



universität
wien

DIPLOMARBEIT

Evaluation of IC₃₁[®] as a vaccine enhancement patch using a protective
model antigen from a mucosal intracellular pathogen

angestrebter akademischer Grad:

Magister der Naturwissenschaften (Mag. rer. nat.)

Autor:	Christian Schenk
Matrikelnummer:	9271373
Studienrichtung:	Molekulare Biologie
Studienkennzahl:	A490
Betreuer:	Benjamin Wizel, Ph.D. & O. Univ.-Prof. Dr. Alexander von Gabain
Institut/Firma:	Intercell AG

Wien, im November 2010

Abstract

Yet, most common vaccines are injected into the deltoid muscle involving the use of needles. Safety concerns have driven research to make use of different immunization routes. The human skin constitutes an immunological rich environment possessing major antigen presenting cells like dendritic cells and Langerhans cells, which are capable of presenting foreign antigen to T and B cell precursors by migration to regional draining lymphnodes. Comprising safe and easy application with potent immunological features of the skin represents an attractive target for future vaccines.

IC₃₁[®] a two component novel adjuvant inducing a mixed Th₁/Th₂ immune response to co-administered antigen is one of only a few adjuvants with an acceptable safety profile capable of inducing CD8⁺ T cell responses.

This thesis now sought to evaluate IC₃₁[®] administered via a patch onto pre-treated murine skin to adjuvant immune responses to a T cell model antigen CopN from *Chlamydia pneumonia*.

I could not detect any adjuvant effects of IC₃₁[®] applied as wet-patch in various formulations and also as single components eliciting detectable CD8⁺ T cell responses to co-injected antigen in multiple experiments. Even co-formulation of IC₃₁[®] with antigen on patch failed to induce noticeable CTL responses.

Analysis of compatibility between IC₃₁[®] and the wet-patch revealed that IC₃₁[®] is adsorbed to adhesive backing of the patches.

An alternative strategy involved rubbing IC₃₁ directly into pre-treated skin without a the use of a patch, which in one experiment lead to significant T cell responses.

Zusammenfassung

Zum status quo werden zahlreiche Impfstoffe mit Hilfe einer Injektionsnadel in den deltoiden Muskel verabreicht, was Risiken in sich birgt. Wissenschaftliche Bemühungen richten sich danach neue Immunisierungsrouten zu erforschen. Die menschliche Haut ist ein immunologisch reichhaltiges Organ, das potente Immunzellen, die dendritischen und Langhans Zellen besitzt. Diese Zellen stellen potente Antigen-präsentierende Zellen dar, welche Fremdanigene durch Migration zu den regionalen Lymphknoten T und B Zellvorläufern präsentieren können.

Die Haut verbindet Einfachheit und Sicherheit der Applikation mit optimalen immunologischen Eigenschaften und ist daher ein attraktives Ziel für zukünftige Impfstoffe.

IC₃₁[®] ist ein neuartiger Adjuvant aus zwei Komponenten, welcher eine gemischte Th₁/Th₂ spezifische Immunantwort zu einem koapplizierten Antigen induzieren kann. IC₃₁[®] ist einer der wenigen verfügbaren Adjuvanten, welcher die Eigenschaft besitzt CD8⁺ T Zellen zu aktivieren und zusätzlich auch ein akzeptables Sicherheitsprofil aufweist.

Meine Diplomarbeit befasst sich nun damit die immunpotenzierende Wirkung von IC₃₁[®] zu evaluieren, wenn es als feuchtes Impfpflaster auf vorbehandelte Haut von Mäusen appliziert wird. Als Modellantigen dient CopN, welches vom intrazellulär lebenden Pathogen *Chlamydia Pneumoniae* stammt.

