelia burgdorferi* named Periplasmatic Oligopeptide-binding protein (oppAV), which was purified in-house. This protein was used for subcutaneous immunizations, as well as for ex-vivo re-stimulations.

OVA:

Chicken Ovalbumin protein obtained from Sigma Aldrich. It was used as an irrelevant stimulus for ex-vivo assays if immunizations with whole proteins were performed during the experiment.

5.1.5 Adjuvants

All topically applied adjuvants were based on Intercell´s IC31® adjuvant or its single components KLK and ODN1a. KLK is a synthetic cationic poly-amino acid with incorporated lysine and leucine [KLK(L)5KLK-COOH]. ODN1a is a, phosphodiester substituted, single stranded oligodeoxynucleotide. Both components are mixed together in a typical

25:1 ratio. The collaborating laboratory at the University of Natural Resources and Life Sciences Vienna (BoKu), lead by Prof. Karola Vorauer-Uhl, kindly provided different gel- and niosomal-based IC31® formulations for further topical (in case of niosomal formulation also nasal) application. The topical adjuvant variants together with their KLK and ODN1a content are shown in Table 5 and 6.

Adjuvant variant	KLK [nM/100µl]	ODN1a [nM/100µl]
IC31® Gel batch 1	150	6
KLK Gel batch 1	150	n.a.
ODN1a Gel batch 1	n.a.	6
Mix of KLK and ODN1a Gels batch 1	150	6
Plain Gel batch 1	n.a.	n.a.
IC31® Gel batch 2	200	8
KLK Gel batch 2	200	n.a.
Mix of KLK and ODN1a Gels batch 2	200	8
Plain Gel batch 2	n.a.	n.a.

Table 6: Different variants of gel adjuvant formulations used for topical application as provided by Prof. Karola Vorauer-Uhl´s laboratory at University of Natural Resources and Life Sciences Vienna and Intercell itself. All gel formulations are buffered in 2.5mM Tris.

Adjuvant variant	KLK [nM/100µl]	ODN1a [nM/100µl]
IC31® Niosomes batch 1	20,5	18,5
KLK Niosomes batch 1	15,4	n.a.
ODN1a Niosomes batch 1	n.a.	17,53
Mix 1 of KLK and ODN1a Niosomes batch 1	11	6,33
Mix 2 of KLK and ODN1a Niosomes batch 1	4,4	15,8
Plain Niosomes batch 1	n.a.	n.a.
IC31® Niosomes batch 2	31,4	28,6
ODN1a Niosomes batch 2	n.a.	28,6
KLK Niosomes batch 2	31,4	n.a.
Mix 1 of KLK and ODN1a Niosomes batch 2	20	17,1
Plain Niosomes batch 2	n.a.	n.a.

Table 7: Different variants of niosomal adjuvant formulations used for topical and intranasal application as provided by Prof. Karola Vorauer-Uhl´s laboratory at University of Natural Resources and Life Sciences Vienna and Intercell itself. All gel formulations are buffered in 2.5mM Tris.

5.1.6 Vaccines

Vaccines for experiments, which included TRP-$2_{180-188}$ were formulated in 2,5mM Tris with a final peptide concentration of 60µg/100µl. Experiments, which included SP1732-3 were formulated in 2,5mM Tris with a final protein concentration of 20µg/100µl. BAP006-2 was formulated in 250mM Sorbitol, 20mM Tris, 150mM NaCl, 300mM L-Arginine to a final concentration of 30µg/100µl.

For all antigens negative controls were prepared which contained only the corresponding buffer. 100µl of the previously mentioned formulations were injected (s.c.) and topical adjuvant was applied subsequently. As positive control IC31® was formulated directly, in the corresponding buffer, with the antigen to a final KLK concentration of 100nM/100µl and ODN1a concentration of 4nM/100µl. It was injected s.c. as a control without any further topical adjuvant treatment. In some experiments an additional positive control, namely Imiquimod cream was applied instead of the IC31® formulations.

In case of intranasal applications Cholera Toxin subunit B (CTB) (Sigma Aldrich) was used as a second positive control and coformulated with the antigen in the corresponding buffer to a final concentration of 1µg/40µl. CTB is a superantigen protein complex of the bacteria *Vibrio cholera.*

5.2 Methods

This section gives an overview of the methods used in this study.

5.2.1 Vaccination of Mice

5.2.1.1 Mice types and Vaccination Schedules

In the course of the studies two different mouse types (*Mus musculus*) were used. Naive female mice were obtained from Charles River laboratories, Germany and used at an age of 8 to 10 weeks. All experiments were conducted at Intercells´ in-house, pathogen-free, animal facility in accordance with the Austrian law. Studies, which included the TRP-$2_{180\text{-}188}$ peptide as a model antigen, involved only C57BL/6 mice (Haplotype H2-b), while studies with protein antigens like BAP006-2 or SP1732-3 involved only C3H mice. The experiments including subcutaneous (s.c.) antigen injections were designed with 5 mice per group and injections at day 0, 14 and 28. Experiments, which included intranasal (i.n.) antigen application, were designed with 8 mice per group and immunized at day 0, 14 and 28. The animals were then sacrificed between days 39-42 by cervical dislocation. Spleens were isolated and stored, pooled in groups, in "Complete Media" including fetal calve serum (FCS) in 24-well flat-bottom plates for further analysis.

5.2.1.2 Topical Skin Vaccination of Mice

Topical skin vaccinations were performed by first anesthetizing the mice with an intraperitoneal (i.p.) injection of a 2% Xylazine (Bayer Healthcare) + 10% Ketamine (Richter Pharma) saline solution. After subsequent shaving and cleaning the mice on their tail-base and part of the back, 100µl of the pre-formulated antigen solution was injected subcutaneously (s.c.). Finally aliquots of the already, by Prof. Karola Vorauers' lab, pre-prepared niosomal (40µl/mouse) - or gel-adjuvant (100µl/mouse) formulations were applied topically on the injection site and around the depot formation on the back, as shown in Figure 4. Over a time-course of 75 minutes approximately 80% of the applied 100µl diffused into the skin. During this diffusion time the mice were separated to ensure the proper uptake of the formulations without disturbance from other individuals. As a positive control 25mg of Aldara 5% cream (Meda) were applied topically instead of the pre-prepared adjuvant formulations. Additionally a control group of five mice with subcutaneous injections of IC31® coformulated with the antigen was included. This control group was also shaved and cleaned as mentioned above to ensure equal treatments of all animals.

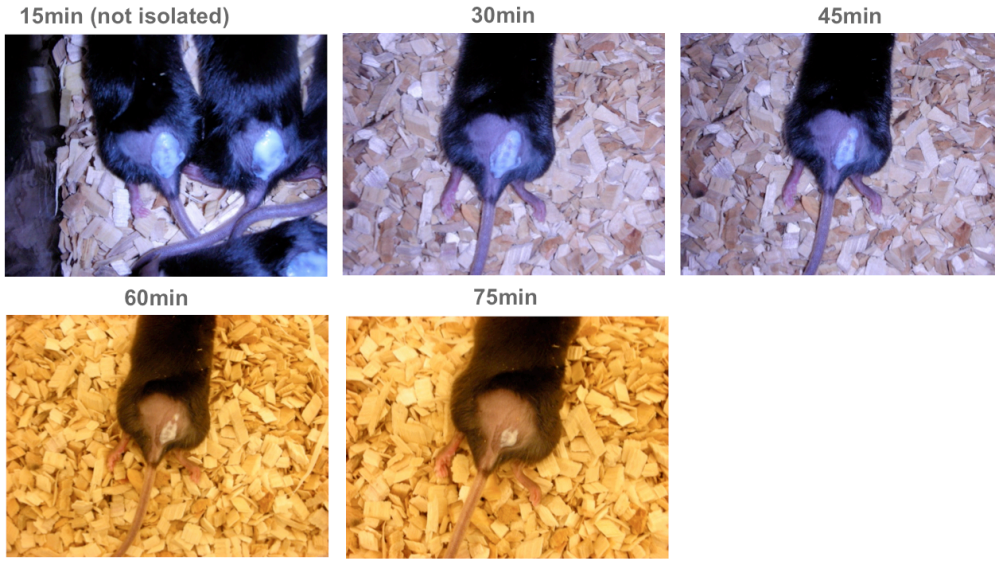


Figure 4: **Topical application of gel-adjuvant formulation on back and tail-base of mice.** Over a time of 75 minutes approximately 80% of the topically applied 100µl solution diffused into the skin. Except for this diffusion study, the mice remained separated during 75 minute time period in all experiments.

5.2.1.3 Intranasal Vaccination of Mice

Intranasal immunizations were performed by anaesthetizing the mice with Isoflurane (Baxter) and applying 40µl of the co-formulated antigen and niosomal adjuvant into the mouse nostrils via pipetting.

5.2.2 Preparation of single cell suspensions

The previously, respective to their groups, pooled organs were taken and crushed through a 70µm single cell strainer (Falcon) with the help of the plunger of a 1ml syringe (Braun). After collecting the resulting cell suspension in a petri dish, both the syringe plunger and cell strainer were washed with additional medium to ensure as few cells as possible

were lost. The cells were pipetted into a 15ml Greiner tube. The petri dish was then washed with another 1 ml of medium, transferred into the same tube, which was then incubated for 5 minutes at room temperature (RT) to sediment larger tissue fragments. After transferring the supernatant into a separate tube the cell suspension was centrifuged at 1600rpm for 5 minutes. The supernatant was removed by aspiration. The pellet was resuspended in 5ml Red Blood Cell Lysis (Sigma) Buffer and incubated for exactly 2 minutes, before adding another 8ml of medium to stop the lysis of the erythrocytes. After subsequent centrifugation of 5 minutes at 1600rpm the pellet was resuspended in 10ml of complete medium and an aliquot is diluted 1:40 for the counting of the cells. 20µl of the aliquot were diluted 1:5 in Trypan Blue stain (GIBCO) and counted in a Neubauer improved hemocytometer counting chamber. By doing so, only life cells could be counted directly, since Trypan Blue only stains cells with disrupted membranes in contrast to living ones. After counting all aliquots the cell suspensions were spinned down another 5 minutes at 1600rpm and the supernatant was removed by aspiration. The pellet was then reconstituted in the calculated amount of medium, so that the resulting cell suspension would have $1x10^8$ cells/ml. Further lower concentrated cell suspensions were prepared according to the experimental setup as needed

5.2.3 Media

For all single cell suspension preparations, long term incubation (more than 12 hours) and ELISpot assays "complete medium" was used. This medium was prepared by adding up the following components directly into a 500ml bottle of DMEM medium (PAA Lab GmbH):

Complete Medium:		
Reagent:	**Company:**	**Amount/500ml:**
DMEM (500ml)	**PAA Lab GmbH**	**n.a.**
(v/v) heat-inactivated FBS	**PAA Lab GmbH**	**25ml**
L-Glutamine(stock: 200mM)	**PAA Lab GmbH**	**5ml**
Sodium Pyruvate (stock: 100mM)	**PAA Lab GmbH**	**5ml**
Non-essential amino-acids (stock: 100x)	**PAA Lab GmbH**	**5ml**
Gentamicin (stock: 10mg/ml)	**PAA Lab GmbH**	**2ml**
2-Mercaptoethanol (stock: 50mM)	**GIBCO**	**0,5ml**

Table 8: Preparation of "Complete Medium" used for single cell preparation, long term incubation (more than 12 hours) and ELISpot assays.

For all flow cytometric assays, which included short term incubation of the cell cultures (below 12 hours), the same medium as above was prepared, however without heat-inactivated fetal bovine serum (FBS). FBS would have disturbed the downstream measurement in the FACS Calibur flow cytometer (BD).

5.2.4 ELISpot Assays

5.2.4.1 IFN-γ ELISpot

All steps until discarding the cells were done under sterile conditions using a laminar flow hood. MAHAS4510 plates (Millipore) were pre-wetted with 100µl sterile 1x PBS (PAA), blotted and then coated with 50µl/well of anti mouse IFN-γ antibody (Becton Dickinson) diluted in coating buffer, which is mentioned in the material section, to a final concentration of 3µg/ml. The plates were incubated over night at 4°C and blocked on the following day with 100µl blocking buffer, prepared as described in the material section, for 1 hour at 37°C. Spleenocytes were plated in triplicates, in different dilutions, for a relevant and an irrelevant stimulus as well as a non-stimulated group (Complete medium added instead) and a positive control (final conc. 10µg/ml Concanavalin A (Con A) (Sigma)) which is not shown in the data sets. After stimulation of the cells with 100µl of the corresponding stimulus (1µg/ml), they were incubated for 16-24h at 37°C, 5% CO_2 over night. On the next day the cells were discarded and the wells were washed 3 times with washing buffer, prepared as described in the material section, at room temperature. Sterile handling was not necessary anymore at this point. All wells were incubated for 2 hours, at 37°C, 5%CO_2, with 50µl of biotinylated anti mouse IFN-γ detection antibody (Becton Dickinson) diluted in 1x PBS (PAA) to a working concentration of 1µg/ml. After another washing step as described above, 50µl of 0,1U/ml Streptavidin-Horseradish Peroxidase (Roche) were added to each well and incubated for 30 minutes at 37°C, 5%CO_2-. Meanwhile, a

solution of 3.3`-Diaminobenzidine (DAB) (1:50) (Sigma) and $NiCl_2$ (1:200) (Sigma) was prepared in 0,1M TrisHCl and filtered to ensure no false positive signals occur from potential particles in the resulting mixture. The plates were then washed again as described above. Shortly before pipetting 50µl into each of the wells, 0.15% H_2O_2 was added to the substrate solution and mixed well. The wells were incubated for approximately 5 minutes at room temperature, before the reaction was stopped by rinsing the plate under cold tap water for 3 times. The protective back covers were removed and the plates were dried at room temperature, protected from light, over night. Final measurement of the spot forming units (SFU), in each well, was performed with the help of a Bioreader 5000 (Bio-Sys) and evaluated with Graphpad Prism 5 displaying results as SFU/$1x10^6$ cells together with the standard error of the mean of the triplicates (SEM).

5.2.4.2 IL-4 ELISpot

IL-4 ELISpot assays were in the same way as described above for IFN-γ ELISpots with the following exceptions. Instead of anti IFN-γ antibodies the corresponding anti IL-4 antibodies (working concentration: 1µg/ml) (Becton Dickinson) were used. Instead of Con A as positive control a stimulation with PMA/Calcium Ionophore (Sigma Aldrich) (working concentration: $1x10^{-7}$M / 1µg/ml) was used. Incubation of the plated and stimulated cells happened for 48 hours.

5.2.4.3 IL-17 ELISpot

IL-17 ELISpot assays were performed in the same way as described above for IFN-γ ELISpots with the following exceptions. Instead of anti IFN-γ antibodies the corresponding anti IL-17 antibodies (working concentration: 1µg/ml) (Becton Dickinson) were used. Incubation of the plated and stimulated cells happened for 48 hours.

5.2.5 CD 154 (CD40L) Surface Staining Assay

All work until the final surface staining after 8 hours incubation time was done under sterile conditions in the laminar flow hood. $1x10^6$ spleen cells/100µl were plated per well in triplicates in 96-well round bottom plates (Nunc). Cells were spinned down at 1600rpm for 5 minutes and rediluted in 100µl of Complete medium – FCS, prepared as described in the material section. The following components were added directly into well:

- 20µl GolgiStop (Becton Dickinson) (working concentration 1x)
- 20µl APC-labelled anti-CD154 (CD40L) or isotype control (both eBioscience) (working concentration 1,25µg/ml)
- 60µl relevant/ irrelevant/ negative control/ positive control (*Staphylococcus aureus* enterotoxin B (SEB) (Sigma Aldrich))/ Stimulus (working concentration 5-10µg/ml)

Cells were then incubated for 8 hours at 37°C, 5% CO_2. After subsequent washing with 100µl MACS Buffer, which is described in the

material section, the triplicates were pooled and centrifuged at 2100rpm, 4°C for 2 minutes. To ensure that no unspecific antibody bindings occur during the downstream staining process the pellet was reconstituted in 100µl/well of anti FcγR antibody (Beckton Dickinson), diluted 1:200 in MACS buffer and incubated for 15 minutes in the dark at 4°C. Following another washing step with 100µl/well MACS Buffer and centrifugation at 2100rpm for 2 minutes, the cells were stained 30 minutes in the dark at 4°C with 100µl/well antibody mix, containing FITC-labeled anti CD4 antibody and 7-AAD viability staining (eBioscience) (1:200 dilutions). Finally, after washing the cells another time as described above, the pellet was fixated in 300µl PFA 4% solution and transferred into FACS tubes (Falcon). The gating strategy is shown in Figure 5. Analysis happened on a FACS Calibur (Beckton Dickinson) using Cellquest as acquisition software. 50.000-100.000 events in the CD4 gate were measured. Further evaluation of the data was done with the help of the FCSexpress 3.0 software.

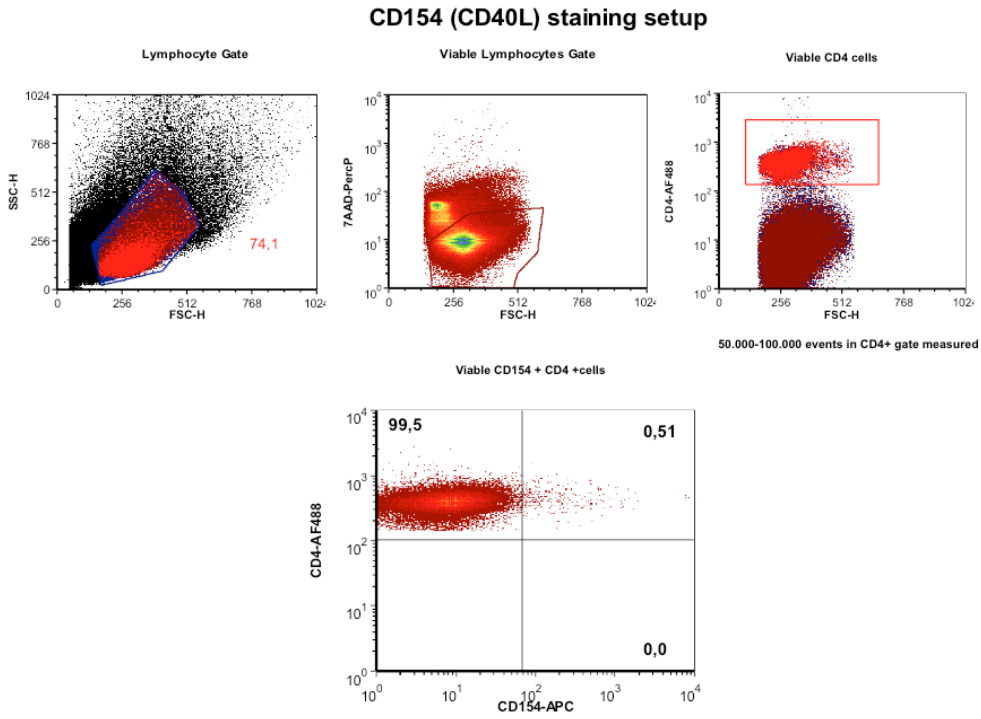


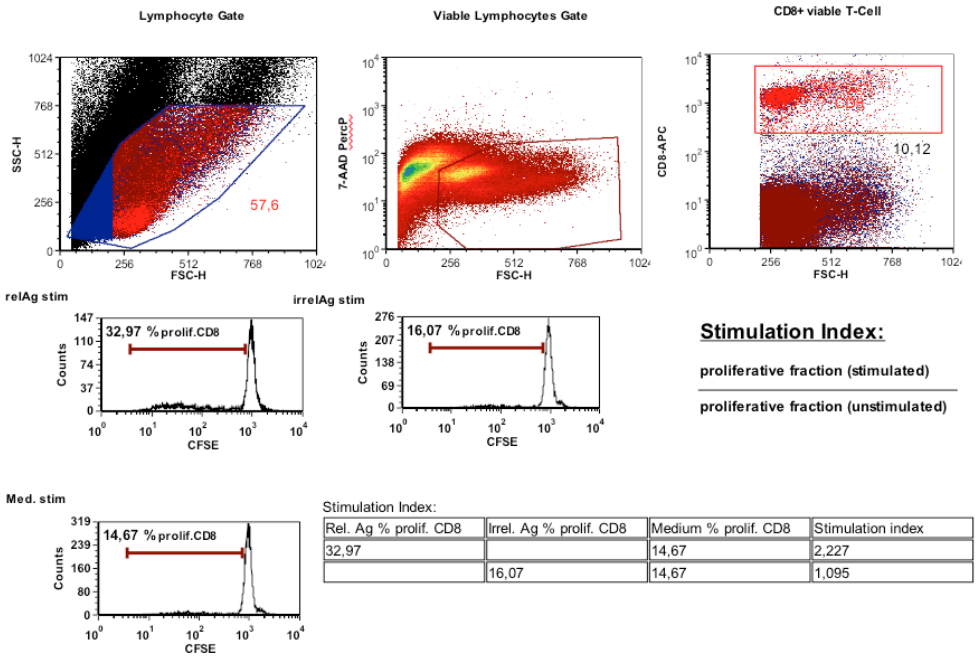
Figure 5: Gating strategy of a CD 154 (CD40L) staining. First the lymphocytes were gated with subsequent selection of viable cells in the non-positive 7-AAD fraction. In the following CD4+ gate 50.000-100.000 events were measured to acquire the final graph shown at the bottom. The x-axis shows the intensity of the CD154 staining whereas the y-axis shows the intensity of the CD4 staining. Double positive signals (upper right quadrant) were visualized in the quadrant with a relative percentage of total CD4 + cells counted.

5.2.6 CFSE Proliferation Assay

All steps until after the 5 days of incubation were done under sterile conditions in the laminar flow hood. $5x10^6$ spleen cells/well/stimuli were collected in a 15 ml tube (Greiner), washed, filtered through a 70µm cell strainer (Falcon) and finally rediluted in 900µl PBS (PAA). The carboxyfluorescein succinimidyl ester (CFSE) proliferation staining (Invitrogen), which initially binds to intracellular molecules of the cell and then halves its intensity by each division, was done at a working concentration of 2µM/ml (enough for about $3\text{-}5x10^7$ cells). The staining

process was performed by adding 100µl of the CFSE solution, which was diluted in PBS, to the 900µl cell dilution of each group while kept vortexing for 1 minute to ensure proper dispersion. The 15 ml tube was then incubated in the dark for 5 minutes at 37°C before the reaction was stopped by adding 1ml fetal bovine serum (FBS) (50%) (PAA). The cells were then washed in PBS and rediluted in Complete medium to a final concentration of $5x10^6$ cells/ml. 1ml/well/stimuli/per group of this cell-dilution was then transferred to a 24-well plate (Nunc). Each well was either stimulated with 1µ-5µg/ml working concentration of positive control (Con A)/ negative control (Complete medium)/ irrelevant antigen/ relevant antigen, by adding 1ml of a double concentrated medium-stimulus solution directly into the well. All plates were incubated for 5 days in the dark at 37°C, 5% CO_2. After 48 hours 1ml of each well was transferred into a separate plate by cautiously taking it from the surface of the liquid to not disturb the cell layer underneath. This 1ml was mixed with 20µl T-cell culture supernatant without ConA (T-stim) (Becton Dickinson) and transferred back into the corresponding well to reach a final working concentration of 1% T-stim. After a total of five days incubation, the cells were transferred into 96-well plates by sequential concentrating the cells in the smaller wells. After subsequent washing with 100µl MACS Buffer, which is described in the material section, the triplicates were pooled and centrifuged at 2100rpm, 4°C for 2 minutes. To ensure that no unspecific antibody bindings happen during the downstream staining process the pellet was reconstituted in 100µl/well of anti FcγR antibody (Beckton Dickinson), diluted 1:200 in MACS buffer and incubated for 15 minutes in the dark at 4°C. Following another washing step with 100µl/well MACS Buffer and centrifugation at 2100rpm for 2 minutes, the cells

were stained 30 minutes in the dark at 4°C with 100µl/well antibody mix, containing APC-labeled rat anti-mouse CD8a antibody and 7-AAD viability staining (eBioscience) (1:200 dilutions). Finally, after washing the cells another time as described above, the pellet was fixated in 300µl PFA 4% solution and transferred into FACS tubes (Falcon). Cell-acquisition happened on a FACS Calibur (Beckton Dickinson) using the Cellquest Sofware and measuring 500.000-1.000.000 total events. Further evaluation of the data was done with the help of the FCSexpress 3.0 software and using the Stimulation index method [90]. This method generates a relative index number by dividing the whole proliferative fraction of the relevant or irrelevant stimulated group through the whole proliferative fraction of the corresponding unstimulated group. The gating strategy and calculation of the stimulation indices are shown in Figure 6. The relevant and irrelevant stimulation indices were then compared and displayed with the Graphpad Prism 4 software.



Stimulation Index:

Rel. Ag % prolif. CD8	Irrel. Ag % prolif. CD8	Medium % prolif. CD8	Stimulation index
32,97		14,67	2,227
	16,07	14,67	1,095

Figure 6: Gating strategy of a CFSE proliferation staining. First the lymphocytes were gated with subsequent selection of viable cells in the non-positive 7-AAD fraction. In the following CD8+ gate the final graphs shown at the bottom of the figure were obtained. The x-axis shows the intensity of the CFSE staining whereas the y-axis shows the event counts. The proliferative fraction beneath the red marker was visualized with a relative percentage of total viable CD8 + cells counted. Total 500.000-1.000.000 events were counted. The relevant and irrelevant Stimulation indexes were calculated by dividing the proliferative fraction of the stimulated group through the unstimulated control. The resulting indices were compared and displayed with the Graphpad Prism 4 software.

5.2.7 Cytrometric Beads Assay

The cytometric beads assay is an easy to perform and effective way to determine absolute amounts of different cytokines in supernatants.

To do so, $1x10^6$ spleenocytes/well were cultured in a 96-well round-bottom plate (NUNC). Each group was stimulated for 24h with relevant, irrelevant antigen as well as complete medium as negative and ConA as positive control with the same concentration as mentioned

in the ELISpot sections. After the restimulation the cells were spinned down and supernatants were collected. The cytometric beads assay (CBA) itself was a ready-to-use prepared Flow Cytomix kit by Bender MedSystems (Cat. BMS820FF). The procedure was performed according to the instruction manual available online. (http://www.ebioscience.com/media/pdf/flowcytomix-software/flowcytomix-pro-software-manual.pdf)

The principles of this assay are beads with attached antibodies against different cytokines, which capture their targets in the supernatant and get detected further via an ELISA-type method with specific biotinylated antibodies. These antibodies are then finally linked to a Streptavidin-PE conjugate, which can be detected via Flow Cytometry. The different populations detected are specific for different cytokines. Data analysis was performed with the FlowCytomix™ Pro 2.4 Software.

6 Results

6.1 Topical Skin Gel formulation studies

The models of the murine melanoma marker TRP-2 peptide 180-188 and the protein SP1732-3 were already established at Intercell AG by previous studies of the Infectious Disease and Animal Models Team via subcutaneous injections in unpublished data, as well as in the literature [85, 86].

6.1.1 TRP-2$_{180-188}$ study with topical Gel formulations batch 1

To determine whether topical Gel formulations containing IC31® or its' single components could elicit a cellular immune response, the different variants of the topically applied gel formulations were compared to subcutaneous injections of 60µg/mouse TRP-2$_{180-188}$ coformulated with IC31® (100nM KLK/ 4nM ODN1a). Immunizations were performed with 60µg/mouse antigen at day 0, 14 and 28. The gels were applied topically on the injection and the depot formation site of the previously administrated peptide. A total number of five naïve six to eight weeks old female C57BL/6 mice were used per group. To compensate the loss of material during the topical application of the gels and to provoke an increased immune response, higher concentration of KLK and ODN1a per 100µl were used, as described in Figure 7. Furthermore, Figure 7 also shows the detailed application procedure and group distribution of the experiment. As negative controls 2.5mM Tris buffer was injected

instead of the antigen. Additionally plain gel, which contained neither IC31 nor it´s single components, was used in one group to determine a possible background adjuvanting effect of the matrix itself. On day 39 the mice were sacrificed by cervical dislocation, the spleens were recovered and analyzed via an IFN-γ ELISpot assay as described in the methods section.

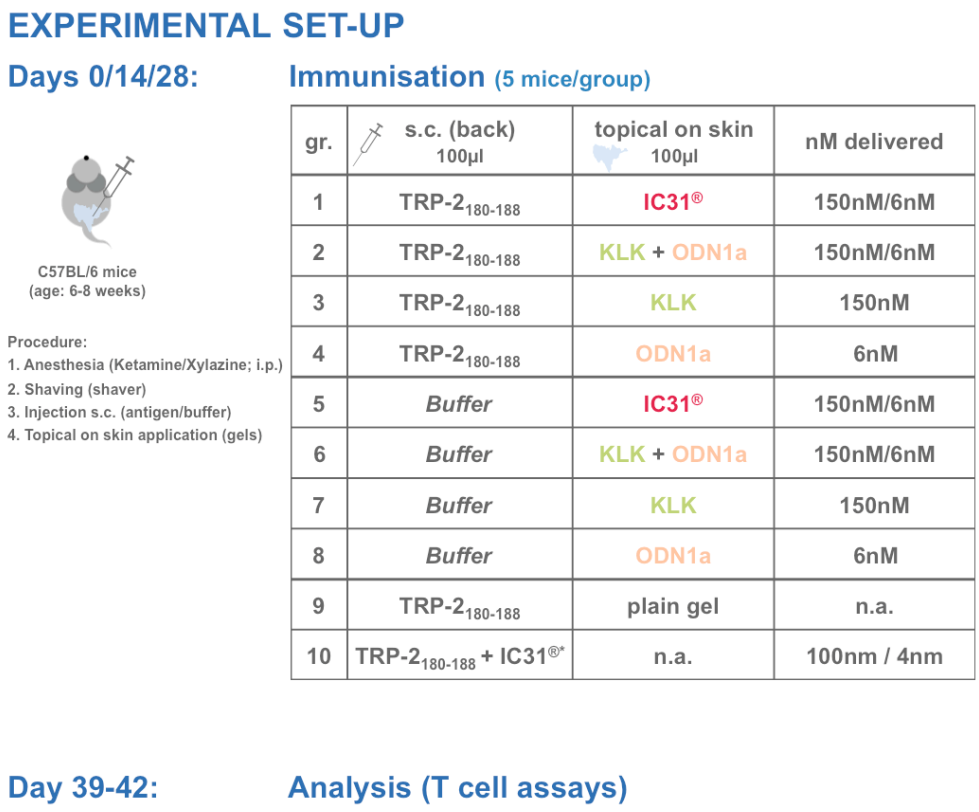


Figure 7: Experimental Setup of TRP-2 $_{180\text{-}188}$ peptide study. 5 female C57BL/6 mice were injected s.c. at day 0,14 and 28 with 60µg/mouse TRP-$2_{180\text{-}188.}$ 100µl/mouse corresponding gel formulation was applied topically. As negative control buffer was injected instead of the antigen or plain gel was used instead of IC31/IC31-single components containing gels. As positive control TRP-$2_{180\text{-}188}$ was coformulated with IC31 and injected s.c. without any topical application. T-cell analysis of the spleen cells was performed between days 39-42.

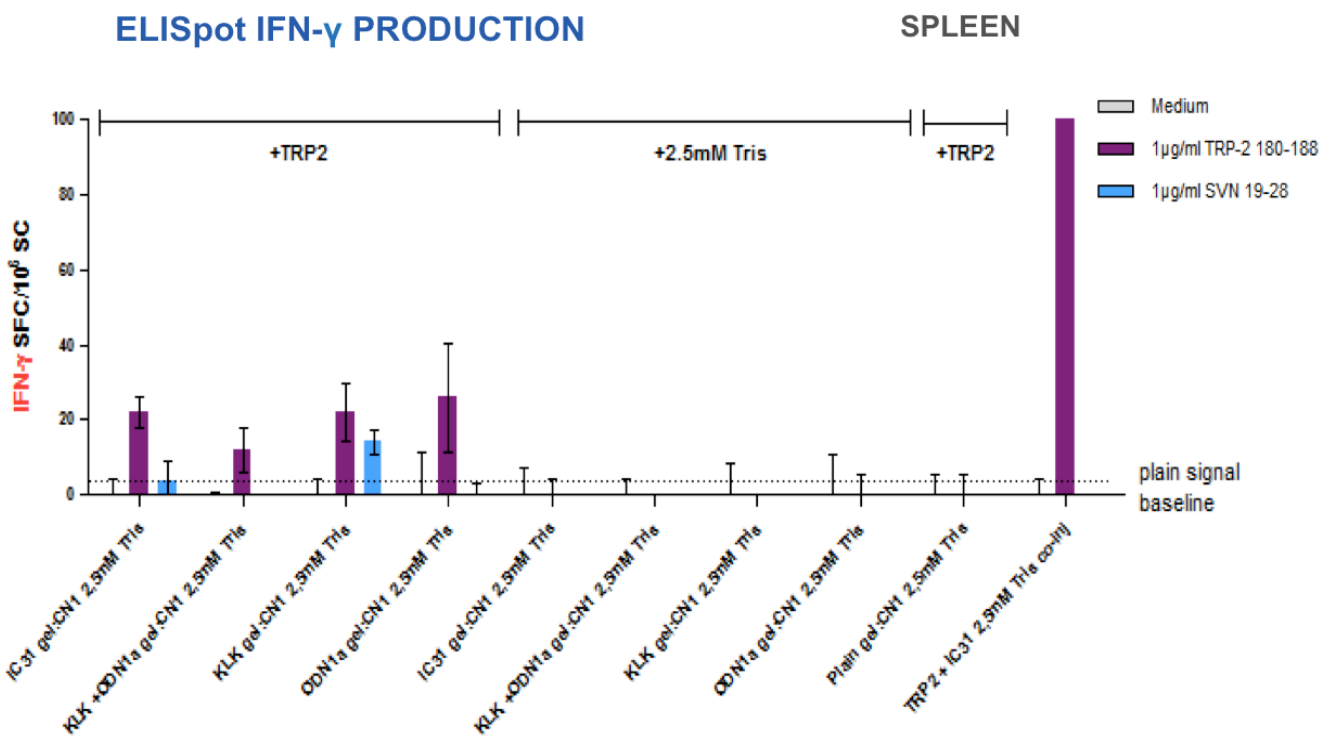


Figure 8: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the TRP2 $_{180-188}$ peptide study. $1x10^6$ spleen cells per well were plated and stimulated for 24h with either medium, relevant antigen (TRP-$2_{180-188}$) or irrelevant antigen (SVN_{19-28}). The differently treated groups on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

Figure 8 shows the mean IFN-γ spot forming units (SFU), together with their SEM, per $1x10^6$ spleen cells. The cells were collected on day 39 after three immunizations and stimulated for 24 hours with Complete Medium + FCS (prepared as described in the material section), 1µg/ml relevant peptide ($TRP\text{-}2_{180\text{-}188}$) or 1µg/ml irrelevant peptide ($SVN_{19\text{-}28}$). The data shown is already corrected with the corresponding medium background signal. Additionally a "plain signal baseline" was selected with the according error of the signal chosen (in this case the irrelevant peptide restimulation signal of Group 1). T-tests were performed with Microsoft Excel 2011 (OSX) to determine whether relevant peptide stimulations were significantly above the plain signal baseline (95% confidence interval). The positive control signal of the $TRP\text{-}2_{180\text{-}188}$ + IC31® coinjection situates significantly above the baseline. Groups 1-4 were injected with antigen and show a slight (but not significant; $p>0,05$) increase of IFN-γ SFUs compared to the background. Nevertheless groups 5-8, which were injected with 2.5mM Tris buffer instead of $TRP\text{-}2_{180\text{-}188}$ solution, showed a lower SFU count than the other groups. Group 9 was injected with the antigen, but topically treated with a plain gel formulation containing only buffer and not IC31® components. This group showed a small signal of the relevant antigen stimulated group but was not significant as well.

In summary the topical gel formulations, in combination with our melanoma peptide model antigen $TRP\text{-}2_{180\text{-}188}$, could only elicit a small but not significant enough systemic Th1 related systemic immune response characterized by IFN-γ ELISpot of the spleen cells.

6.1.2 SP1732-3 study with topical Gel formulations batch 1

Since the selected peptide antigen model could not elicit a sufficient immune response in comparison to the co-injection containing antigen + IC31®, a protein model was tested. The selected model protein, an in-house purified one, is closely related to Serine/Threonine Kinase protein of *Streptococcus pneumonia* (StkP). Additionally to the co-injection of antigen and IC31® an additional positive control was introduced to the experiment. In group 4 the mice received a SP1732-3 s.c. injection in the same way as the other groups did, but instead of applying a topical gel formulation, approx. 25mg of Aldara 5% cream (Imiquimod) were administrated on the back. Imiquimod already proved effective in topical application experiments and was therefore used here as well [46, 91]. Since the gel formulation containing ODN1a showed the highest variation in the previous experiment (Figure 8) it was left out here and replaced with the Imquimod positive control. The basic setup of the SP1732-3 protein antigen study in combination with topical gel formulations remained the same (see Figure 9). For this antigen C3H mice were used instead of C57BL/6 as already done in the literature [92].

Days 0/14/28: **Immunisation** (5 mice/group)

C3H mice
(age: 6-8 weeks)

Procedure:
1. Anesthesia (Ketamine/Xylazine; i.p.)
2. Shaving (shaver)
3. Injection s.c. (antigen/buffer)
4. Topical on skin application (gels)

gr.	s.c. (back) 100µl	topical on skin 100µl	nM delivered
1	SP1732-3	IC31®	150nM/6nM
2	SP1732-3	KLK + ODN1a	150nM/6nM
3	SP1732-3	KLK	150nM
4	SP1732-3	Imiquimod	n.a.
5	*Buffer*	IC31®	150nM/6nM
6	*Buffer*	KLK + ODN1a	150nM/6nM
7	*Buffer*	KLK	150nM
8	*Buffer*	Imiquimod	n.a.
9	SP1732-3	plain gel	n.a.
10	SP1732-3 + IC31®***	n.a.	100nm / 4nm

Day 39-42: **Analysis (T cell assays)**

Figure 9: Experimental Setup of SP1732-3 protein study. 5 female C3H mice were injected s.c. at day 0, 14 and 28 with 20µg/mouse SP1732-3. 100µl/mouse corresponding gel formulation was applied topically. As negative control buffer was injected instead of the antigen or plain gel was used instead of IC31/IC31-single components containing gels. As positive control SP1732-3 was coformulated with IC31 and injected s.c. without any topical application. An additional positive control group was injected with the antigen and topically treated with Imiquimod. T-cell analysis of the spleen cells was performed between days 39-42.

Figure 10 shows the IFN-γ spot forming units (SFU) per $1x10^6$ spleen cells and thus indicates a potential systemic Th1 immune response. The data is again already medium-stimulated SFU corrected and a plain signal baseline has been set at the signal of Group 9. This group shows a highly specific signal upon restimulation with 5µg/ml SP1732-3 (relevant antigen) in contrast to 5µg/ml OVA (irrelevant stimulation). Since this Th1-type immune response was provoked already by the antigen itself or boosted by a potentially adjuvanting effect of the plain gel the baseline was set at this signal. Every response above the baseline

and its' corresponding error, thus has to be the result of the IC31® components and is not correlated with the "background". Both positive controls are significantly higher than the baseline as a direct result of the boosting effect of IC31® injected s.c. or Imiquimod applied topically. The topical application even almost reaches the level of the s.c coinjection. The only group above the baseline is Group 3, containing only the KLK, the microbial peptide component of IC31®. KLK interestingly seemed to be the driver of this immune response since the additional ODN1a component lowered the signal below the baseline in Groups 1 and 2. Nevertheless, all topical applications of IC31®, or the single components incorporated within a gel matrix, could not significantly increase the systemic immune response above the plain signal baseline.

Only Imiquimod and IC31® s.c. coinjected were able to raise the level of SFUs above the plain gel signal baseline. A possible explanation for the failure to elicit a higher immune response could be the fact that not enough material of the IC31® components was delivered through the skin in time to boost and direct the immune response. This is supported by the fact that the IC31® coinjection was able to provoke a potent response in contrast to irrelevant in vitro restimulation. A topical delivery of adjuvanting material in a gel matrix seems possible as well, since Imiquimod could promote a higher IFN-γ SFU production and proved to be almost as high as the s.c coinjection of adjuvant and antigen in Group 4.