In mehreren Versuchen konnte ich keine immunmodulierende Wirkung von IC₃₁[®] in verschiedenen Pufferformulierungen oder durch seine Einzelkomponenten feststellen, wenn es durch ein feuchtes Impfpflaster zu einem zuvor injizierten Antigen verabreicht wurde. Sogar eine Koformulierung von IC₃₁ und dem Antigen appliziert als Pflaster führte zu keiner veränderten Wirkung.

Bei einer Analyse der Kompatibilität von IC₃₁[®] mit dem Impfpflaster stellte sich heraus, dass IC₃₁[®] von der adhesiven Abdeckung des Pflaster adsorbiert wird.

Als alternative Strategie wurde IC₃₁[®] ohne Hilfe eines Pflasters direkt auf die vorbehandelte Haut appliziert, was in einem Experiment zu einer signifikanten T Zell Aktivierung führte.

Acknowledgments

Especially I want to thank my advisor, Benjamin Wizel PhD, for giving me the opportunity to work on this very diploma thesis, involving diversified work at the mouse house and performing a large spectrum of immunological assays in the laboratory. The time at Intercell was a great experience for me which radically improved my technical and intellectual scientific skills. Although the proof-of-concept study with IC₃₁ on patch failed in multiple experiments, Ben did an excellent job encouraging me and coming up with new approaches to solve this particular problem. Thank you for this great support.

Olga Lorenz PhD, technical assistant, did a great job introducing my colleague diploma student and me to the technical work with the mice and performance of the various immunological assays. She was always available, when questions came up or immediate help was needed.

Petra Schlick PhD, staff scientist, acted as great consultant, providing me with feedback concerning my results and sharing thoughts.

Dominik Aschenbrenner, my colleague diploma student, was a great partner working with, sharing thoughts and supporting each other.

Special thanks go to the Senior Vice President & Head of Pre-clinical Research, Eszter Nagy and Intercell founder and now Strategic Advisor to the board, Alexander von Gabain, who both despite their essential function for the company took their time to provide sophisticated feedback to all diploma and Ph.D. students at the monthly student seminars and were always available for consultation.

Finally I want to thank the IDM Team for various technical assistance, the AFD team for providing me with IC₃₁[®] stocks, Markus Hanner and the MMB/PEP team for providing rCopN and the Intercell USA team for all patch related information and material.

Table of contents

ABSTRACT	2
ZUSAMMENFASSUNG	3
ACKNOWLEDGMENTS	4
TABLE OF CONTENTS	5
1 INTRODUCTION	6
1.1 SKIN IMMUNOLOGY	6
1.2 TRANSCUTANEOUS IMMUNIZATION	6
1.3 PATCH TECHNOLOGY	8
1.4 ADJUVANTS	9
1.5 IC31®	10
1.6 EXPERIMENTAL BACKGROUND	11
1.7 THESIS AIMS	11
2 RESULTS	13
2.1 ADJUVANTICITY OF IC31® APPLIED MUCOSALLY	13
2.2 ADJUVANTICITY OF IC31® APPLIED ON PATCH	16
2.3 ADJUVANTICITY OF ETEC-LABILE TOXIN APPLIED ON PATCH	21
2.4 ADJUVANTICITY OF SINGLE IC31® COMPONENTS APPLIED ON PATCH	23
2.5 ADJUVANTICITY OF rCopN CO-FORMULATIONS WITH IC31®, KLK OR LT APPLIED ON PATCH	25
2.6 RETENTION OF IC31® ON THE PATCH	28
2.7 ADJUVANTICITY OF IC31®, SINGLE IC31® COMPONENTS AND LT RUBBED ON SKIN	30
2.8 ANTIBODY-RESPONSE TO INTRANASALLY, PATCH-APPLIED AND SKIN-RUBBED IC31®	34
3 DISCUSSION	38
3.1 GENERAL	38
3.2 PROBLEMS INVOLVING THE RUBBED-INTO-SKIN APPROACH	39
3.3 WHAT ARE POTENTIAL REASONS FOR A LACK OF IC31® ADJUVANTICITY APPLIED ON SKIN?	39
3.4 SUGGESTIONS FOR FURTHER EXPERIMENTS	40
3.5 ALTERNATIVE DELIVERY STRATEGIES	40
4 MATERIALS AND METHODS	42
4.1 VACCINATION INGREDIENTS	42
4.2 MODE OF VACCINATION	42
4.3 MICE	43
4.4 CELL ISOLATION AND PREPARATION	43
4.5 BLEEDINGS	43
4.6 IMMUNOLOGICAL ASSAYS	44
5 REFERENCES	46

1 Introduction

Injection into the deltoid muscle constitutes a widely used route for immunization. Great blood supply in the muscle allows quick systemic spreading. Furthermore the mode of application is easy and large volumes can be injected.

In recent years research sought to make use of different routes. The human skin, covering about 2m^2 constitutes an excellent target for novel vaccines.

1.1 Skin immunology

Numerous bacterial and viral pathogens enter the body via the skin, which therefore constitutes an immunologically rich environment presenting an attractive target for immunization.

The skin acts as a physical and chemical barrier, impermeable to most substances. The barrier function is realized by the uppermost layer of the epidermis, the stratum corneum. Keratinocytes differentiating from deeper strata finally into corneocytes filled with keratin are forming flat stacks of dead cells. Lipid compounds act like a glue between the corneocytes.

In the underlying strata of the epidermis Langerhans cells (LCs) are interconnected by their long dendrites forming a tight network of immune system sentinels. These cells are professional antigen presenting cells in an immature state. Once they prime foreign antigen, they mature and migrate via lymphatic vessels in the underlying dermis to the regional draining lymphnodes, presenting the antigen to T cell precursors.

The underlying dermis is dominated by connective tissue comprising bundles of collagen fibers containing blood and lymphatic vessels. Immature dermal dendritic cells (DCs) are capable of migrating to the regional draining lymphnodes for antigen presentation.

1.2 Transcutaneous immunization

Safety concerns have driven research to needle-free vaccine application routes. Injection involves danger of blood-borne diseases due to re-use of needles or improper disposal and requires trained personnel for application. Furthermore quick access to the blood flow bears the danger of a systemic shock. The advantage of transcutaneous

immunization relies on its easy and safe administration route to an immunological rich environment. (1)