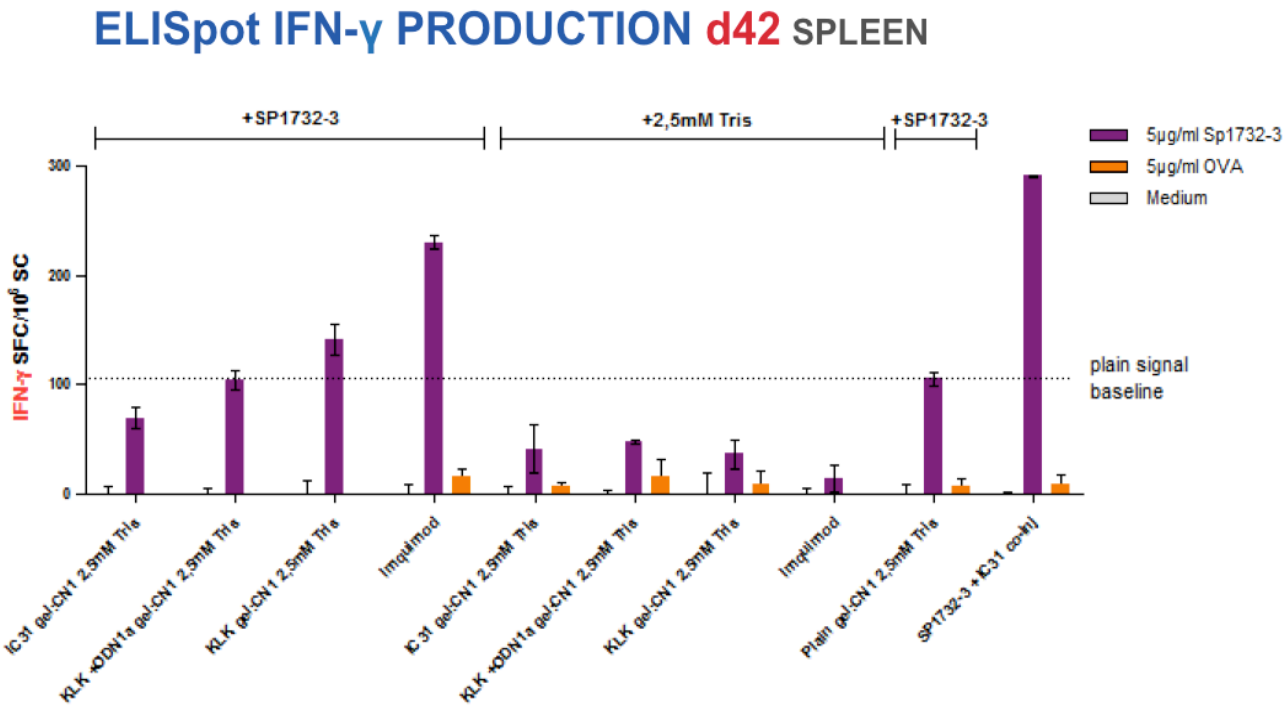


Figure 10: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. 1x10^6/well were plated and stimulated for 24h with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA). The differently treated groups on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

6.1.3 SP1732-3 study with topical Gel formulations batch 2

Since SP1732-3 seemed to have the potential to be adjuvanted via IC31® s.c. and partly topically applied, as well as topically via Imiquimod, the next step was to try to increase this response. The best way to do this is to increase the concentration of delivered adjuvant through the skin. Thus a second batch of gel formulations were provided to Intercell AG by the BoKu Vienna and a repeat of the SP1732-3 study has been performed, with higher concentration of KLK and ODN1a incorporated per 100µl applied to the back of the mice. Details and the experimental setup are shown in Figure 11.

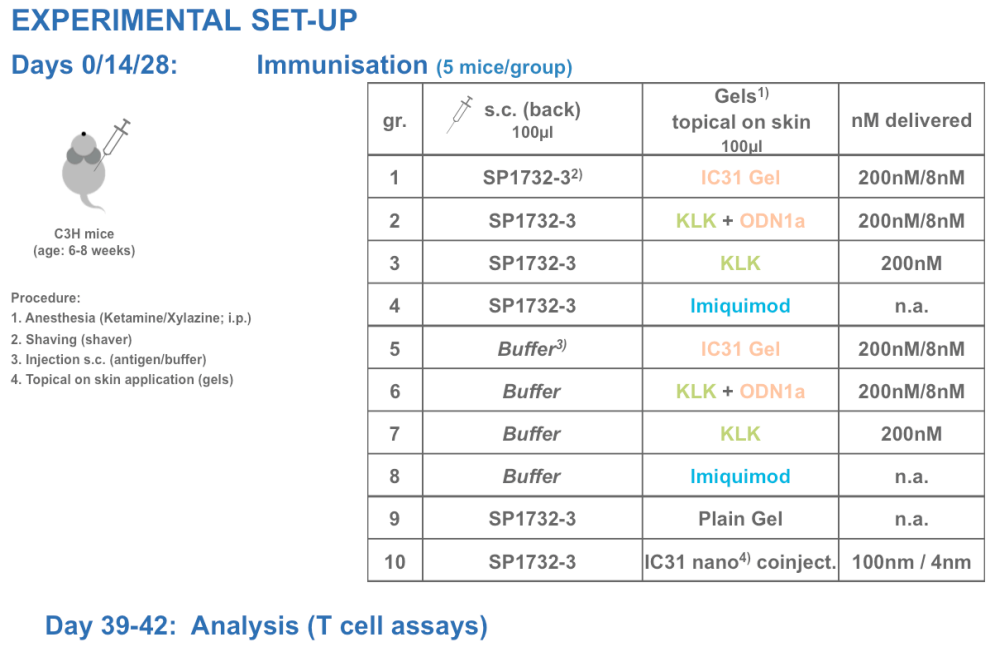


Figure 11: Experimental Setup of SP1732-3 protein study. 5 female C3H mice were injected s.c. at day 0, 14 and 28 with 20µg/mouse SP1732-3. 100µl/mouse corresponding gel formulation was applied topically. As negative control buffer was injected instead of the antigen or plain gel was used instead of IC31/IC31-single components containing gels. As positive control SP1732-3 was coformulated with IC31 and injected s.c. without any topical application. An additional positive control, one group was injected with the antigen and topically treated with Imiquimod. T-cell analysis of the spleen cells was performed between days 39-42.

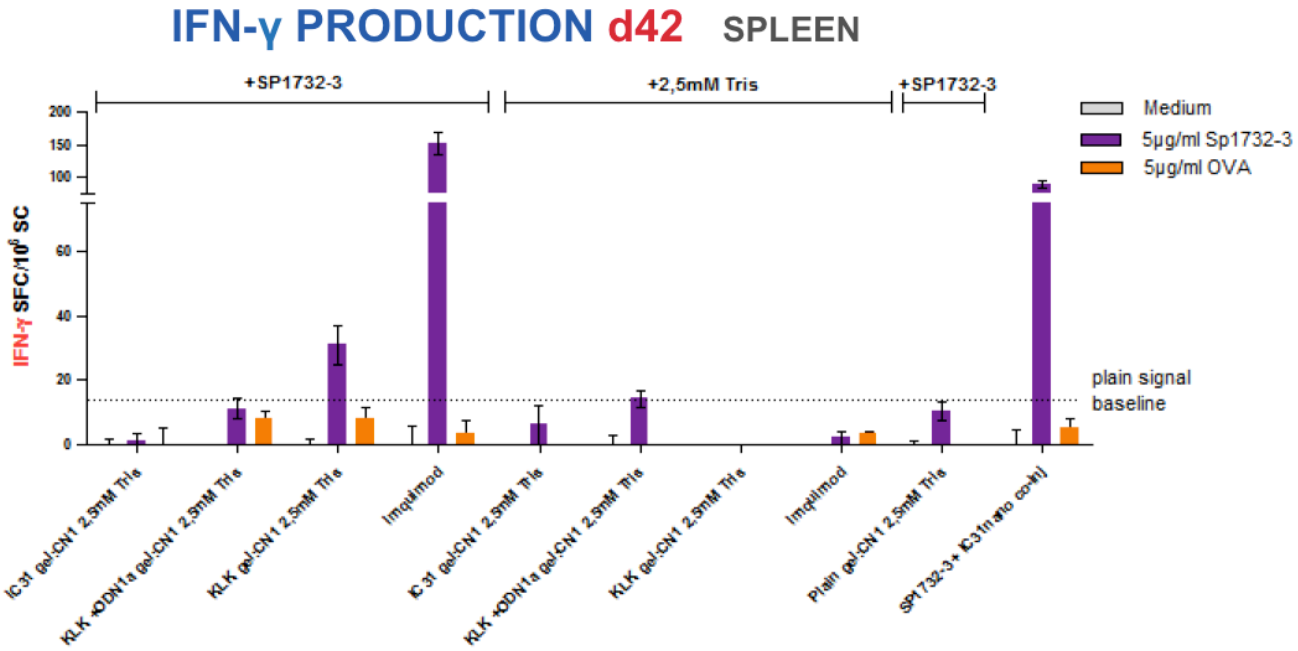


Figure 12A: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA). The categories on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

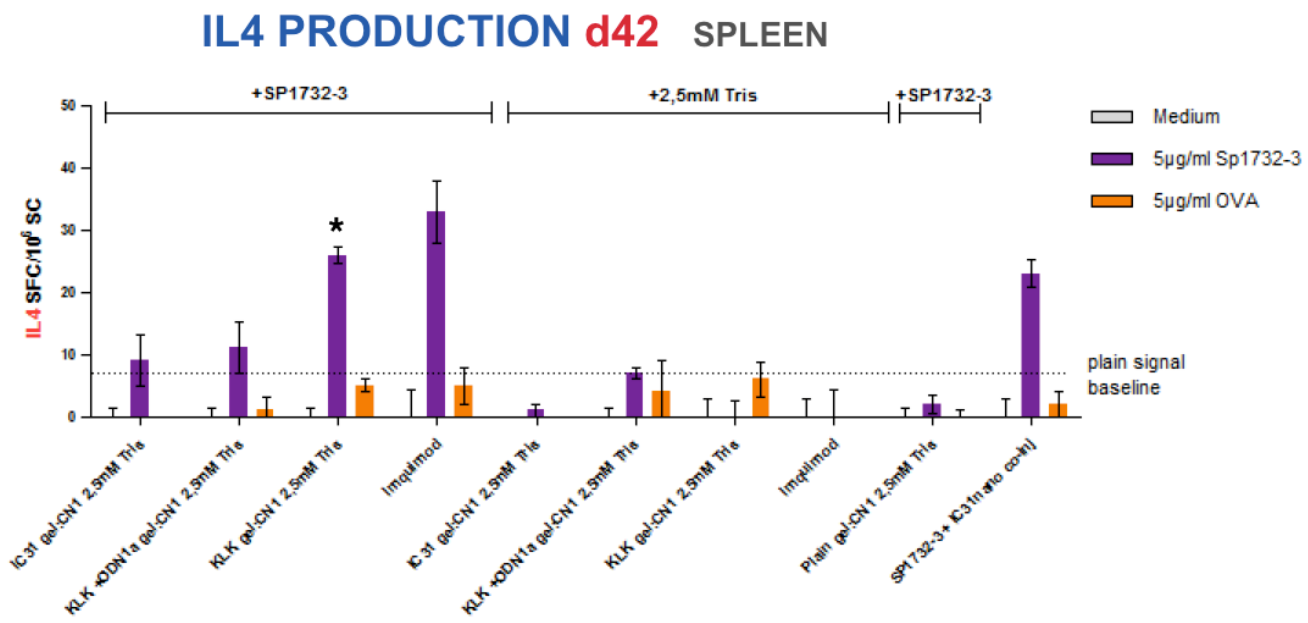


Figure 12B: IL4 Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells /well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA). The categories on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

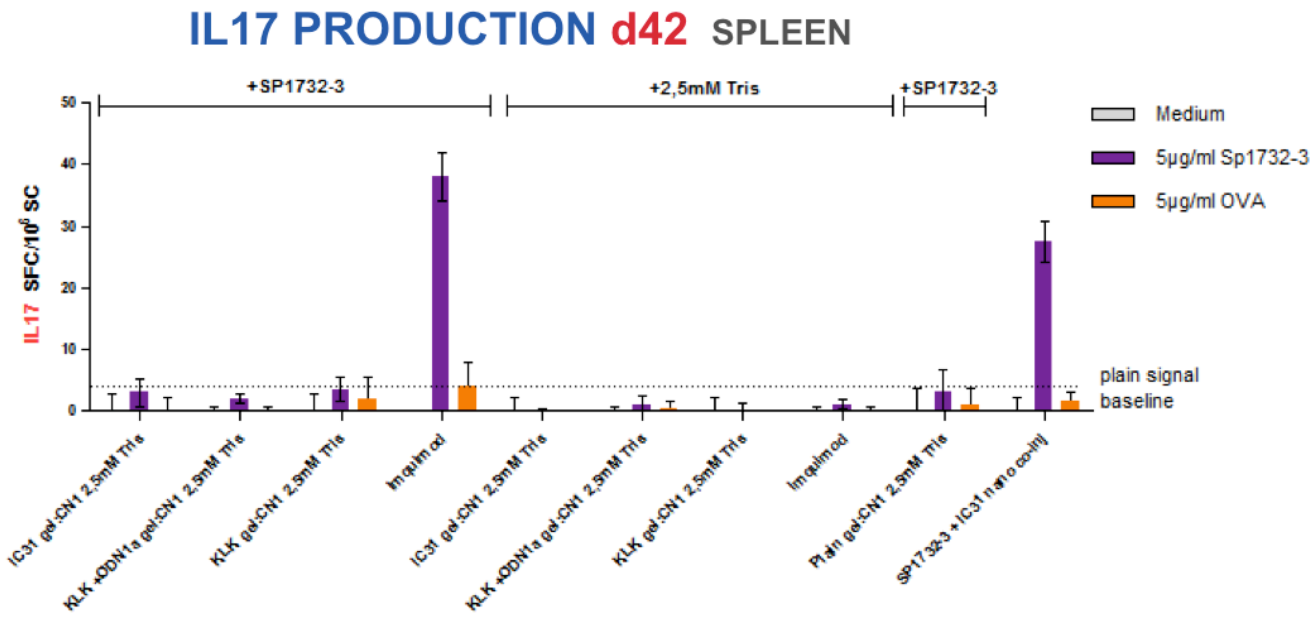


Figure 12C: IL17 Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells /well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA). The categories on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

The results of the ELISpot study of this experiment are shown in Figure 12A displaying the IFN-γ, Figure 12B displaying the IL4 and Figure 12C displaying the IL17 SFUs/1x10^6 spleen cells to characterize a Th1, Th2 and Th17 immune response of the mice against SP1732-3. The expectation of the experiment was to deliver a similar result as the previous study, but with slightly higher SFU counts of Groups 1-3 due to the higher KLK and ODN1a concentration delivered per dose. Additionally to the IFN-γ and IL17 ELISpot an IL4 ELISpot was performed to ensure a wider coverage of the spectrum of T-cell responses to the antigen. The SFU count differs from the previous experiment (see Chapter 5.1.2), but this is due to two experimental approaches, as well as due to the group-corresponding medium-stimulated SFU substractions. The overall outcome pattern still remained the same as expected. As shown in Figure 12A the SFU difference between the baseline and the only signal above it, namely the group with SP1732-3 s.c. injected and a KLK containing gel formulation, increased in contrast to the former experiment. Yet, it still fails to be significant enough in the t-test ($p>0.05$). Figure 12C shows a similar pattern, like witnessed in the first SP1732-3 experiment (data not shown), since no topical applied adjuvant formulation, beside Imiquimod, was able to elicit a potent IL17 response. Finally figure 12B delivers a different picture. Here, the same group, which could previously not elicit a Th1 response above the background level, shows a significant IL4, thus Th2-type, response to the antigen (see asterisk Figure 12B). Since one pathway inhibits the other, it makes sense that the IFN-γ signal appears in a smaller extent, but would finally be suppressed by the Th2 pathway. Unfortunately no IL4 data is available

for the lower KLK and ODN1a concentration of the first experiment. The result indicates a potential of the KLK containing gel formulations to elicit a Th2-type immune response via topical application and s.c. injection of SP1732-3.

Nevertheless, a systemic protection against *Streptococcus pneumonia* via injection of SP1732-3 and topical application of the KLK containing gel formulation seems unlikely, since one of the main defense mechanisms against it is a potent Th17 immune response, which helps to decrease the carriage time, as indicated already in previous studies. [86]. In fact a vaccine should provide antibody production as shown and a Th17 response as well, to promote factors like epithelial protection and so on.[13] Only Imiquimod was able to trigger a broad adaptive systemic immune response against the antigen via topical application, thus could potentially lead to a protective vaccination, beside IC31® plus antigen s.c. coinjections.

6.2 Topical Skin niosomal formulation studies

Niosomes are vesicular, liposome-like, structures with several advantages in contrast to normal Liposomes. They are able to encapsulate and protect various kinds of substances. They have also already shown to be able to enhance, just by themselves, transdermal as well as intranasal drug delivery as already described in the literature [24, 70, 83]. Since one of the key factors of the topical vaccination is the problem of efficient delivery of enough material through a potent defense barrier like mucosal tissue or the *Stratum Corneum* of the skin, it is pivotal to find a vector, which not only protects the adjuvant from degradation, but also increases the transport across these obstacles. To do so Niosomes, due to their high deformability and adjuvanting effect, are a method, which promises high potential as an alternative to the gel formulations already tested. They are able to encapsulate a certain amount of ODN1a and adsorb KLK, but still remain a nanometer (nm) scale of size to diffuse through the skin.

6.2.1 TRP-2$_{180\text{-}188}$ study with topical Niosome formulations batch 1

To be able to compare the adjuvanting effect of IC31® components gels and niosomal formulations another TRP2$_{180\text{-}188}$ peptide study similar to the first (see chapter 5.1.1) was conducted. Mice were treated with antigen injections of 60µg/mouse at day 0, 14 and 28. Spleenocytes were collected. Instead of 100µl gel formulations, 40µl niosomal formulation, containing either only buffer or ODN1a encapsulated together with or without KLK adsorbed to the surface. Additionally, different mixtures

of KLK-only and ODN1a-only containing Niosomes were tested. ELISpot assays were performed to characterize the immune response. Due to technical issues it was not possible to deliver the same concentration of KLK or ODN1a as in the gel formulations. Details of the delivered amounts of KLK and ODN1a as well as the experimental setup are described in Figure 13.

EXPERIMENTAL SET-UP

Days 0/14/28: **Immunisation** (5 mice/group)

C57BL/6 mice
(age: 6-8 weeks)

Procedure:
1. Anesthesia (Ketamine/Xylazine; i.p.)
2. Shaving (shaver)
3. Injection s.c. (antigen/buffer)
4. Topical on skin application (gels)

gr.	s.c. (back) 100µl	Niosome topical on skin ~40µl	nM delivered
1	TRP-$2_{180-188}$	ODN1a	6nM
2	TRP-$2_{180-188}$	KLK	6,7nM
3	TRP-$2_{180-188}$	KLK + ODN1a	6,1nM / 6,7nM
4	TRP-$2_{180-188}$	KLK + ODN1a	2,5nM / 4,4nM
5	TRP-$2_{180-188}$	KLK + ODN1a	6nM / 1,7nM
6	*Buffer*	ODN1a	6nM
7	*Buffer*	KLK	6,7nM
8	*Buffer*	KLK + ODN1a	6,1nM / 6,7nM
9	TRP-$2_{180-188}$	Plain Niosome	n.a.
10	TRP-$2_{180-188}$/IC31®	n.a.	n.a.

Day 39-42: **Analysis (T cell assays)**

Figure 13: Experimental Setup of TRP-2 $_{180-188}$ peptide study. 5 female C57BL/6 mice were injected s.c. at day 0, 14 and 28 with 60µg/mouse TRP-$2_{180-188}$.40µl/mouse corresponding Niosome formulation was applied topically. As negative control buffer was injected instead of the antigen or plain Niosomes were used instead of IC31/IC31-single components containing Niosomes. As positive control TRP-$2_{180-188}$ was coformulated with IC31 and injected s.c. without any topical application. T-cell analysis of the spleen cells was performed between days 39-42.

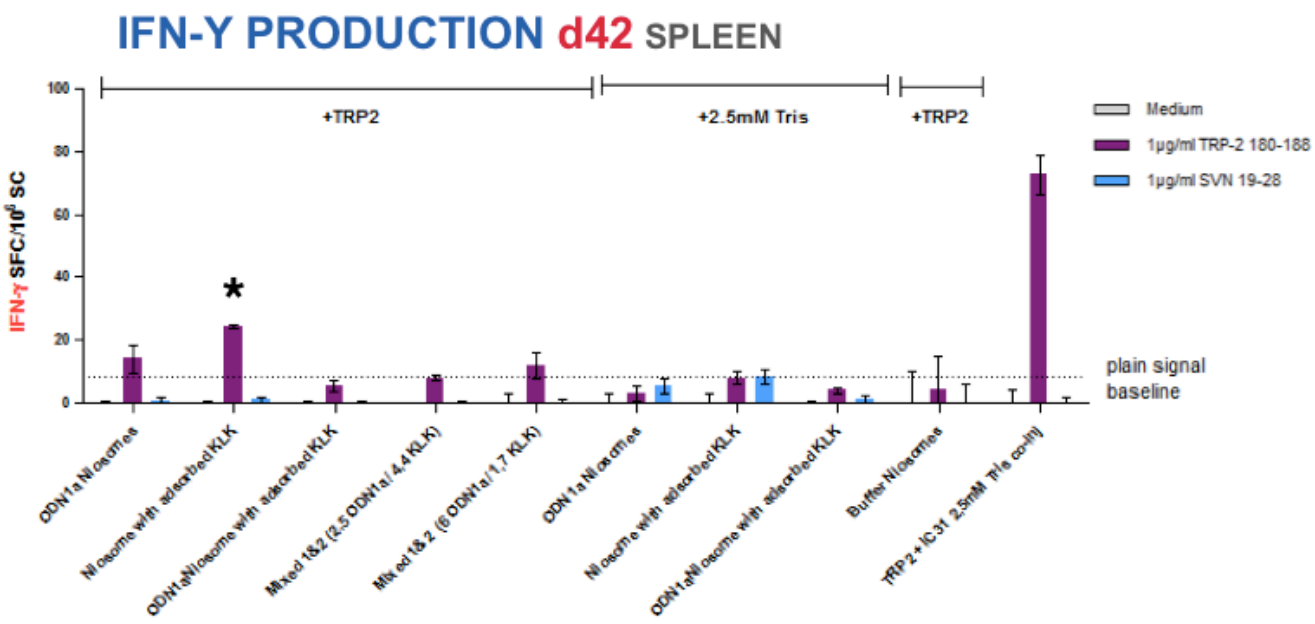


Figure 14: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the TRP2 $_{180-188}$ peptide study. $1x10^6$ spleen cells/well were plated and stimulated for 24h with either medium, relevant antigen (TRP-$2_{180-188}$) or irrelevant antigen (SVN_{19-28}). The categories on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

In this experiment (see Figure 14) the plain signal baseline was defined by the SFU´s of the irrelevant peptide stimulated signal of Group 7, together with its error. Despite the lower concentration of ODN1a and KLK (about 1/6 of the concentration included in the gel formulations) applied per 35µl, there was a similar adjuvanting effect on the IFN-γ levels measured in the ELISpot assay above. The only group, which shows a significant increase in adjuvanticity, above the plain signal background baseline, was Group 2 (see asterisk Figure 14). This group was treated topically with Niosomes, which had KLK (6,7nM/35µl) adsorbed to their surface. This low amount was already sufficient to elicit a specific Th1-type immune response against TRP-$2_{180\text{-}188}$. The relevant antigen signal of the IC31 + antigen s.c. coinjection is still about three times higher than the topically one. A further increase of delivered material could potentially improve the signal and boost it above the background. Neither the mixed niosomal formulations of different types, nor the ODN1a-only ones could produce a similar strong immune response.

6.2.2 TRP-$2_{180\text{-}188}$ study with topical Niosome formulations batch 2

After overcoming some of the technical issues during the production process of the niosomal formulations, the amount of IC31® components incorporated in and on the vesicles could be increased, without compromising their stability. Since the previous experiment (see Chapter 5.2.1) demonstrated the importance of KLK in the production of a detectable and significant IFN-γ immune response, the formulations containing this synthetic microbial peptide were further

investigated. Experimental details and setup are described in Figure 15. Unfortunately any further increase of this component was not possible due to the fact that KLK destabilizes the vesicular structure. This could be seen indirectly by the size of the vesicles. Niosome vesicle sizes, which only contained adsorbed KLK increased up to approximately 150nm, while empty Niosomes had an average size of approximately 115nm. Interestingly the so-called IC31® Niosomes, which contained ODN1a inside the vesicle and KLK adsorbed at the surface were smaller than the KLK-only ones. Their size reduced to approximately 115nm, suggesting a stabilizing effect of the ODN1a DNA backbone-like structure inside the vesicle.

EXPERIMENTAL SET-UP

Days 0/14/28: Immunisation (5 mice/group)

C57BL/6 mice
(age: 6-8 weeks)

Procedure:
1. Anesthesia (Ketamine/Xylazine; i.p.)
2. Shaving (shaver)
3. Injection s.c. (antigen/buffer)
4. Topical on skin application (gels)

Day 39-42: Analysis (T cell assays)

gr.	s.c. (back) 100µl	Niosome topical on skin 35µl	nM delivered
1	TRP-$2_{180-188}$	ODN1a	10nM
2	TRP-$2_{180-188}$	KLK	11nM
3	TRP-$2_{180-188}$	KLK + ODN1a	11nM / 10nM
4	TRP-$2_{180-188}$	KLK + ODN1a	7nM / 6nM
5	TRP-$2_{180-188}$	Imiquimod	n.a.
6	*Buffer*	ODN1a	6nM
7	*Buffer*	KLK	6,7nM
8	*Buffer*	KLK + ODN1a	11nM / 10nM
9	*Buffer*	KLK + ODN1a	7nM / 6nM
10	*Buffer*	Imiquimod	n.a.
11	TRP-$2_{180-188}$	Plain Niosome	n.a.
12	TRP-$2_{180-188}$	IC31 nano coinject.	100nm / 4nm

Figure 15: Experimental Setup of TRP-2 $_{180-188}$ peptide study. 5 female C57BL/6 mice were injected s.c. at day 0,14 and 28 with 60µg/mouse TRP-$2_{180-188}$. 35µl/mouse corresponding Niosome formulation was applied topically. As negative control buffer was injected instead of the antigen or plain Niosomes were used instead of IC31/IC31-single components containing Niosomes. As positive control TRP-$2_{180-188}$ was coformulated with IC31 and injected s.c. without any topical application. T-cell analysis of the spleen cells was performed between days 39-42.

Figure 16 shows the result of the TRP-2$_{180-188}$ study in combination with Niosome formulations containing higher IC31® components concentrations. Group 2 showed a statistically significant, specific systemic immune response against the antigen above the baseline (see asterisk Figure 16). Interestingly, despite the actual delivered nM amount could almost be doubled in comparison to the first experiment (see Chapter 5.2.1.), a signal increase of only about 25-30% could be achieved within group 2, which was treated with a KLK-only niosomal formulations. Two groups, which were topically treated with different variants of Niosomes containing ODN1a as well as KLK could not elicit an IFN-γ response above the background level, similar to the previous experiment.

In summary these two TRP-2$_{180-188}$ studies, in combination with topically applied Niosome formulations of IC31® on the skin, show that only KLK adsorbed to vesicle structure is able to provoke a significant systemic Th1 immune response in the spleen cells. Yet this response is still lower than a direct s.c. injection of the same amount of peptide, coformulated with IC31®. This could be due to reason that only a fraction of the 35-40µl applied on the back of the mice actually diffuses through the skin and reaches its destination at the "depot" of the s.c. injected antigen.

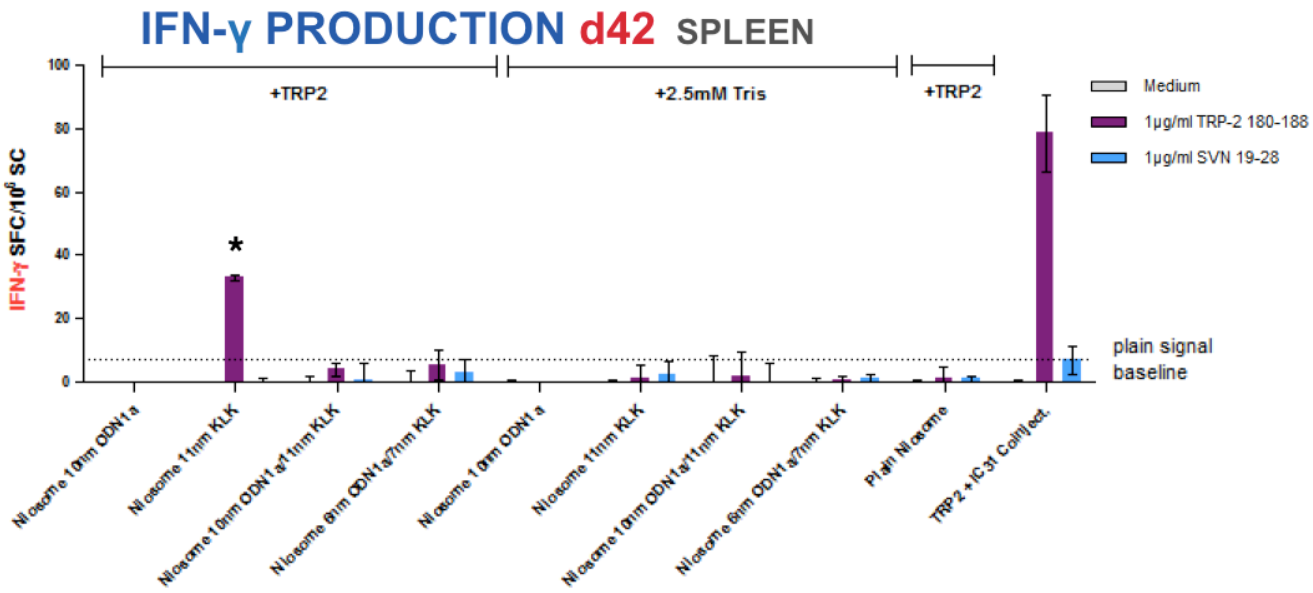


Figure 16: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the TRP2 $_{180\text{-}188}$ peptide study. 1x10^6 spleen cells/well were plated and stimulated for 24h with either medium, relevant antigen (TRP-$2_{180\text{-}188}$) or irrelevant antigen ($SVN_{19\text{-}28}$). The categories on the x-axis are also numbered from left to the right starting with 1. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

6.2.3 SP1732-3 study with topical Niosome formulations batch 2

Both batches of niosomal formulations of IC31 components delivered a statistical significant systemic adjuvanting effect above the background signal, when used in combination with a defined T-cell epitope peptide antigen. Especially batch 2 (see Chapter 5.2.2), with its increased concentration of KLK showed to be effective, but still lower than the s.c. injected control. The logical next step was to conduct an experiment analyzing the effect of the same niosomal formulations in combination with a protein antigen. In this case SP1732-3 was used to gain a direct comparison to the gel formulation experiments already performed. Again 20µg/mouse of SP1732-3 was injected s.c. in the back (as described in the methods section) of female C3H mice, of an age of 6-8 weeks, at day 0, 14 and 28. 35µl of Niosome formulations were applied on the injection and depot formation sites and rubbed in with the cap of an Eppendorf tube. An additional positive control group which was topically treated with 1,25mg of Imiquimod (Aldara 5% cream) was also included. Details and experimental setup, as well as group distribution are described in Figure 17.

EXPERIMENTAL SET-UP

Days 0/14/28: Immunisation (5 mice/group)

C3H mice
(age: 6-8 weeks)

Procedure:
1. Anesthesia (Ketamine/Xylazine; i.p.)
2. Shaving (shaver)
3. Injection s.c. (antigen/buffer)
4. Topical on skin application (gels)

Day 39-42: Analysis (T cell assays)

gr.	s.c. (back) 100µl	Niosomes[1] topical on skin 35µl	nM delivered
1	SP1732-3[2]	ODN1a	10nM
2	SP1732-3	KLK	11nM
3	SP1732-3	KLK + ODN1a	11nM/10nM
4	SP1732-3	Imiquimod	n.a.
5	*Buffer*[3]	ODN1a	10nM
6	*Buffer*	KLK + ODN1a	11nM
7	*Buffer*	KLK	11nM/10nM
8	*Buffer*	Imiquimod	n.a.
9	SP1732-3	Plain Niosomes	n.a.
10	SP1732-3	IC31 nano[4] coinject.	100nm / 4nm

Figure 17: Experimental Setup of SP1732-3 protein study. 5 female C3H mice were injected s.c. at day 0, 14 and 28 with 20µg/mouse SP1732-3. 35µl/mouse corresponding niosomoal formulation was applied topically. As negative control buffer was injected instead of the antigen or plain Niosomes were used instead of IC31/IC31-single components containing Niosomes As positive control SP1732-3 was coformulated with IC31 and injected s.c. without any topical application. An additional positive control, one group was injected with the antigen and topically treated with Imiquimod. T-cell analysis of the spleen cells was performed between days 39-42.

The summarized ELISpot data collected from this study is a visualized in Figure 18. Figure 18A shows the IFN-γ, 18B the IL4 and 18C the IL17 SFU production per $1x10^6$ Spleenocytes after 24 or 48h of in-vitro restimulation with relevant, irrelevant antigen and Complete Medium.

The plain signal baseline, which determines the threshold for a signal not provoked by the pure Niosomes (which already have the potential to produce an adjuvanting effect by themselves) or the SP1732-3 protein itself, was set at the mean SFU count of group 9 (± SEM). Group 9 was treated with s.c. antigen injections and topical skin application of Niosomes encapsulating only buffer and no IC31 components. The outcome of this experiment first seems similar to the comparable gel formulation study, but features a few key differences.

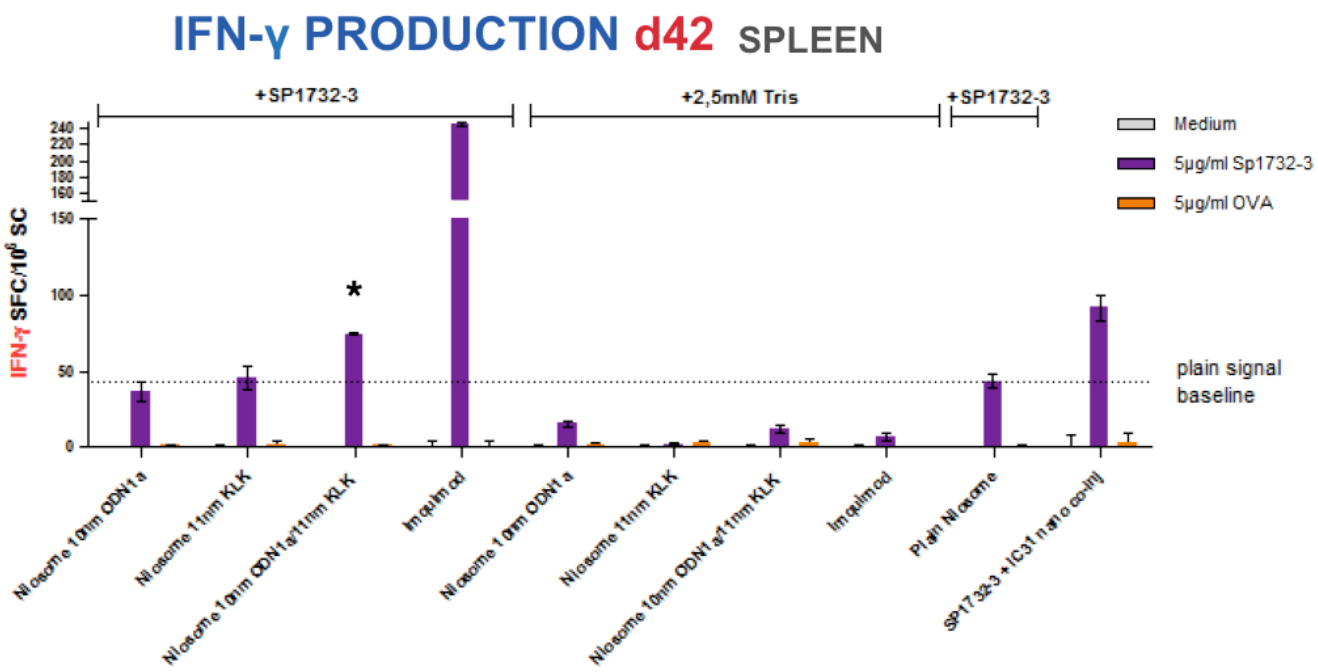


Figure 18A: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

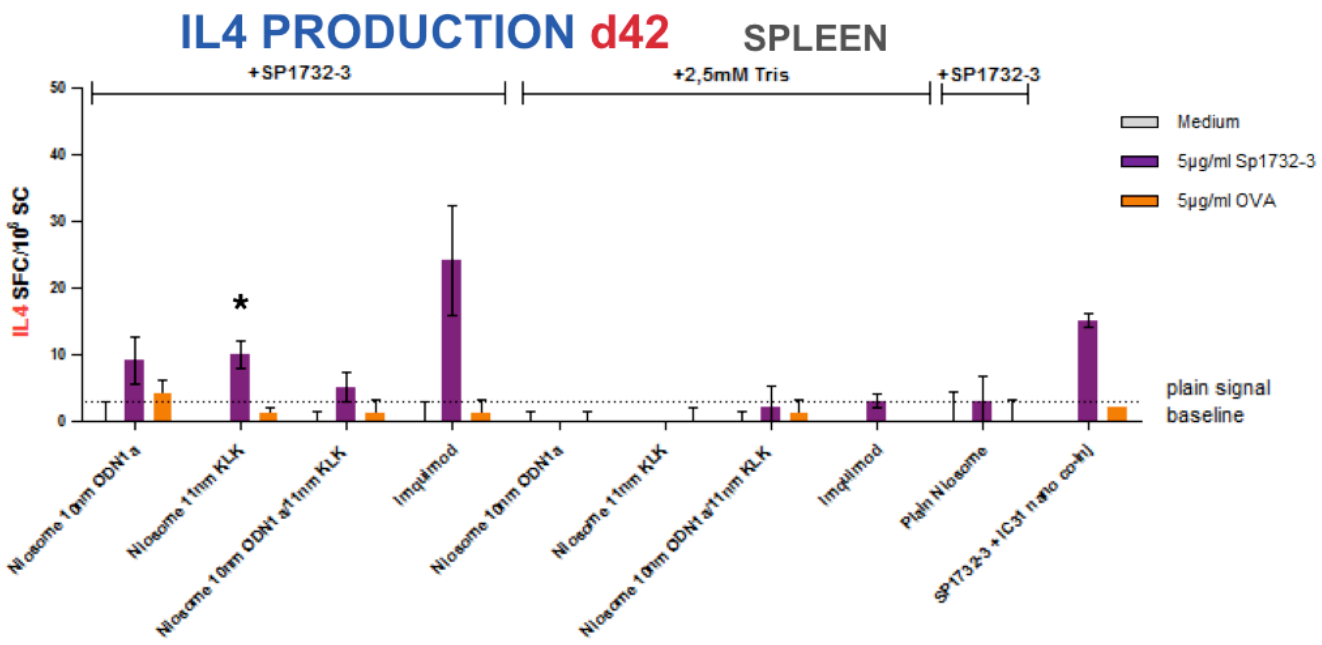


Figure 18B: IL4 Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

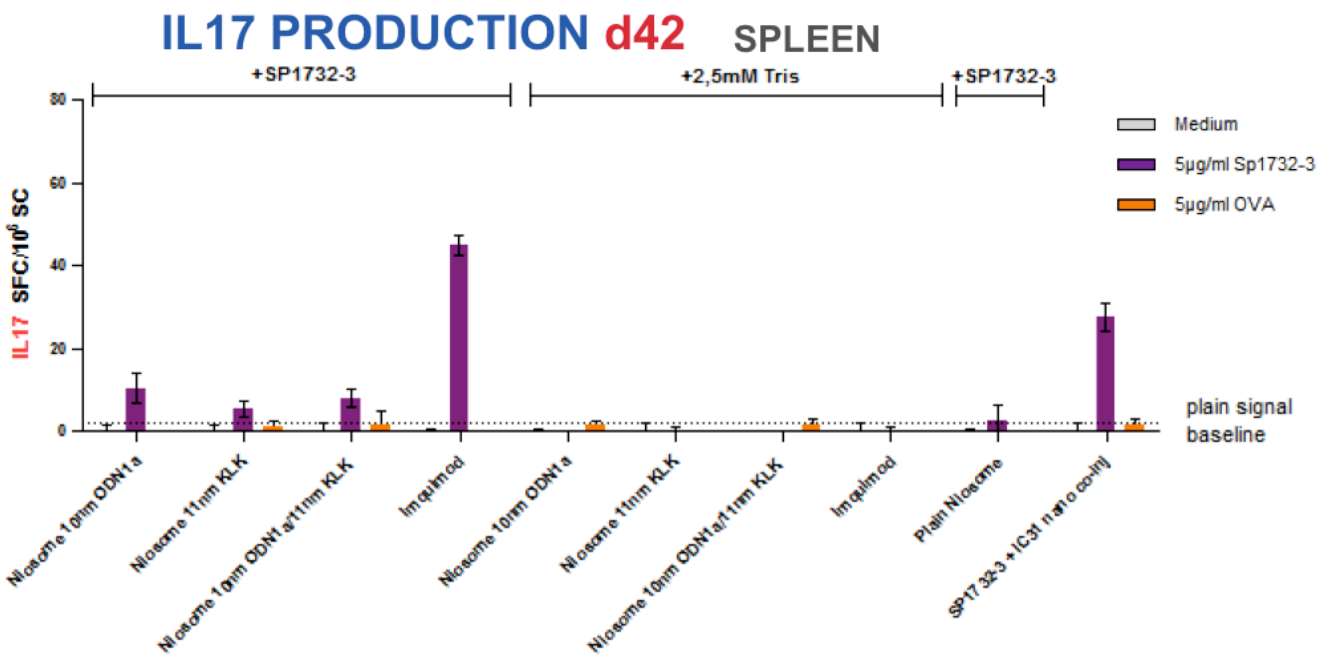


Figure 18C: IL17 Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the SP1732-3 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (SP1732-3) or irrelevant antigen (OVA) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