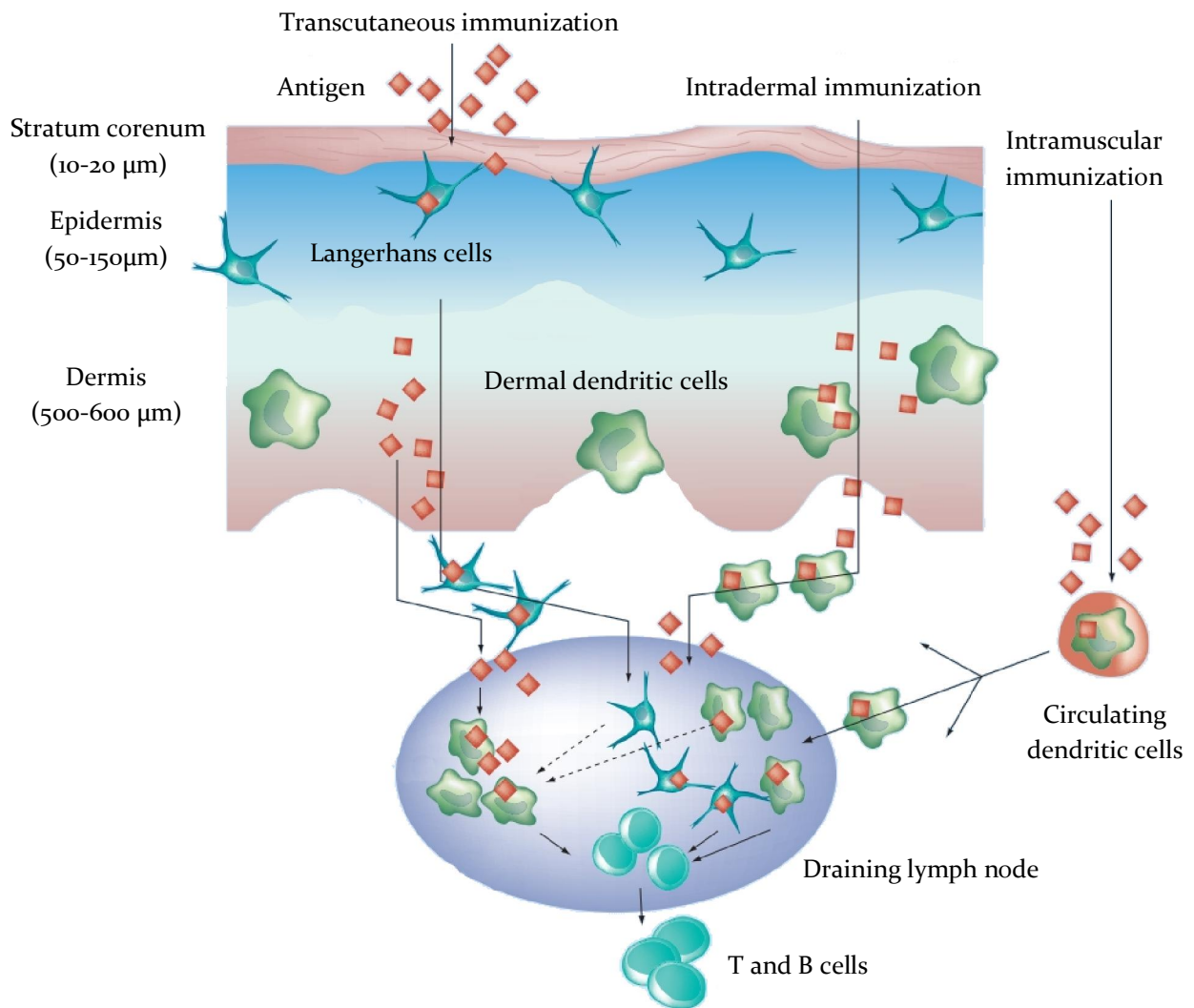


Figure 1: Antigen delivery via transcutaneous, intradermal and intramuscular immunization targets antigen-presenting cells (APCs), i.e. Langerhans cells and dendritic cells. The APCs migrate via lymphatic vessels to regional draining lymphnodes, where they present the antigen to T and B cell precursors.¹

Transcutaneous immunization bypasses gastrointestinal degradation and hepatic first pass elimination. Patient compliance is dramatically improved in case of trypanophobic² vaccinees, due to a needle-free approach including eliminated

¹ Illustration adopted from Nicolas and Bruno, 2008 was modified.

² Def.: fear of medical procedures involving injections

injection site pain. On a global scale speedier and safer vaccination reducing the number of trained healthcare personnel is a major goal of the WHO in regard of mass vaccinations. (2)

Different approaches targeting the skin for immunization currently exist. Electrical procedures involve low-frequency ultrasound sonication (3-7), iontophoresis or electroporation (8-10). Physical approaches try to conquer the stratum corneum by a microneedle array (11-14) or physical removal with abrasive tapes. Chemical vehicles comprise liposomal gels, ethosomes or transferosomes. (15-23)

1.3 Patch technology

Enterotoxigenic *Escherichia coli* (ETEC) causes travellers' diarrhoea. ETEC labile toxin (LT), the heat-labile enterotoxin sharing 82% sequence homology with cholera toxin (CT) has shown to enhance systemic and mucosal immune responses to co-administered antigens, when given via the nasal or oral route. (24) However toxicity of LT given via the nasal route is unacceptable and therefore safer transcutaneous delivery was suggested. (25)

The former Iomai Corp. developed a novel vaccine delivery system using a patch. The procedure involved removal of stratum corneum with an abrasive device, like adhesive tape, emery paper or pumice swab. Then antigen and adjuvant were simply pipetted in liquid form onto a patch-gauze, backend by an adhesive overlay and placed over the pretreated skin. The patch was worn for several hours and then removed. For practical use a dried solid-state patch-format was implemented later on, making use of evaporating water of the skin to dissolve the patch-contents. This way high physical and thermo-stability are achieved. (26) Combined with a novel Skin Prep System (SPS), an easy to use abrasive device subsequent immunization lead to sustained anti-LT IgG antibody titers. (27)

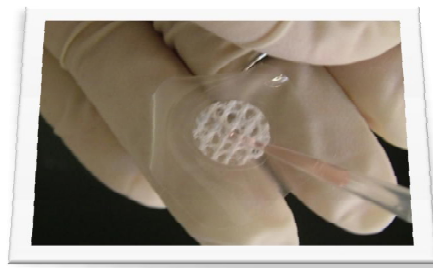


Figure 2: A wet-patch being applicated with vaccine.



Figure 3: A Skin-Prep-System device and a LT dry-patch.

A human clinical trial revealed that LT administered via patch induced a 97% seroconversion with high levels of anti-LT IgG and IgA and therefore a systemic and also mucosal response. (25) Another Phase II field trial affirmed a protective efficacy of 75% against travellers' diarrhoea in endemic countries.(28)

Iomai Corp. was overtaken by Intercell AG in 2008. At present a travellers' diarrhea vaccine patch is in a phase III study. A pandemic influenza vaccine patch, which acts as a vaccine enhancement patch placed over the injection site of a pandemic influenza vaccine, is currently in phase II.³

1.4 Adjuvants

An ideal adjuvant is easily applied in conjunction with a target antigen, capable of potentiating an immune response towards the antigen, but with little or even better no response against itself. It should be non-toxic, biodegradable, cheap to produce and easy to apply or co-formulate with antigen. (29)

Adjuvants are of particular need for sub-unit vaccines, like proteins and peptides that merely do not trigger a proper immune response by themselves. The use of adjuvants sought to induce a sustained immune reaction of a specific Type 1, Type 2 or even mixed direction. Furthermore a reduction of antigen on the one hand and higher efficacy of vaccines in newborns, elderly, or immunocompromised persons on the other hand can be achieved. (29)

Typical adjuvants include mineral salts, emulsions, bacterial compounds or nucleic acids. Unfortunately most adjuvants in research failed to be used in novel vaccines due to an unacceptable safety profile. (30)

³ For more information visit www.intercell.com

Aluminum salts were the first adjuvants used in broad range, first demonstrated in the 1920s (29). In the past decade new adjuvants were approved for human use: MF59 (Novartis) and ASo₃, both oil-in-water emulsions and ASo₄ (GSK), a monophosphoryl lipid A and aluminum hydroxide combination. (30, 31)

The precise modes of action for most adjuvants are unclear. Typical features involve increased APC uptake of antigen, by the adjuvant acting as a depot for antigen at the injection site, or as vehicle towards the APC and modulation of the immune system, by favoring a particular cellular, humoral or mixed response.

1.5 IC31®

Intercell AG developed a novel dual-component adjuvant IC31®, compiled of KLK, an anti-bacterial peptide and ODN1a, an oligodeoxynucleotide.