The IFN-γ ELISpot shows that only group 3, which was treated with a niosomal formulation with ODN1a inside and KLK adsorbed to vesicle surface, could elicit a significant positive signal above the baseline (see asterisk Figure 18A). These results stand in contrast to the corresponding gel experiment. There (see Figure 12A) none of the groups could produce a significant enough IFN-γ signal to be within a 95% confidence interval, tested with a student's T-test. In case of the IL4 SFUs, in both experiments (see asterisk in Figures 12B & 18B), the groups treated with KLK–only Niosomes were able to induce a significant Th2-type immune response ($p<0.05$). The results indicate that the gel formulations seem to work better here than the niosomal formulations. Overall Niosomes, which incorporate both KLK and ODN1a tend to induce a significant Th1-type response. This could be due to the fact that ODN1a as a synthetic TLR9 agonist dominates the scenery here, as it is securely delivered to the Langerhans cells via the Niosomes. If a KLK only gel formulation is used on the other hand, the immune response switches more to a Th2-type one, which goes along with the features known about KLK.[37] Still both responses don´t meet the magnitude of the s.c. injected IC31® control or the topically applied Imiquimod cream. Since SP1732-3, alias STkP, is potentially able to induce either a Th1- or a Th2-type T-cell response together with a Th17 one, such a diverse outcome was not surprising[86]. Unfortunately though, none of the tested topical niosomal IC31 component formulations was able to induce a significant IL17 response (shown in Figure 18C). As already shown in previous experiments, only the s.c injected controls and Imiquimod were able to induce a significant IL17 signal. Thus it seems unlikely that a vaccine based on niosomal

formulations, which contain the currently used amount of ODN1a and KLK, in combination with the used antigen would be able to deliver a high systemic protection against *Streptococcus pneumonia.*

6.2.4 BAP006-2 study with topical Niosome formulations batch 2

To ensure a wider range of tested antigen models, BAP006-2 (Intercell AG) was tested in an additional study. BAP006-2 is a homologue to oppAV (Periplasmatic oligopeptide-binding protein) of *Borrelia burgdorferi,* which is transmitted by infected ticks. OppAV has previously shown to be able to elicit a potent Th2-type immune response, when injected i.p. (intraperitoneal) in combination with Freunds complete adjuvant.[89]

For the BAP006-2 protein study 5 female C3H mice per group, age 6-8 weeks, were immunized at day 0, 14 and 28 with 30µg/mouse protein solution s.c. and topically treated with 35µl topically applied Niosome formulations of batch 2. Spleenocytes were gathered at day 42 after the first injection and T-cell assays were performed. Further experimental and group details are shown in Figure 19. As positive controls group 4 was injected with the antigen and treated topically with 1,25mg Imquimod. Additionally, Group 8 was injected s.c. with BAP006-2 coformulated with IC31® nano.

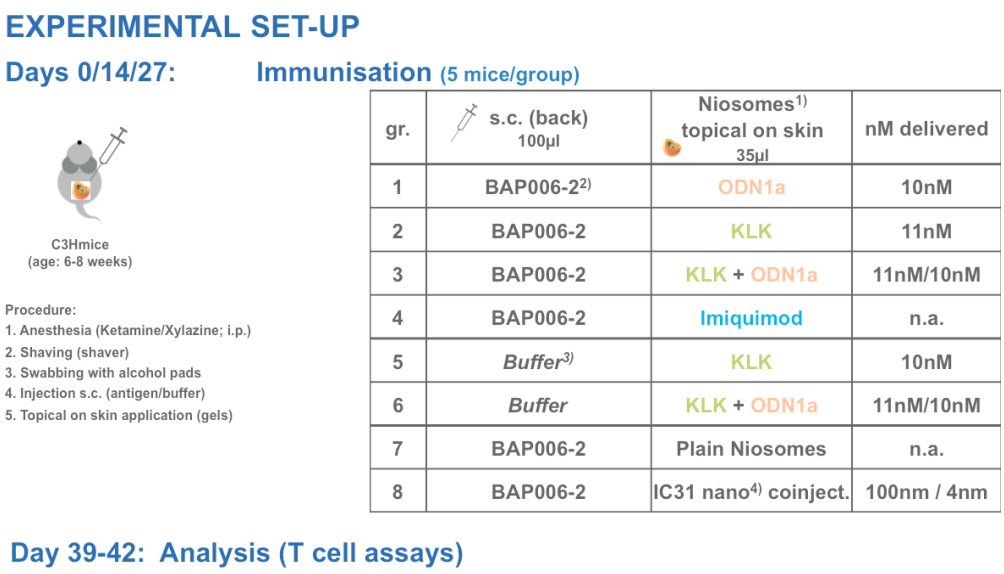


Figure 19: Experimental Setup of BAP006-2 protein study. 5 female C3H mice were injected s.c. at day 0, 14 and 28 with 30µg/mouse BAP006-2. 35µl/mouse corresponding niosomoal formulation was applied topically. As negative control buffer was injected instead of the antigen or plain Niosomes were used instead of IC31/IC31-single components containing Niosomes. As positive control BAP006-2 was coformulated with IC31 and injected s.c. without any topical application. As additional positive control, one group was injected with the antigen and topically treated with Imiquimod. T-cell analysis of the spleen cells was performed between days 39-42.

Figure 20 show the SFU production of the IL4 and IFN-γ ELISpots of Spleenocytes, after in vitro restimulation with either 5µg/ml BAP006-2 as relevant, or 5µg/ml OVA as irrelevant protein. Beside in the control groups, both IL4 and IFN-γ seemed to be produced in a low amounts. Only group 1 and 3 showed a statistically significant difference ($p<0.05$) to the selected baseline, above the background adjuvanting level of the Niosomes or antigen themselves. It seems unlikely though that ODN1a could elicit a potent Th2-type immune response, like it is suggested by this data. The IL4 signal of the group treated with KLK-only niosomal formulations is just too low to induce a significant immune response (see Figure 20B). Group 3 gave a borderline-significant signal though (see asterisk Figure 20B).

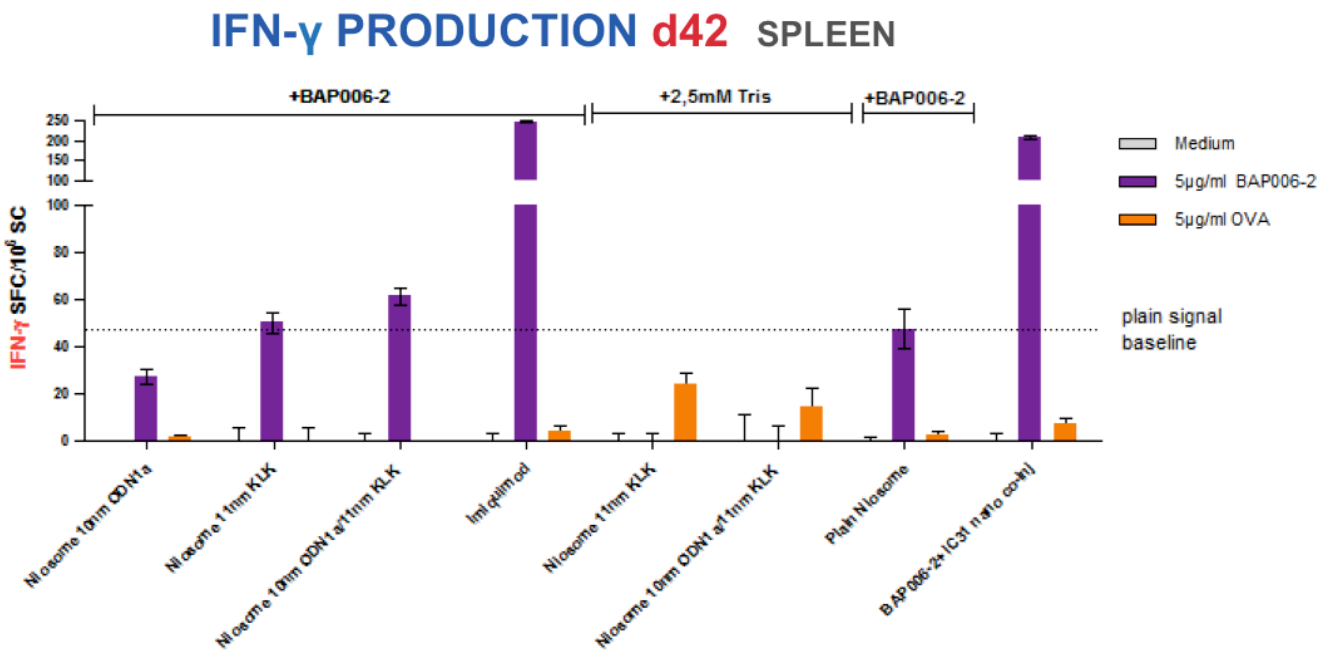


Figure 20A: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the BAP006-2 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (BAP006-2) or irrelevant antigen (OVA) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

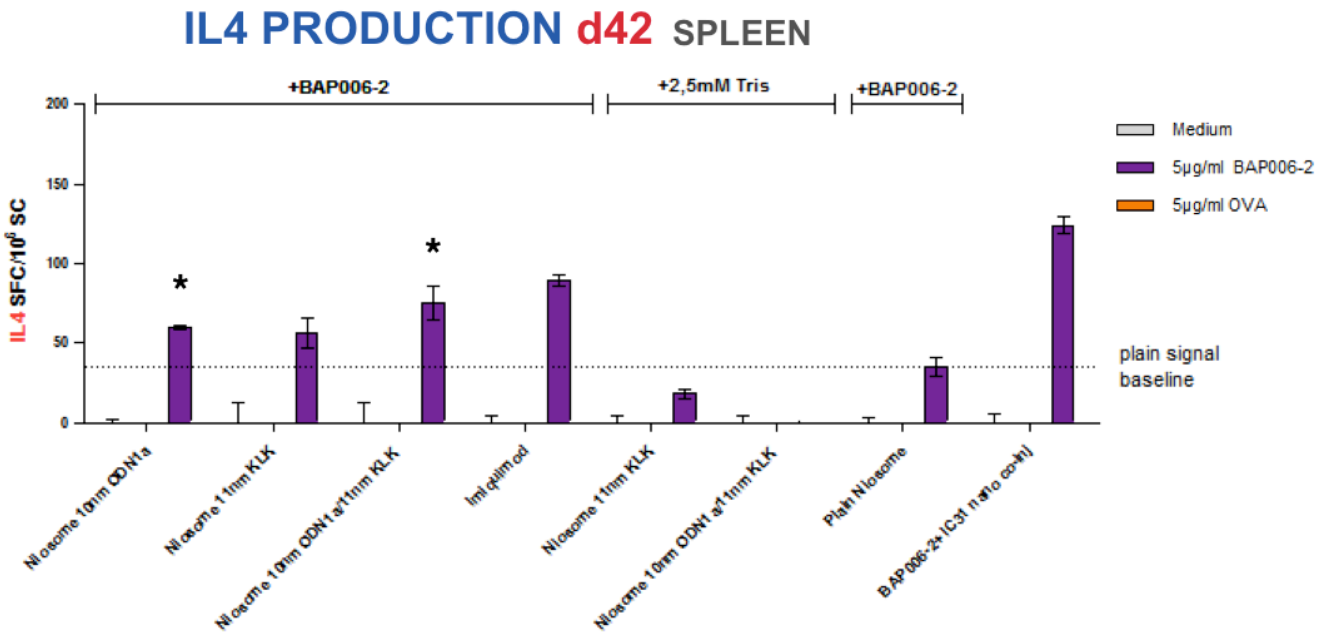


Figure 20B: IL4 Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the BAP006-2 protein study. $1x10^6$ spleen cells/well were plated and stimulated with either medium, relevant antigen (BAP006-2) or irrelevant antigen (OVA) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

Since the IL4 ELISpot assay was not fully conclusive here, different additional assays had to be performed. Figure 21 shows the result of a CD154/CD40L surface marker staining of CD4 T-cells in a spleen cell population. This staining can be used to identify CD4+ memory T-cells, which have the potential to promote mainly a Th2- but ultimately also a Th1-type immune response by differential cytokine secretion. CD154 is part of the second signal pathway used to activate APCs, as well as B-cells. The gating strategy, which involved 7´AAD viability gating, as well as further direct gating of the CD4+ population, is shown in detail in the methods section (chapter 4.2.5). During the data collection 500.000-1.000.000 total events were measured and the relative percentages of CD154+ CD4+ T-cells, out of the total CD4+ population, are displayed in the upper right quadrant of each dotplot. Each column represents a certain in-vitro stimulus, whereas *Staphylococcus aureus* enteroxin subunit B (SEB) was used as positive control and medium without FCS, as well as OVA as negative controls. The rows indicate the type of topical treatment the group received. The X-axis displays the CD154-APC-coupled staining, whereas the y-axis shows the CD4-AF488-coupled staining. The topically applied Imiquimod positive control showed a relative double positive population of 1.6%, whereas the direct s.c. IC31+Antigen coinjection reached only 0.52%, upon restimulation with 5µg/ml BAP006-2. Group 2, which was treated with KLK-only Niosomes, was the only one to reach above the background level of 0.34% double positive population (see red circle Figure 21).

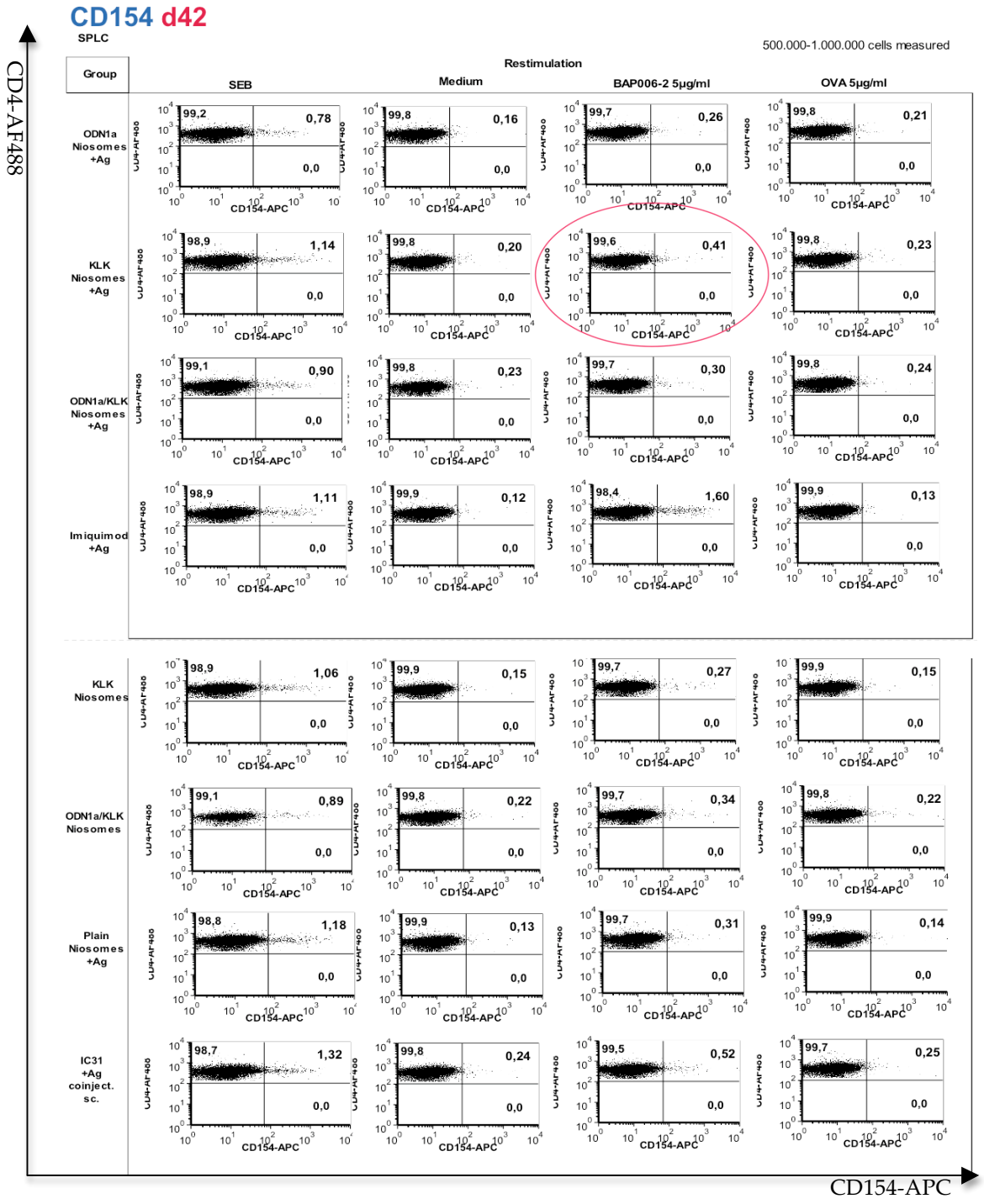


Figure 21: Flow cytometric CD154/CD40L surface staining of CD4+ T-cells. 50000-1000000 events of the spleen cell culture in the CD4+ gate were measured. The columns represent the stimulus each group received, whereas SEB was used as positive control and medium without FCS as well as OVA as negative controls. The rows describe the differently vaccinated groups. Each graphs x-axis represents a CD154-APC linked surface staining, whereas the two right quadrants describe CD154+ T-cells. The corresponding y-axis displays the CD4-AF488 linked staining, whereas the cells above the horizontal baseline are considered CD4+. The upper right quadrant resembles the CD4+CD154+ population which is also shown

in relative percentage of the overall CD4+ population.. The circled graph represents a small positive signal of group 2 above the general background upon in-vitro restimulation with relevant antigen.