KLK, a KLKL₅KLK sequenced cationic peptide mimicks structural elements of sapecin B, an anti-microbial peptide of the flesh fly. (32) KLK has shown to exhibit potent adjuvant effects to co-injected ovalbumin (OVA), inducing potent IgG1a antibody-titers, IL-4 and IL-5 producing splenocytes and eliciting a Th2 directed response. Furthermore enhancement of antigen uptake and depot formation at the injection site could be observed. (33)

ODN1a is compiled of dimeric repeats of deoxy-inosine and deoxy-cytosine connected by a phosphodiester backbone. Unmethylated CpG dinucleotides in bacterial DNA have been shown to exhibit immunostimulatory activity via TLR-9 recognition, MyD88 signaling and NFκB activation, inducing Th1-like innate and adaptive immune responses. (34) Even though they induce systemic side effects, like high levels of IL-6 and TNF-α potentially contributing to a toxic shock syndrome, which can be avoided by adding Poly-L-arginin. (35-37)

In order to evade proinflammatory side-effects ODN1a was combined with KLK in a ratio of 1:25 to form the novel adjuvant IC31®.

IC31® could exhibit potent antigen-specific CTL responses, as well as a mixed Th1/Th2 directed antibody response. Studies with rats showed no systemic toxic effects of IC31® and attested well tolerability. Additionally adjuvanticity of IC31® was not accompanied by detectable levels of pro-inflammatory cytokines as IL-6 and TNF-α. (38)

Analyzing the mechanism of action, KLK acts as a depot at the injection site, gradually releasing antigen, but also as a delivery system, enhancing uptake of antigen and ODN1a into the APC. As mentioned before KLK induces a Th2 dominated immune response. ODN1a on the other hand promotes a Type 1 response by activation of APCs via the TLR9/MyD88 pathway. (39)

Co-formulation of IC₃₁[®] with a tuberculosis vaccine candidate resulted in significant levels of protection in a mouse aerosol protection model and potent long-lived T cell responses against *Mycobacterium tuberculosis* in human. (40, 41)

Another study demonstrated induction of serum haemagglutinin inhibition titers, IL-4 and IFN- γ production by CD4⁺-T cells and potent IgG1a and IgG2a serum antibody titers after vaccination with the Agrippal[®]S1 (Novartis Vaccines) influenza vaccine combined with IC₃₁[®]. (42)

1.6 Experimental background

The mouse model of *Chlamydia pneumonia* is well-established at Intercell. The chlamydial outer protein N (CopN) in conjunction with LT has been shown to protect against pulmonary *Chlamydia pneumoniae* infection in mouse model, inducing potent lymphocyte proliferation and IFN- γ production. (43) Induction of T lymphocytes is essential for protection against a *Chlamydia pneumoniae* infection and especially CD8⁺ T cells are of vital importance. (44)

Experiments immunizing mice intranasally or by injection with CopN in conjunction with IC₃₁[®] carried out at Intercell showed significant protection against pulmonary challenge with *Chlamydia pneumoniae*. An internal screening identified strong immunogenic CD8⁺ T cell restricted H-2K^d epitope peptides of CopN, shown in **Table 2**.

Due to a high antigenic response to the adjuvant LT itself delivered via a patch, LT is not presenting an optimal vaccine adjuvant. IC₃₁ on the other hand is not activating T or B cells by itself and is well-tolerated. (38)

The classic IC₃₁[®] formulation used at Intercell contains salt and forms particles of about 2-25 μ m size, which are too large in order to penetrate the skin, even after occlusion by hydration and removal of stratum corneum. Therefore Intercell developed novel nanoparticulate IC₃₁ in new salt-less buffer formulations forming nano-sized particles sized 80-200nm. Find all formulations with details in **Table 1**.

1.7 Thesis aims

The diploma thesis sought to evaluate application of IC₃₁[®] as a vaccine enhancement patch, adjuvanting an immune response to co-injected or co-formulated CopN antigen.

In a first approach adjuvanticity of mucosally delivered novel nanoparticulate IC₃₁[®] formulations should be assessed in terms of T cell and antibody responses.

A second approach aimed at evaluating adjuvant activity of classical and nanoparticulate IC₃₁[®] formulations applied as wet-patch to co-injected CopN in comparison to CopN injected alone without adjuvant.