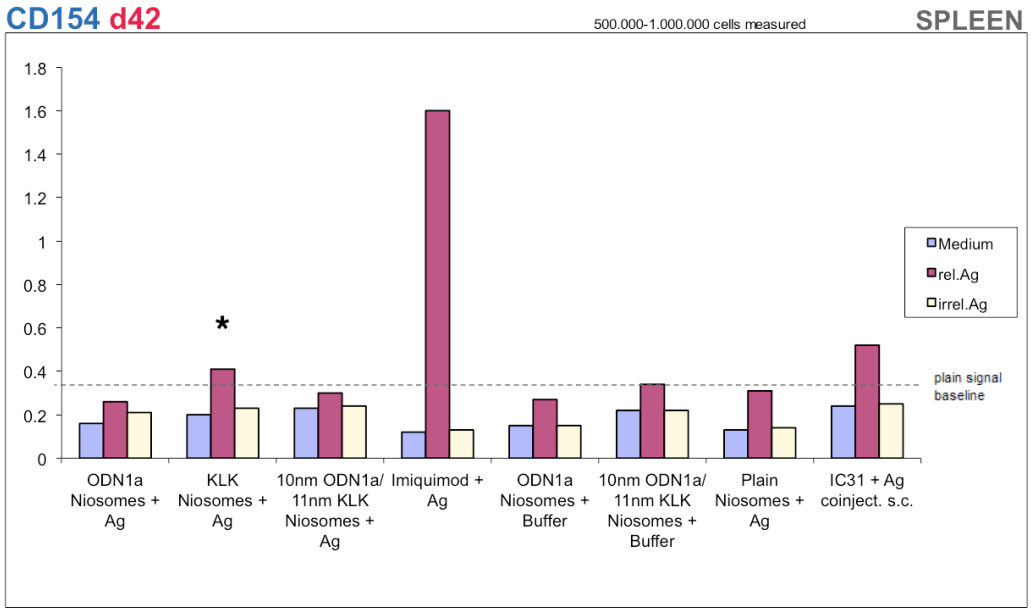


Figure 22: Flow cytometric CD154/CD40L surface staining of CD4+ T-cells visualized in a graph. 50000-1000000 events of the spleen cell culture in the CD4+ gate were measured. The columns represent the stimulus each group received. The x-axis represents the different groups, whereas the y-axis displays the relative percentage of CD4+ CD154+ T-cells out of all CD4+ cells. Groups 1-4 were treated with Niosomal formulation or Imiquimod in combination with s.c. antigen injections. Groups 5-7 received buffer injections instead. Group 8 received only s.c. injections without any topical treatment. Group 2 shows a small positive signal above the selected plain signal baseline background.

The same results are shown, visualized, in Figure 22 in a more comparable way. The slightly increased CD154/CD40L signal is an indicator for a stronger CD4+ memory cell activation, which ultimately leads to a class switch in B-cells from IgM to IgG type antibodies, therefore eliciting a "stronger" and more specific Th2- type immune response (see asterisk Figure 22).

In Figure 23 the results of a CFSE proliferation of the CD8+ T-cells are shown. This analysis indicates directly whether niosomal IC31 component formulations would be able to promote a stronger Th1-type immune response. Proliferating CD8+ T-cells, co-cultured with CD4+ T-cells, can be an effective indicator for protection against intracellular pathogens [93]. The assay was performed as already described in the methods section. Each column again represents the way of in-vitro restimulation in a similar way as in the CD154 staining. Instead of SEB, Concanavalin A (Con A) was used as a positive control for the proliferation assay. After five days of culturing, the cells were collected and measured in the FACS Calibur Flow cytometer. The total counts (y-axis) vs. CFSE intensities (x-axis) are displayed in the graphs. Each CFSE peak resembles a generation of T-cells, whereas the first generation has the highest CFSE intensity (most right), which then halves by each division. The Con A positive control underwent at least six to seven cell division cycles before being measured. Since the peaks could not be distinguished properly in the other columns a proliferative fraction of CD8+ T-cells was set beneath the red marker and displayed as relative percentage of the total living CD8+ T-cell population. With this data the stimulation indices of the columns, stimulated with relevant antigen and medium as control as well as irrelevant antigen and medium as control, were calculated and displayed in Figure 24.

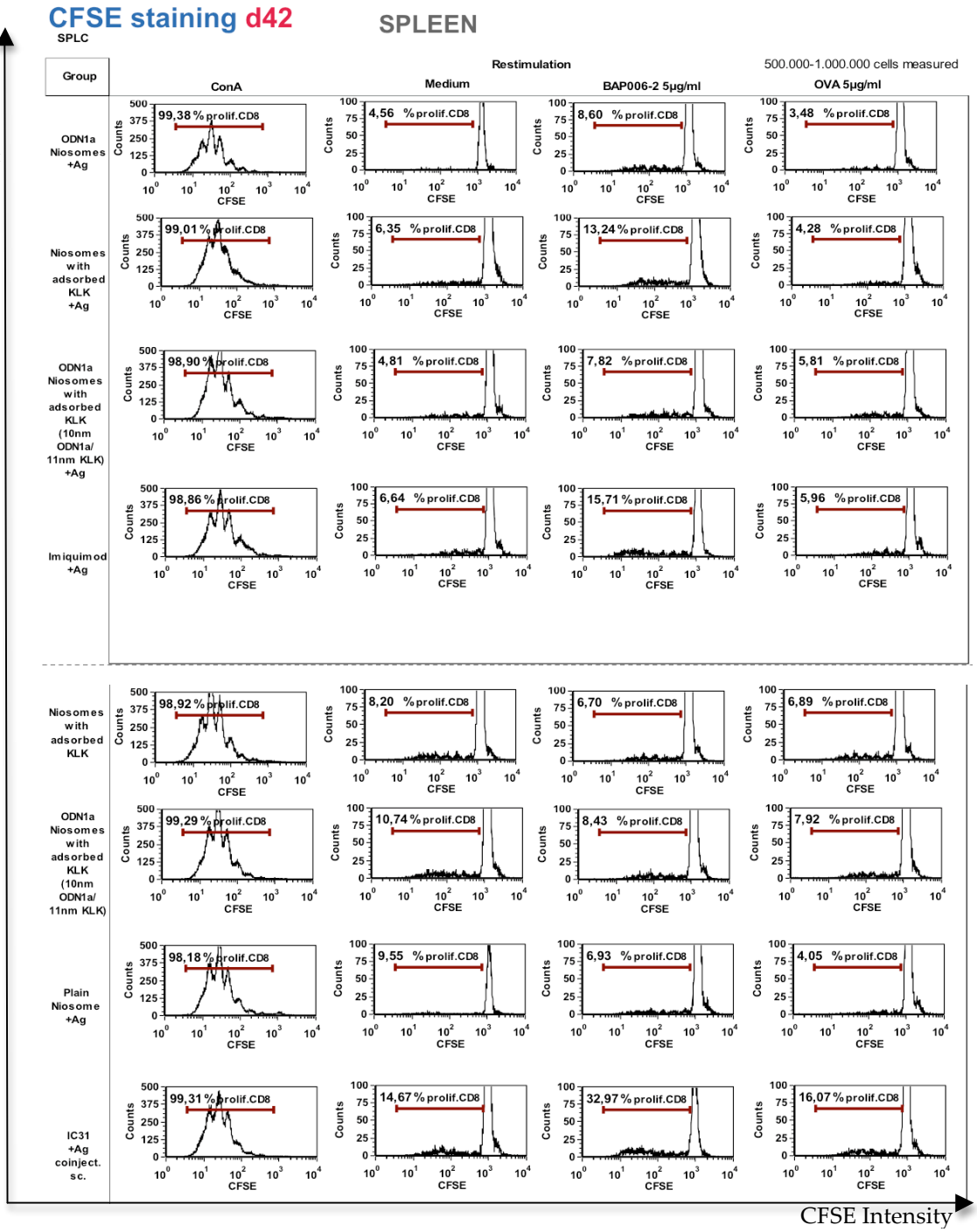


Figure 23: Flow cytometric CFSE proliferation staining of CD8+ T-cells. 500000-1000000 total events of the spleen cell culture were measured. The columns represent the stimulus each group received whereas Con A was used as positive control and medium without FCS as well as OVA as negative controls. The rows describe the differently vaccinated groups. Each graphs x-axis represents the intensity of the CFSE staining, whereas the events below the red marker show the proliferative fraction of the CD8+ T-cells. The corresponding y-axis displays the event count of gated CD8+ cells. The proliferative fraction is also shown as relative percentage of total CD8+ T-cells measured.

The stimulation indices (y-axis) shown in Figure 24 were calculated as described in the methods section and in the related literature [90]. The "plain signal baseline" was set at the highest background value of the irrelevant protein-restimulated Group 3.

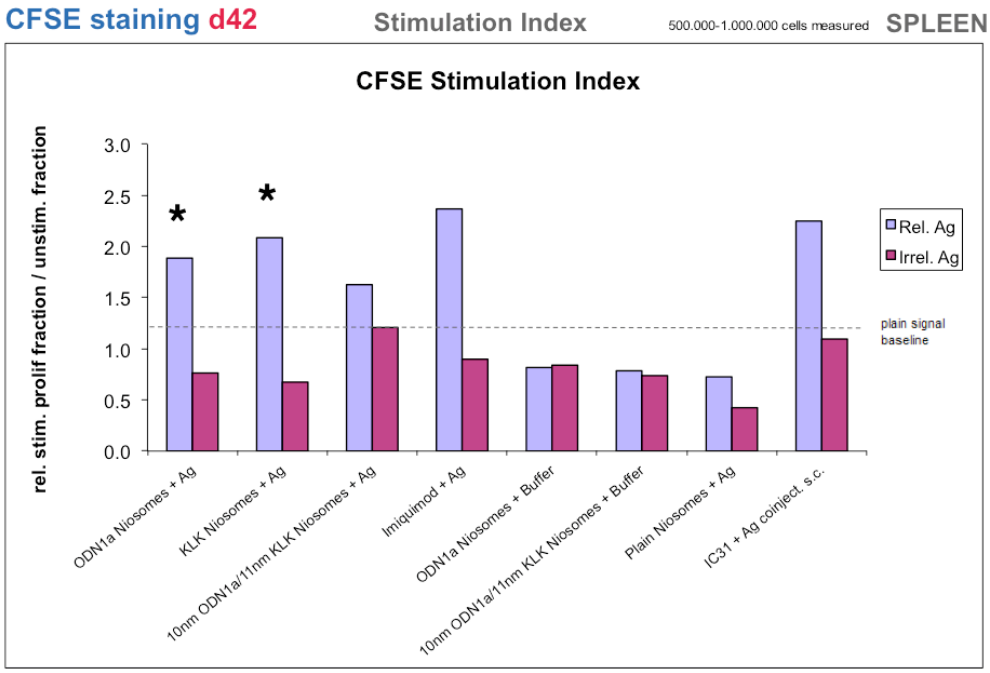


Figure 24: Flow cytometric CFSE proliferation staining Stimulation Index of CD8+ T-cells. 500000-1000000 total events of the spleen cell culture were measured. The columns represent the stimulation index of each group either calculated out of the relevant antigen restimulation together with the non-stimulated group or irrelevant antigen restimulation together with the non-stimulated group. The x-axis shows the different topical formulation used to treat the groups. Groups 1-4 received s.c. BAP006-2 injections. Groups 5 / 6 did receive buffer injections. Group 7 / 8 also received the antigen. The background signal baseline was set above the irrelevant antigen background signal.

The groups, which were treated with either KLK-only or ODN1a-only Niosomes as adjuvants, showed proliferation indices significantly above the baseline. In case of ODN1a-only formulations this makes sense, since ODN1a is known to promote a Th1-type immune response

as TLR9 agonist as described in literature [36]. Group 2, treated with KLK-only niosomal formulations, also showed a high increase of the CD8+ proliferation index in contrast to the baseline and especially its own group specific background. The stimulation index even almost reached up to the level of the positive controls, indicating a potential of the KLK-only Niosomes to elicit an immune response in combination with BAP006-2, which is not strictly restricted in either direction in the first place, but would most likely turn over into a specific Th1- or Th2-type response. The group treated with IC31® Niosomes (Group 3) was considered not significant, since the signal of the irrelevant restimulation almost reached the same stimulation index value.

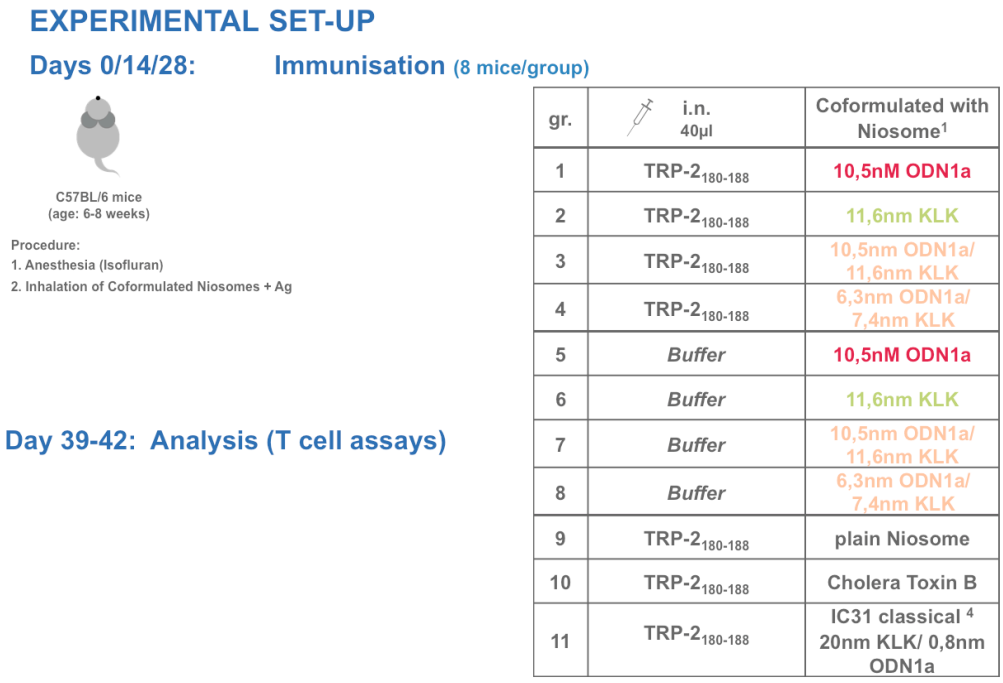
The ELISpot assays were not found to be fully conclusive enough in this case. The findings of the CD154 and CFSE assay stay in contrast to the IL4 or IFN-γ SFU data. Taking into consideration that the BAP006-2 homologue was already described as being able to elicit a specific Th2 response [89] and that KLK already showed the ability to provoke a, potentially, stronger immune response against protein antigens, it seems logical to believe in a false negative signal of the IL4 ELISpot SFU data of the KLK-only group since this group also showed an increased CD154+ CD4+ population within the spleenocytes, as well as more CD8+ proliferation when co-cultured with CD+4 cells. The ELISpot signals of the KLK/ODN1a Niosome Group 3 and the KLK-only Group 2 are more consistent throughout the whole range of experiments than ODN1a-only ones. Taking all this into consideration the KLK-only Niosomes still seem to be the best candidate to provoke both an increased Th1- or Th2-type systemic immune response specific to protein antigens like BAP006-2.

6.3 Intranasal niosomal formulation study

6.3.1 TRP-2$_{180-188}$ study with intranasal Niosome formulations batch 2

Besides the already tested topical skin application, Niosomes have also shown to be an effective delivery mechanism via the intranasal route (i.n.) [83]. Due to their vesicular structure they are able to provide increased protection and delivery efficiency as they fuse with the host cell membrane by endocytosis. Since the formulations showed the potential to deliver antigen more or less effectively across the skin, the intranasal route was picked as an alternative application pathway and tested with the melanoma peptide antigen TRP-2$_{180-188}$. 8 female C57BL/6 mice per group were immunized on day 0, 14 and 28. T-cell analysis of the spleens happened on day 42 after the first injection. In contrast to the topical application experiments, no antigens were injected s.c. All groups received co-formulations of the tested adjuvant system and 60µg/40µl/mouse TRP-2$_{180-188}$ solution. The mice were anaesthetized with Isofluran and 40µl of the vaccine preparations were applied intranasal with a pipette. Negative control groups received only adjuvant formulations co-formulated with 2.5mM Tris buffer to ensure the same concentration of IC31® components was delivered to all corresponding groups. Two different positive control groups were introduced into the experiment. First, instead of IC31® nano particle formulation, the in-house produced IC31® classical formulation (µm range particle size) was used, which also contains Tris buffer. In this group 20nM KLK/ 0,8nM ODN1a were delivered during every immunization. Second, 1µg *Vibrio Cholerae* Toxin subunit B (CTB) was coformulated with antigen and applied i.n. CTB was already used as a

potent adjuvanting system in vaccine studies with viral antigens and has shown to be able to induce a significant mucosal immune response when applied intranasal [44, 45]. Further experimental details, like actual adjuvant amounts (nM) delivered per mouse and a summary of the descriptions mentioned above can be found in Figure 25.



gr.	i.n. 40µl	Coformulated with Niosome[1]
1	TRP-$2_{180-188}$	10,5nM ODN1a
2	TRP-$2_{180-188}$	11,6nm KLK
3	TRP-$2_{180-188}$	10,5nm ODN1a/ 11,6nm KLK
4	TRP-$2_{180-188}$	6,3nm ODN1a/ 7,4nm KLK
5	*Buffer*	10,5nM ODN1a
6	*Buffer*	11,6nm KLK
7	*Buffer*	10,5nm ODN1a/ 11,6nm KLK
8	*Buffer*	6,3nm ODN1a/ 7,4nm KLK
9	TRP-$2_{180-188}$	plain Niosome
10	TRP-$2_{180-188}$	Cholera Toxin B
11	TRP-$2_{180-188}$	IC31 classical [4] 20nm KLK/ 0,8nm ODN1a

Figure 25: Experimental Setup of the intranasal TRP-2 $_{180-188}$ peptide study. 8 female C57BL/6 mice received i.n. vaccinations at day 0, 14 and 28 with 60µg/mouse TRP-$2_{180-188}$.40µl/mouse corresponding Niosome + antigen co-formulation was applied. As negative control buffer was used instead of the antigen. As positive control TRP-$2_{180-188}$ was co-formulated with either IC31 classical (µm range particle size) or 1µg *Cholera Vibriae* Toxin Subunit B (CTB). T-cell analysis of the spleen cells was performed between days 39-42.

The IFN-γ SFU production acquired by the ELISpot assay (see Figure 26) shows a very interesting outcome of the experiment. All groups treated i.n. with a combination of KLK containing Niosomes and antigen produced a significant Th1-type systemic immune response above the background level. The CTB positive control group also responded very strong in contrast to the other control group with classical IC31® in combination with the TRP-2 peptide. Between the previously mentioned Niosome-treated groups no significant difference could be detected, since the specific SFU counts differed too much. The immune response itself is dependent on KLK, since the ODN1a-only Niosomes were not able to elicit a similar response. The small differences between the group 2 and 4 can be explained by the general error of the ELISpot technique and can be seen as statistically equivalent. Group 3 shows a slight decrease here. The higher concentration of incorporated ODN1a seems to hinder a further increase of the signal.

To gain additional information about the immune response a CBA assay was performed with the collected supernatants (see Figure 27). This assay unveils, that groups 3 and 4 could not produce an increased level of Th2 or Th17 associated cytokines like IL6 IL10 or IL 17 (see Figure 27A, B & C).

Group 2 shows a very interesting immune profile in the ELISpot and CBA assays. It seems like the cytokine levels are all above the plain signal baseline. This niosomal formulation was able to elicit a broad immune response, whereas the Th1-type still seems to be dominant, as expected for the antigen.