Third, once the proof-of-concept studies succeeded, protective efficacy studies sought to be carried out, comparing patch-applied IC₃₁[®] upon CopN injection to patch-delivered IC₃₁[®] in co-formulation with CopN.

Finally local inflammatory responses in the skin ought to be examined via histopathology studies.

2 Results

2.1 Adjuvanticity of IC31[®] applied mucosally

The mouse infection model of Chlamydia Pneumoniae is well-established at Intercell. The chlamydial outer protein N (CopN) has shown to elicit a strong T cell activating response when administered co-formulated with the adjuvant IC31[®] via the intranasal (i.n.) and subcutaneous route (s.c.). (45)

IC31[®], a novel adjuvant is available at Intercell in 2 classical formulations that contain salt: classical IC31[®] PBS and classical IC31[®] Tris. As these formulations form micron-sized particles due to ionic interactions, new nanoparticulate IC31[®] was generated that only forms sub-micron sized particles. These formulations without salt include nanoparticulate IC31[®] Tris, nanoparticulate IC31[®] His and nanoparticulate IC31[®] Tris/Sorbitol. Find detailed information in **Table 1**.

Formulation	Buffer	pH	Stock concentration (KLK/ODN1a)[mM]
Classical IC31 [®] PBS	1xPBS	7.4	2000 / 80
Classical IC31 [®] Tris	10mM Tris, 135mM NaCl	7.5	3300 / 132
Nano IC31 [®] Tris	5mM Tris	6.5	3300 / 132
Nano IC31 [®] His	20mM Histidine	6	3300 / 132
Nano IC31 [®] Tris/Sorbitol	10mM Tris, 270mM Sorbitol	6.5	3300 / 132

Table 1: List of available IC31[®] formulations

In order to compare the immunopotential of various IC31[®]-formulations applied i.n. in terms of CTL responses, groups of 5 naïve BALB/c mice aged 6-8 weeks were immunized 3 times on days 0, 14 and 28 with 40µg of recombinant CopN (rCopN) mixed with IC31[®] (20nmol KLK/0.4 nmol ODN1A) in various buffer-formulations. As negative control, 2 groups of mice were immunized with IC31[®] only and rCopN only respectively. Find a detailed experimental scheme in **Table 3**.

10 days after the third immunization, spleens of 3 mice per group were isolated and analyzed for antigen-specific CD8⁺ T cell responses in an interferon gamma (IFN-γ) ELISpot. Isolated T cells were stimulated 24h with strong immunogenic CopN CD8⁺ H-

2K^d epitope peptides B8, B8.1, E8.1, listed in **Table 2** and Medium only. Consequently IFN-γ spot-forming cells (SFC) were quantified and are shown in **Figure 4**.

Peptide	Sequence	
B8	LADKYASGNSEISGQ	15mer
B8.1	KYASGNSEI	9mer
B8.2	YASGNSEIS	9mer
E8.1	DYFESRVPI	9mer

Table 2: CopN H-2K^d-restricted CD8⁺ T cell epitope peptides.

Group	Antigen + Adjuvant	Route
1	rCopN (40μg) + Classical IC31 [®] PBS (20/0.4 nmol)	i.n.
2	rCopN (40μg) + Classical IC31 [®] Tris (20/0.4 nmol)	i.n.
3	rCopN (40μg) + Nano IC31 [®] Tris (20/0.4 nmol)	i.n.
4	rCopN (40μg) + Nano IC31 [®] His (20/0.4 nmol)	i.n.
5	Classical IC31 [®] Tris (20/0.4 nmol)	i.n.
6	rCopN (40μg)	i.n.

Table 3: Experimental scheme; immunizations were taking place on days 0, 14, 28 with 5 mice per group.

All tested IC31[®] formulations exhibited potent and relatively equal adjuvant activity following 3 immunizations in terms of CD8⁺ T cell responses. Stimulating the isolated splenocytes for 24h with the peptides B8, B8.1 and E8.1 generally resulted in an equal number of IFN-γ expressing CD8⁺ T cells. Classical IC31[®] Tris alone has no T cell activating potential, whereas immunization with rCopN alone elicits a moderate CD8⁺ T cell response.