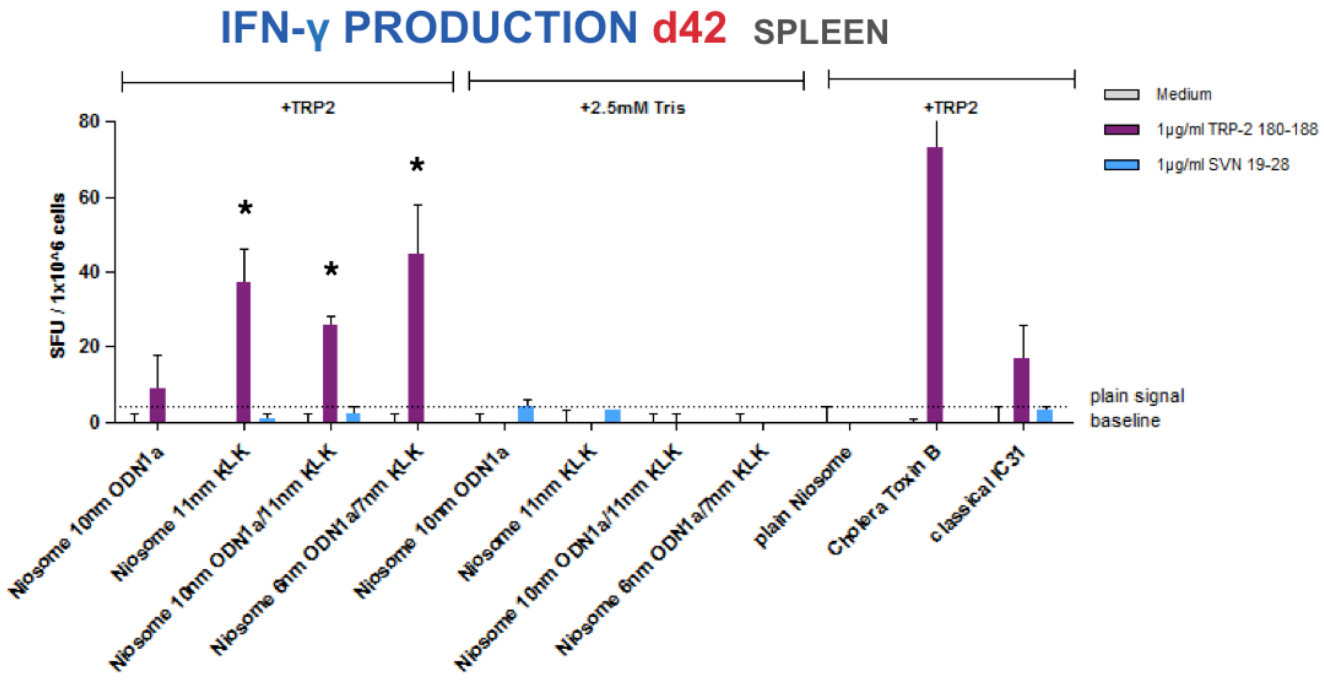
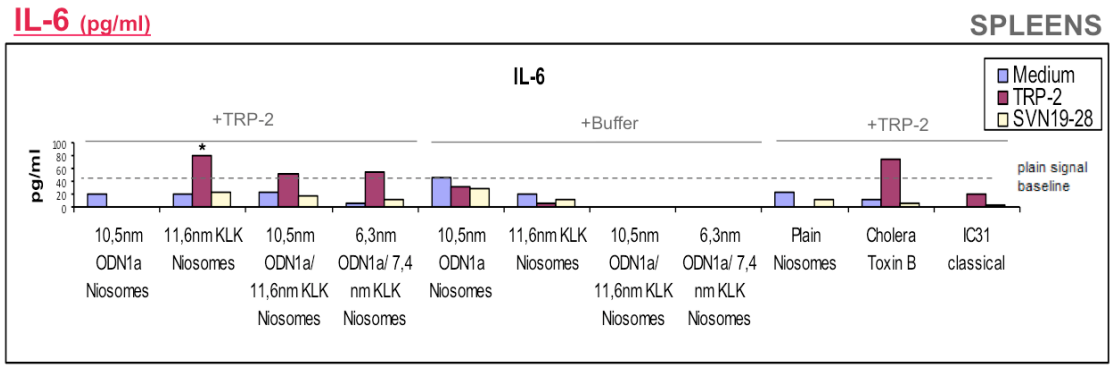
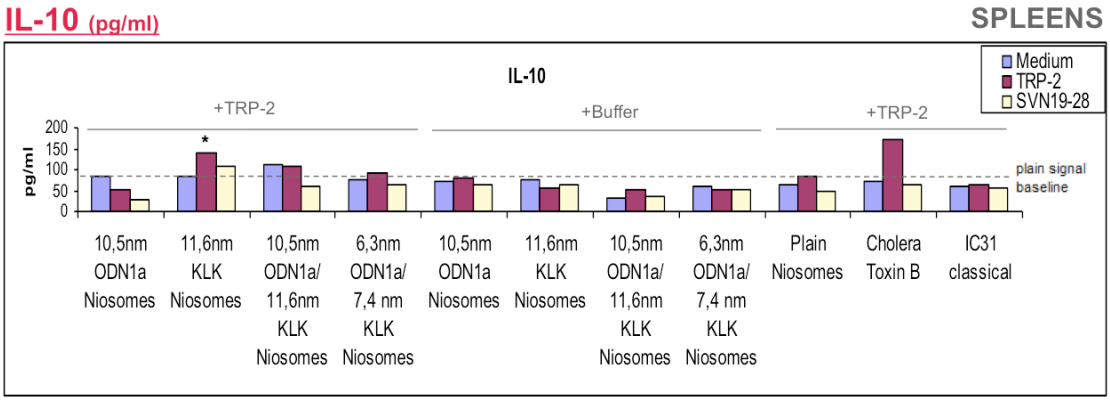


Figure 26: IFN-γ Net-Spot forming units (SFU) after corresponding medium-stimulated signal substraction of the intrasal TRP2 $_{180-188}$ peptide study. $1x10^6$ spleen cells/well were plated and stimulated for 24h with either medium, relevant antigen ($TRP\text{-}2_{180-188}$) or irrelevant antigen (SVN_{19-28}) and performed as described in the methods section. The y-axis shows the mean number of spots derived from triplicates (error bars indicate ± standard error of the mean (SEM)). The data is already medium corrected via Graphpad Prism 4 and a baseline together with its error marks the remaining background signal. Significance of the signals above the baseline was determined with a t-test with Microsoft Excel 2011 (95% confidence interval)(data not shown).

A



B



C

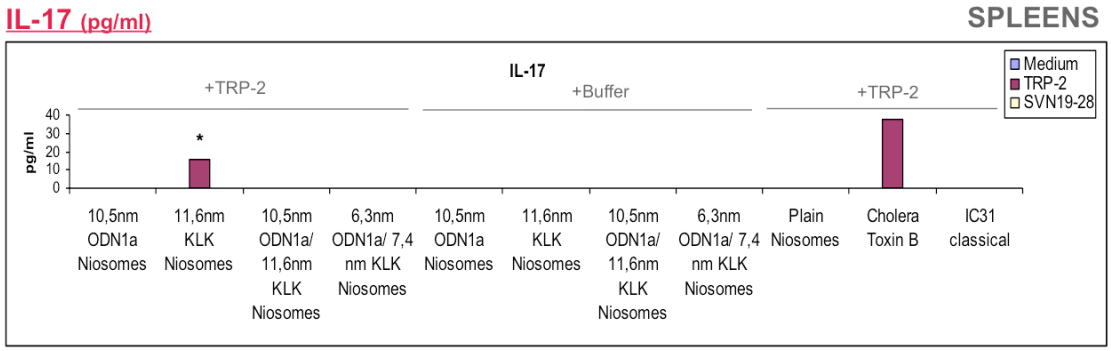


Figure 27: Cytokine concentrations (pg/ml) measured by Cytometric Beads Assay (CBA) of the intrasal TRP2 $_{180\text{-}188}$ peptide study. 1x10^6 spleen cells/well were plated and stimulated for 24h with either medium, relevant antigen (1µg/ml TRP-2$_{180\text{-}188}$) or irrelevant antigen (1µg/ml SVN$_{19\text{-}28}$) and performed as described in the methods section. Y-axis shows the absolut concentration of the corresponding Cytokine (IL6 in A; IL10 in B; IL17 in C).

The TRP-2 peptide has already shown to be a potent Th1-type immune response inducing antigen in previous experiment, but Figure 27 (asterisk marks) shows a significant increased IL6, IL10 and IL17 response, which could be due to the Th2-type immune response promoting abilities of KLK, or the different application route of the antigen. Another explanation could be that intranasal immunization in general could also elicit an increased Th-2 type response to ensure that not only CD8 T-cells but also secreted antibodies fight off a possible pulmonary pathogen [94]. In this case this seems unlikely though, since not all groups showed this effect. Thus a KLK-associated effect must be the driver of the additional Th2-type immune stimulatory effect.

In summary the KLK only containing niosomal formulation proved to be a broad adjuvant together with the used intranasal model peptide antigen. Groups 3 and 4 have also shown to be able to elicit a significant Th1-type (Figure 26) response, but only the KLK Niosomes were also able to induce a broader immune stimulatory effect. The TRP-2 melanoma peptide mainly requires a potent CD8+ T-cell response to be protected against, but wider activation of the immune system could proof useful as well if it does not exceed certain levels.

7. Discussion

7.1 IC31®/IC31® components topical Gel formulation studies

The tested formulations of IC31® and its single components, incorporated into a gel matrix generally gave suboptimal immune responses in comparison to the s.c. injected combination of antigen plus IC31®.

No significant systemic Th1-type immune response could be detected in combination with the determined CD8+ T-cell target melanoma peptide TRP-$2_{180\text{-}188}$ [85] (see Figure 8).

The model protein antigen used (SP1732-3 alias StkP) showed just a small, but not significant enough, immune response with the first batch of gel formulations used (see Figure 10). The fact that higher concentrations of the IC31® components could improve this signal was shown in the repeated experiment with a second, modified, batch of the same formulations. The gel formulations containing only KLK could elicit a potent systemic Th2-type immune response, characterized by an IL4 ELISpot assay (see Figure 12B). This is already a pivotal step in gaining protection against the pathogen and corresponds well with the known mechanisms of action of KLK in literature [37, 95]. It still seems unlikely though that this formulation could be used as an adjuvant to induce a protective immune response against the antigen, since no IL17 and therefore no Th17-type immunity was detected (see Figure 12C).

This type is characteristic as an defensive immunity against the antigen [86].

In general, ODN1a gel formulations showed no significant immune stimulatory effect, when applied topically on the skin. This could be due to the reason that ODN1a is a synthetic DNA-backbone-like structure and thus sensitive to nucleases, which are abundant on the skin [56]. Another explanation for the failure of the gel formulations to elicit a sufficiently strong systemic immune response, could be the fact that the transition time of the gel through the skin is simply too long (about 75 minutes for 80% of the gel; see Figure 4). Due to the slow transition, the depot formation effect of IC31, discovered by Schellack et al., is lost and too much antigen is already degraded by proteases or dispersed into the surrounding tissue [96].

7.2 IC31®/IC31® components Niosome formulation studies

In contrast to the gel formulations, which can widen pores in the *Stratum Corneum* of the skin, but still could not increase the uptake of the IC31 components, Niosomes are flexible vesicular structures. They can mediate the transport and the uptake of material across the layers of the skin [24]. The potential increase of uptake speed and the small size of the used vesicles (size between 150-180nm) served as rationales for this delivery technique. Beside this effect Baillie et al. found that, Niosomes are also able to increase material transfer across cell membranes, as a consequence potentially increasing the uptake of IC31® components into the cell via endocytosis [67].

Generally the Niosomes formulations were able to elicit stronger and more significant immune responses than the gel formulations throughout the studies. In particular this means a significant systemic Th1-type immune response against the melanoma peptide antigen TRP-$2_{180\text{-}188}$ (see Figures 14 & 16), which was however still three times lower than the positive controls. This appears logical, since the peptide is known to elicit a strong CD8 T-cell response [85]. Interestingly though, this was only observed in the groups treated with KLK-only Niosomes. KLK is known to mediate Th2-type responses [37]. It is possible that the niosomal-mediated uptake of KLK into the antigen presenting cells played a role in this shift of the immune response, or that KLK is able to provoke it on its own.

The streptococcal protein SP1732-3 study showed similar outcomes with the niosomal and the gel formulations (see Figures 10, 12A, 12B, 12C & Figures 18A, 18B, 18C). In contrast to the gel formulations, the IC31® – Niosomes, that contained ODN1a inside and KLK adsorbed to the surface and could elicit a significant IFN-γ, thus Th1-type immune response in the spleen cells (see Figure 18A). It seems that the protective effect of niosomal vesicles, in combination with the increased uptake mediated by the Niosomes themselves and KLK, lead to a functional and strong enough delivery of ODN1a that overcame obstacles like endonucleases, the skin and the cell membrane of antigen presenting cells. Further research has to be done to find out whether a Th1-type or Th2-type immune response alone would be sufficient to produce a protective vaccine against the *Streptococcus pneumonia* target protein SP1732-3. In the literature a potent Th17-type would be characteristic

beside one of the other types [87]. In this study no niosomal formulation was able to provoke a significantly higher IL17 SFU production, in contrast to the s.c. injection controls used (see Figure 18C). A further increase of IC31® material, delivered trough the skin, could potentially change this and get closer to the immune stimulatory effect of the IC31® co-injections or even the topically applied Imiquimod positive control cream.

The second protein antigen tested was BAP006-2 (*Borrelia burgdoferi*). Since the ELISpot data (shown in Figures 20A and 20B) was inconclusive here, further flow cytometric tests were performed and showed an increased proliferation of CD8 T-cells co-cultured with other T-cells in the groups treated with ODN1a-only or KLK-only niosomal formulations (see Figure 24). As recently described in literature by Lingnau et al., ODN1a, once delivered successfully and undegraded into the cells, is able to provoke a Th1-type immune response [36]. A proliferation of CD8+ cells induced by KLK seems to be unlikely at first, since KLK is known to elicit a characteristic Th2-type immune response [37]. The CD154 (CD40L) assay, portraying CD4 memory cell populations, brought more insight into the potential Th-1 immune response. The CD154 (CD40L+) CD4+ memory T-cell population was increased in the KLK-only niosomal formulation-treated groups (see Figure 22). Consequently it can be suggested, that a higher number of CD4 memory T-cells were also able, beside their B-cell activating function, to induce a higher CD8 T-cell proliferation, by secretion of IFN-γ and indirectly via activation of cocultured macrophages as found by Olazabal et al. [97]. It seems likely that higher concentrations of delivered KLK, via niosomal formulations, would be able to cause a

protective immune response against the *Borrelia burgdorferi* protein BAP006-2 (StkP). The currently tested batch showed a small but still suboptimal version of this.

Intranasal applied niosomal-like formulations have already shown to be very effective in delivering ODNs and other substances pulmonary. They were also able to elicit significant immune responses of different types, characterized by Interleukins and antibodies [82, 83]. The performed TRP-$2_{180\text{-}188}$ peptide study, showed a similar and very promising result. All used formulation variants, beside the ODN1-only, could elicit a strong and clearly significant Th1-type immune response characterized by IFN-γ ELISpot assays (see Figure 26). The failure of the ODN1a niosomal formulations to be effective enough could be due to the various reasons. Firstly, the physical difference of the vesicle used in contrast to the ones described in literature [82]. Secondly, it could be the concentration of the adjuvant or niosomal material used.

Clearly, in the intranasal study, all KLK-containing formulations were superior to the ODN1a-only ones. They could elicit not only a strong Th1-type immune response, but also showed a high potential in the CBA assays (see Figure 27). Here the KLK-only formulations also provoked increased levels of IL6, IL10 and IL17, suggesting a possible additional immune stimulatory effect of the adjuvant system towards a systemic Th2 or Th17-type immune response.

7.3 Topical application of IC31®

Summarizing the results of all IC31® studies performed, it seems that topically applied gel formulations and niosomal formulations have the potential to elicit a significantly stronger immune response against protein antigens compared to the background. Peptide antigens could only be significantly adjuvanted with niosomal formulations, when tested topically. Despite already very high concentrations of IC31® in gel formulations, they could not elicit an immune response close to the injection controls or topically applied Imiquimod control cream. Thus IC31® gel formulations barely show potential in the current configuration in comparison to s.c. injections or the synthetic topically applied TLR7 agonist Imiquimod. Further increase of the delivered IC31® component concentrations in Niosomes one the other hand, which could not be done during this study due to technical issues, might improve the immune responses and make them even more effective. Niosomal formulations of IC31® also showed highest potential, when applied i.n., to adjuvant the tested melanoma peptide antigen TRP-$2_{180\text{-}188}$ on a systemic level. Thus i.n. IC31® niosomal formulations are able to compete against other highly effective i.n. adjuvant systems like CTB.

7.3 Outlook

Additional intranasal have to be performed to confirm the effectiveness of niosomal IC31® formulations not only with peptides but also protein antigens. The experiments included in this study indicate already great potential for this use-case. Further studies have to be done to improve the topical effectiveness of the niosomal IC31® formulations. Especially the concentration of KLK, as main mediator of the provoked immune response, should be increased. ODN1a could also act here as stabilizer of the vesicular structures and additional mediator of the immune response. Alternative bi-layered vesicular topical delivery systems could be used to overcome the technical issues faced when incorporating higher KLK concentrations. The KLK amount, due to its membrane-destabilizing effect, could potentially be increased in Transferosomes, since they have proven to be more flexible than Niosomes. They also show a higher potential to deliver bigger amounts of incorporated agents into the cells via a similar mechanism of endocytosis. Additionally, Transferosomes show an even higher tendency to cross the skin barrier than Niosomes due to their deformability.[72, 98]

This study showed that Intercells´ IC31® niosomal formulations, once further improved, have the potential to create a non-invasive, potent and easy-to-use topical/intranasal adjuvant system that can boost immune responses to future vaccines and ultimately foster world-wide application by improving the acceptance of vaccines in the population.

8. Appendix

8.1 List of tables

Table 9: List of tables

8.2 List of figures

Table 10: List of figures

8.3 References

1. Bloch, H., Edward Jenner (1749-1823). The history and effects of smallpox, inoculation, and vaccination. Am J Dis Child, 1993. 147(7): p. 772-4.
2. Bohm, H., [On the history of smallpox vaccination]. Z Gesamte Hyg, 1964. 10(8): p. 576-82.
3. Dunn, P.M., Dr Edward Jenner (1749-1823) of Berkeley, and vaccination against smallpox. Arch Dis Child Fetal Neonatal Ed, 1996. 74(1): p. F77-8.
4. Huerkamp, C., The history of smallpox vaccination in Germany: a first step in the medicalization of the general public. J Contemp Hist, 1985. 20(4): p. 617-35.
5. The Vaccination History of Small-Pox Cases. Br Med J, 1902. 2(2166): p. 67-8.
6. Culbertson, C.G., F.B. Peck, Jr., and H.M. Powell, Duck-embryo rabies vaccine; study of fixed virus vaccine grown in embryonated duck eggs and killed with beta-propiolactone (BPL). J Am Med Assoc, 1956. 162(15): p. 1373-6.
7. Isomura, S., et al., A long-term follow-up study on the efficacy of further attenuated live measles vaccine, Biken CAM vaccine. Biken J, 1986. 29(1): p. 19-26.
8. Baxter, R., et al., Immunogenicity and safety of an investigational quadrivalent meningococcal ACWY tetanus toxoid conjugate vaccine in healthy adolescents and young adults 10 to 25 years of age. Pediatr Infect Dis J, 2011. 30(3): p. e41-8.

9. Eyigun, C.P., et al., A comparative trial of two surface subunit recombinant hepatitis B vaccines vs a surface and PreS subunit vaccine for immunization of healthy adults. J Viral Hepat, 1998. 5(4): p. 265-9.
10. Alikasifoglu, M., et al., Comparison study of the immunogenicity of different types and dosages of recombinant hepatitis B vaccine in healthy neonates. J Trop Pediatr, 2001. 47(1): p. 60-2.
11. Konishi, E., et al., Japanese encephalitis DNA vaccine candidates expressing premembrane and envelope genes induce virus-specific memory B cells and long-lasting antibodies in swine. Virology, 2000. 268(1): p. 49-55.
12. Akis, S., et al., Factors associated with parental acceptance and refusal of pandemic influenza A/H1N1 vaccine in Turkey. Eur J Pediatr, 2011. 170(9): p. 1165-72.
13. Malley, R., Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. J Mol Med (Berl), 2010. 88(2): p. 135-42.
14. Shorvon, S. and A. Berg, Pertussis vaccination and epilepsy--an erratic history, new research and the mismatch between science and social policy. Epilepsia, 2008. 49(2): p. 219-25.
15. Powles, T., et al., The long-term risks of adjuvant carboplatin treatment for stage I seminoma of the testis. Ann Oncol, 2008. 19(3): p. 443-7.
16. Omer, S.B., et al., Vaccine refusal, mandatory immunization, and the risks of vaccine-preventable diseases. N Engl J Med, 2009. 360(19): p. 1981-8.
17. Buonaguro, F.M., M.L. Tornesello, and L. Buonaguro, New adjuvants in evolving vaccine strategies. Expert Opin Biol Ther, 2011. 11(7): p. 827-32.

18. Vogel, F.R., Immunologic adjuvants for modern vaccine formulations. Ann N Y Acad Sci, 1995. 754: p. 153-60.

19. Kenney, R.T. and R. Edelman, Survey of human-use adjuvants. Expert Rev Vaccines, 2003. 2(2): p. 167-88.

20. Kool, M., et al., Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med, 2008. 205(4): p. 869-82.

21. Audibert, F., [Use of muramyl dipeptides in models of synthetic vaccines]. Boll Ist Sieroter Milan, 1985. 64(2): p. 95-102.

22. Skidmore, B.J., et al., Immunologic properties of bacterial lipopolysaccharide (LPS): correlation between the mitogenic, adjuvant, and immunogenic activities. J Immunol, 1975. 114(2 pt 2): p. 770-5.

23. Cluff, C.W., Monophosphoryl lipid A (MPL) as an adjuvant for anti-cancer vaccines: clinical results. Adv Exp Med Biol, 2010. 667: p. 111-23.

24. Schreier H, e.a., Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. Journal of Controlled Release, 1994. 30(1): p. 1-15.

25. Guo, J.X., Q.N. Ping, and T. Wu, [Transdermal delivery mechanisms of lecithin nanoparticles with cyclosporin A through mice skin]. Yao Xue Xue Bao, 2000. 35(10): p. 782-5.

26. Morein, B., Iscoms. Vet Microbiol, 1990. 23(1-4): p. 79-84.

27. Morein, B. and K.L. Bengtsson, Immunomodulation by iscoms, immune stimulating complexes. Methods, 1999. 19(1): p. 94-102.

28. Newman, M.J., et al., Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. J Immunol, 1992. 148(8): p. 2357-62.

29. Allison, A.C., Squalene and squalane emulsions as adjuvants. Methods, 1999. 19(1): p. 87-93.

30. Wang, Y., et al., The Toll-like receptor 7 (TLR7) agonist, imiquimod, and the TLR9 agonist, CpG ODN, induce antiviral cytokines and chemokines but do not prevent vaginal transmission of simian immunodeficiency virus when applied intravaginally to rhesus macaques. J Virol, 2005. 79(22): p. 14355-70.

31. El Andaloussi, A., et al., Stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors. Glia, 2006. 54(6): p. 526-35.

32. Mattner, F., et al., Vaccination with poly-L-arginine as immunostimulant for peptide vaccines: induction of potent and long-lasting T-cell responses against cancer antigens. Cancer Res, 2002. 62(5): p. 1477-80.

33. McKee, A.S., M.W. Munks, and P. Marrack, How Do Adjuvants Work? Important Considerations for New Generation Adjuvants. Immunity, 2007. 27(5): p. 687-690.

34. Aichinger, M.C., et al., Adjuvating the adjuvant: facilitated delivery of an immunomodulatory oligonucleotide to TLR9 by a cationic antimicrobial peptide in dendritic cells. Vaccine, 2011. 29(3): p. 426-36.

35. Aichinger, M., et al., Unique membrane-interacting properties of the immunostimulatory cationic peptide KLKL5KLK (KLK). Cell Biology International, 2008. 32(11): p. 1449-1458.

36. Lingnau, K., K. Riedl, and A. von Gabain, IC31 and IC30, novel types of vaccine adjuvant based on peptide delivery systems. Expert Rev Vaccines, 2007. 6(5): p. 741-6.

37. Fritz, J.H., et al., The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a TH2-type immune response to co-injected antigens. Vaccine, 2004. 22(25-26): p. 3274-84.

38. Riedl, K., et al., The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice. Vaccine, 2008. 26(27-28): p. 3461-8.

39. Cheng, C., et al., Induction of protection in mice against a respiratory challenge by a vaccine formulated with the Chlamydia major outer membrane protein adjuvanted with IC31(R). Vaccine, 2011. 29(13): p. 2437-43.

40. Kamath, A.T., et al., Adult-like anti-mycobacterial T cell and in vivo dendritic cell responses following neonatal immunization with Ag85B-ESAT-6 in the IC31 adjuvant. PLoS ONE, 2008. 3(11): p. e3683.

41. Blank, N., et al., Cholera toxin binds to lipid rafts but has a limited specificity for ganglioside GM1. Immunol Cell Biol, 2007. 85(5): p. 378-82.

42. Hansen, G.H., et al., Cholera toxin entry into pig enterocytes occurs via a lipid raft- and clathrin-dependent mechanism. Biochemistry, 2005. 44(3): p. 873-82.

43. Dawson, R.M., Characterization of the binding of cholera toxin to ganglioside GM1 immobilized onto microtitre plates. J Appl Toxicol, 2005. 25(1): p. 30-8.

44. Kim, H.J., et al., Intranasal vaccination with peptides and cholera toxin subunit B as adjuvant to enhance mucosal and systemic immunity to respiratory syncytial virus. Arch Pharm Res, 2007. 30(3): p. 366-71.

45. Prabakaran, M., et al., Protective immunity against influenza H5N1 virus challenge in mice by intranasal co-administration of

baculovirus surface-displayed HA and recombinant CTB as an adjuvant. Virology, 2008. 380(2): p. 412-20.

46. Huang, S.J., et al., Imiquimod enhances IFN-gamma production and effector function of T cells infiltrating human squamous cell carcinomas of the skin. J Invest Dermatol, 2009. 129(11): p. 2676-85.

47. Audisio, T., F.C. Roca, and C. Piatti, Topical imiquimod therapy for external anogenital warts in pregnant women. Int J Gynaecol Obstet, 2008. 100(3): p. 275-6.

48. Gill, V.L., et al., Use of imiquimod 5% cream (Aldara) in cats with multicentric squamous cell carcinoma in situ: 12 cases (2002-2005). Vet Comp Oncol, 2008. 6(1): p. 55-64.

49. Bernardini, B., et al., Imiquimod for the treatment of classical Kaposi's sarcoma. Acta Derm Venereol, 2010. 90(4): p. 417-8.

50. Huang, S.W., et al., Imiquimod simultaneously induces autophagy and apoptosis in human basal cell carcinoma cells. Br J Dermatol, 2010. 163(2): p. 310-20.

51. Greenberg, H.L., et al., Severe reaction to 5% imiquimod cream with excellent clinical and cosmetic outcomes. J Drugs Dermatol, 2007. 6(4): p. 452-8.

52. Akkilic-Materna, M., C. Massone, and P. Komericki, Imiquimod and lymphatic field clearance: a new hypothesis based on a remote immune action on skin cancer. Acta Derm Venereol, 2011. 91(4): p. 432-5.

53. Benbow, M., The skin. 1. Its structure and functions. Nurs Times, 2002. 98(25): p. 43-6.

54. Butnaru, C.A. and J. Kanitakis, Structure of normal human skin. Eur J Dermatol, 2002. 12(6): p. II-IV.

55. Menon, G.K., New insights into skin structure: scratching the surface. Adv Drug Deliv Rev, 2002. 54 Suppl 1: p. S3-17.

56. Tabachnick, J. and R. Freed, Demonstration of nucleases on mammalian skin surface and in saline extracts of hair. Nature, 1961. 190: p. 921-2.

57. Bos, J.D., et al., The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. J Invest Dermatol, 1987. 88(5): p. 569-73.

58. Merad, M., F. Ginhoux, and M. Collin, Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. Nat Rev Immunol, 2008. 8(12): p. 935-47.

59. Miller, L.S., Toll-Like Receptors in Skin. Advances in Dermatology, 2008. 24: p. 71-87.

60. Parker, V., Jet gun or syringe? A trial of alternative methods of BCG vaccination. Public Health, 1984. 98(6): p. 315-20.

61. Draghia-Akli, R. and A.S. Khan, In vivo electroporation of gene sequences for therapeutic and vaccination applications. Recent Pat DNA Gene Seq, 2007. 1(3): p. 207-13.

62. Dimache, G., et al., Intradermal typhoid vaccination in men by jet-injector. Immunological estimation by laboratory tests. Arch Roum Pathol Exp Microbiol, 1977. 36(3-4): p. 227-32.

63. Kim, S.A., et al., DNA vaccination against foot-and-mouth disease via electroporation: study of molecular approaches for enhancing VP1 antigenicity. J Gene Med, 2006. 8(9): p. 1182-91.

64. Matsuo, K., et al., Transcutaneous vaccination using a hydrogel patch induces effective immune responses to tetanus and diphtheria toxoid in hairless rat. J Control Release, 2011. 149(1): p. 15-20.

65. Cheng, J.Y., et al., Transcutaneous immunization by lipoplex-patch based DNA vaccines is effective vaccination against Japanese encephalitis virus infection. J Control Release, 2009. 135(3): p. 242-9.

66. Buchan, S., et al., Electroporation as a "prime/boost" strategy for naked DNA vaccination against a tumor antigen. J Immunol, 2005. 174(10): p. 6292-8.

67. Baillie, A.J., et al., The preparation and properties of niosomes--non-ionic surfactant vesicles. J Pharm Pharmacol, 1985. 37(12): p. 863-8.

68. Coderch, L., et al., The effect of liposomes on skin barrier structure. Skin Pharmacol Appl Skin Physiol, 1999. 12(5): p. 235-46.

69. Guo, J., Q. Ping, and L. Zhang, Transdermal delivery of insulin in mice by using lecithin vesicles as a carrier. Drug Deliv, 2000. 7(2): p. 113-6.

70. Shatalebi, M.A., S.A. Mostafavi, and A. Moghaddas, Niosome as a drug carrier for topical delivery of N-acetyl glucosamine. Res Pharm Sci, 2010. 5(2): p. 107-17.

71. El Maghraby, G.M., A.C. Williams, and B.W. Barry, Skin delivery of oestradiol from lipid vesicles: importance of liposome structure. Int J Pharm, 2000. 204(1-2): p. 159-69.

72. Prem N. Gupta, et al., Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. International Journal of Pharmaceutics, 2004. 293: p. 73-82.

73. Brandtzaeg, P., Mucosal Immunity: Induction, Dissemination, and Effector Functions. Scandinavian Journal of Immunology, 2009. 70(6): p. 505-515.

74. Fric, J., et al., Phenotype, immunological reactivity and cytokine production profile of Peyer's patch cells from mice immunized orally with allogeneic cells. Folia Biol (Praha), 2000. 46(3): p. 119-25.

75. Koornstra, P.J., et al., The Waldeyer ring equivalent in the rat. A model for analysis of oronasopharyngeal immune responses. Acta Otolaryngol, 1991. 111(3): p. 591-9.

76. Kuper, C.F., et al., Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study. Cell Tissue Res, 1990. 259(2): p. 371-7.

77. Kuper, C.F., et al., The role of nasopharyngeal lymphoid tissue. Immunol Today, 1992. 13(6): p. 219-24.

78. Hameleers, D.M., et al., An immunohistochemical study on the postnatal development of rat nasal-associated lymphoid tissue (NALT). Cell Tissue Res, 1989. 256(2): p. 431-8.

79. Csencsits, K.L., M.A. Jutila, and D.W. Pascual, Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. J Immunol, 1999. 163(3): p. 1382-9.

80. de Haan, A., et al., Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. Vaccine, 1995. 13(2): p. 155-62.

81. Yokoyama, Y. and Y. Harabuchi, Intranasal immunization with lipoteichoic acid and cholera toxin evokes specific pharyngeal IgA and systemic IgG responses and inhibits streptococcal adherence to pharyngeal epithelial cells in mice. Int J Pediatr Otorhinolaryngol, 2002. 63(3): p. 235-41.

82. Alcon, V.L., et al., Intranasal immunization using biphasic lipid vesicles as delivery systems for OmlA bacterial protein antigen and CpG oligonucleotides adjuvant in a mouse model. J Pharm Pharmacol, 2005. 57(8): p. 955-62.

83. Terzano, C., et al., Non-phospholipid vesicles for pulmonary glucocorticoid delivery. Eur J Pharm Biopharm, 2005. 59(1): p. 57-62.

84. Henderson, A., et al., Mucosal immunization with liposome-nucleic acid adjuvants generates effective humoral and cellular immunity. Vaccine, 2011. 29(32): p. 5304-12.

85. Kochenderfer, J.N., et al., Synergism between CpG-containing oligodeoxynucleotides and IL-2 causes dramatic enhancement of vaccine-elicited CD8+ T cell responses. J Immunol, 2006. 177(12): p. 8860-73.

86. Schmid, P., et al., Th17/Th1 biased immunity to the pneumococcal proteins PcsB, StkP and PsaA in adults of different age. Vaccine, 2011. 29(23): p. 3982-9.

87. Moffitt, K.L., et al., T(H)17-based vaccine design for prevention of Streptococcus pneumoniae colonization. Cell Host Microbe, 2011. 9(2): p. 158-65.

88. Wang, X.G., et al., Analysis of Differences in the Functional Properties of the Substrate Binding Proteins of the Borrelia burgdorferi Oligopeptide Permease (opp) Operon. Journal of Bacteriology, 2003. 186(1): p. 51-60.

89. Barbour, A.G., et al., A Genome-Wide Proteome Array Reveals a Limited Set of Immunogens in Natural Infections of Humans and White-Footed Mice with Borrelia burgdorferi. Infection and Immunity, 2008. 76(8): p. 3374-3389.

90. Rimaniol, A.C., et al., Evaluation of CD4+ T cells proliferating to grass pollen in seasonal allergic subjects by flow cytometry. Clin Exp Immunol, 2003. 132(1): p. 76-80.

91. Adams, S., et al., Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. J Immunol, 2008. 181(1): p. 776-84.

92. Giefing, C., et al., The pneumococcal eukaryotic-type serine/threonine protein kinase StkP co-localizes with the cell division apparatus and interacts with FtsZ in vitro. Microbiology, 2010. 156(6): p. 1697-1707.

93. Steven, R.F., Autologous CD4/CD8 co-culture assay: a physiologically-relevant composite measure of CD8+ T lymphocyte function in HIV-infected persons. J Immunol Methods, 2007. 327(1-2): p. 75-81.

94. Jones, H.P., et al., The pulmonary environment promotes Th2 cell responses after nasal-pulmonary immunization with antigen alone, but Th1 responses are induced during instances of intense immune stimulation. J Immunol, 2001. 167(8): p. 4518-26.

95. Trzcinski, K., et al., Protection against nasopharyngeal colonization by Streptococcus pneumoniae is mediated by antigen-specific CD4+ T cells. Infect Immun, 2008. 76(6): p. 2678-84.

96. Schellack, C., et al., IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. Vaccine, 2006. 24(26): p. 5461-5472.

97. Olazabal, I.M., et al., Activation outcomes induced in naive CD8 T-cells by macrophages primed via "phagocytic" and nonphagocytic pathways. Mol Biol Cell, 2008. 19(2): p. 701-10.

98. Xu, J., Y. Ding, and Y. Yang, Enhancement of mucosal and cellular immune response in mice by vaccination with respiratory syncytial virus DNA encapsulated with transfersome. Viral Immunol, 2008. 21(4): p. 483-9.

8.4 Curriculum vitae

Alexander Frühwirth

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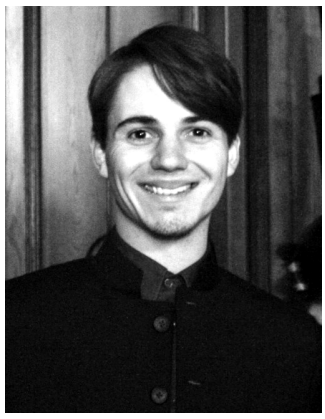
PROFILE



Name	Alexander FRÜHWIRTH
Date of Birth	02-03-1986
Marital Status	not married
Nationality	Austria

EDUCATION

2009 *January* → *July 2009*
Sweden, Lund University **Exchange Semester**
Master-Courses in Immunology with the Erasmus-Program of the European Union.

2005 *October* → *ongoing*
University of Vienna **Study Genetics/Microbiology**
Specializing in Genetics on Immunology/Virology.

2005 *October* → *ongoing*
University of Vienna **Study Anthropology**
Specializing in Anthropology on Human Genetics.

1996 *September* → *June 2004*
Konrad Lorenz Gymnasium Gänserndorf **High School Graduation**
Natural science high school graduation with distinction in the subjects Biology, Chemistry, Mathematics, German and English

EXPERIENCE

2010 *October* → *Feb 2012*

Tutor — **University of Vienna**

Tutoring students in the Laboratory Practical: Biochemistry II (Prof. BarbaraHamilton)

2010 *July* → *April 2011*

Master Thesis — **Intercell AG**

Profiling the immune reaction of the companies own adjuvant IC31 applied topically together with different antigens using various kinds of immunological state of the art techniques like intracellular , surface and proliferation flow cytometric stainings, ELISA, ELISPOT,... (Supervisor: Prof. Alexander von Gabain)

2010 *April* → *May 2010*

Research Assistant — **Singapore Immunology Network (SIgN) A*Star**

Research Assistant in the Singapore Immunology Network Department (SIgN) of the Agency for Science, Technology and Research (A*Star), studying the Subversion of Toll-like receptor signaling by Pathogens with the help of new virulence factors like TcpC via rt-PCR. (Principal Investigator: Dr. Catharina Svanborg)

2009 *December* → *June 2010*

Research Assistant — **Immunological Dpt. Baxter Bioscience**

Developing and characterizing a mouse model considering the brake of immune tolerance against blood coagulation factor VII, as well as performing different other kinds of experiments like RNA-isolation, PCR and ELISA. (Principal Investigator: Doz. Dr. Birgit Reipert)

2009 *August* → *October 2009*

Research Assistant — **Immunological Dpt. Baxter Bioscience**

10-weeks internship in the Animal models group establishing new and approved immunological methods on surveying Beta-Sheet Aggregates in pharmaceutical products, like FACS, Multiplex, ELISPOT, Thioflavin-T assay, tPA-activation/binding assay and ELISA. (Principal Investigator: Doz. Dr. Birgit Reipert)

2008 *July*
→ October 2008

Research Assistant — **Preclinical Research and Development, Baxter Bioscience**

10-weeks internship in the Biochemical Product Characterization department. Structural und functional analysis of different recombinant blood coagulation factors with the help of common and new developed immunological assays (Western Blot, SDS-Page, ELISA, Chromogenassays, Cell-based Assays…) (Principal Investigator: Dr. Katalin Varadi)

2007 *November*
→ April 2008

Laboratory support — **Ecowork Laboratories Consulting GmbH**

Supportive works in the laboratory.

2007

Maintenance — **Planpool**

Maintenance of disinfection systems in the spa- and wellness area.

2004 *June*

Event management — **Association for new Media Marchfeld**

Organisation and coordination of public youth events in the district of Gänserndorf, Lower Austria. Last event with 200people was localized in the event hall „Optimum Matzen".

2004 *October*
→ October 2005

Civil Service — **Lebenshilfe Niederösterreich**

Work with disabled people in a protected facility and driving service in Lower Austria.

AWARDS

& Stipends

2009 *November* — **Singapore International Pregraduate Award of A*Star Singapore (Agency for Science, Technology and Research Singapore)**

2009 *January* — **Erasmus Outgoing Stipend, European Union**

2009 *January* — **Top-Stipend for Outgoing Students, Lower Austria**

2006 *November* — **Top-Stipend for Students, Lower Austria**

ORGANISATIONS

ÖGGGT — Austrian association for genetics and gene technology

STV Biologie — Student representatives for biology

DB — Association for new Media Marchfeld

TZW — Diving Center Vienna

SOCIAL

& Volunteering

2009 *April* → *lasting* — **Erasmus Student Network**
Mentoring for exchange students in Vienna in the "buddy-network" program.

2008 *November* — **University of Vienna**
Applied "Reef Check" program in a coral reef in Dahab, Egypt.

2006 *October* → *lasting* — **Student representatives for Biology**
Tutoring and mentoring for students. Search committee for the professorship for "In Silico Genomics". Organization of information events.

2000 *June* → *lasting* — **Association for new Media Marchfeld**
Organization of private IT-Workshops.

LANGUAGES

English	Excellent in speech and writing, TOEFL certificate (112/120), Numerous language stays in e.g. New Orleans, Eastbourne, St. Barbara, Singapore and an exchange term in Lund, Sweden.
French	High school level
Swedish	Basics

CONFERENCES

attended

2009	**2nd Symposium on HAMLET and Tumor-Killing Proteins Lund, Sweden**
2010	**World Day of Immunology 2010, A*Star, Singapore**
2010	**4th Elsevier Vaccine and ISV Annual Global Congress, Vienna, Austria**
2011	**5th Semmering Vaccine Symposium, Baden, Austria**
2011	**3rd International PhD Workshop, IAI, Medical University Vienna, Austria**

INTERESTS

& Motivation

My main scientific interests are Immunology, Virology and Vaccine development. I am also engaging myself as a student representative in biology and in the “buddy-network” of ESN for international exchange students. International orientation and mobility is of great professional and even more personal importance to me. I enjoy very much being challenged by other people, be it at work, in a diverse team, provocative projects or in academic studies – I take great inspirations and satisfaction from such challenges and consider myself a downright communicative person.

8.5 Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich diese Diplomarbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Stellen meiner Arbeit, die dem Wortlaut oder dem Sinn nach anderen Werken entnommen sind, habe ich in jedem Fall unter Angabe der Quelle als Entlehnung kenntlich gemacht. Dasselbe gilt sinngemäß für Tabellen, Karten und Abbildungen.

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Weiters bin ich damit einverstanden, dass ein Exemplar meiner Diplomarbeit in der Bibliothek ausgeliehen werden kann.

Wien, am

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Unterschrift