This data suggests that IC31[®] bears a strong immunopotentiating effect in terms of CTL activation after mucosal application, irrespective of the formulation. CopN seems to be a potent immunogen by itself, probably comprising strong T cell activating epitopes.

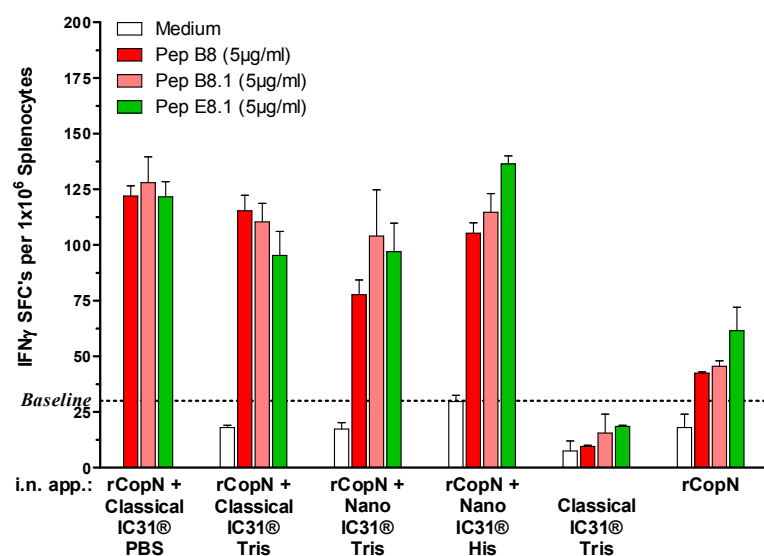


Figure 4: Adjuvanticity of IC₃₁[®] applied mucosally; groups of 5 mice were immunized i.n. on days 0, 14, 28 with 40μg of CopN mixed with classical and nanoparticulate IC₃₁[®] (100nmol KKK/4nmol ODN1a) in various buffer formulations. CTL responses were assessed in an IFN-γ ELISpot with spleen cells from 3 mice stimulated with strong immunogenic CopN CD8⁺ Tcell epitope peptides B8, B8.1 and B8.2. and Medium only 10 days after the third immunization. Results are expressed as spot forming cells (SFCs) per 1x10⁶ splenocytes.

2.2 Adjuvanticity of IC31[®] applied on patch

Enterotoxigenic *Escherichia Coli* Labile Toxin applied as patch has shown to be a strong immune-enhancing adjuvant inducing systemic and mucosal immune responses to co-administered antigens. (24, 46, 47) However, the generation of a strong antibody (Ab) response to LT precludes its repeated use and widespread applicability as a vaccine adjuvant.

Replacing LT by IC31[®] could result in a new vaccine enhancement strategy that combines the simplicity of patch application and potentially safe transcutaneous delivery of a potent adjuvant.

For assessing immunogenicity of IC31[®] delivered via a patch, several experiments were carried out testing and comparing multiple IC31[®] formulations applied as wet-patch upon intradermal injection of rCopN:

In a first experiment 5 naïve BALB/c mice per group were dorsally shaved, wetted with sterile water, stroked 10 times with sandpaper, immunized with 40µg of rCopN, which was injected intradermally in the middle of the back, followed by a release of 30.3µl of IC31[®] (100nmol KLK/4nmol ODN1a) on the patch gauze and patch-application directly above the injection side. IC31[®] was tested in classical IC31[®] Tris and nanoparticulate IC31[®] Tris formulation. Patches were removed from skin after 24h.

To rule out immunological effects of the skin-pretreatment itself, one group of 5 mice was immunized with CopN i.d., but an empty patch without IC31[®] was applied on top. Furthermore two other sets of 5 mice were injected with buffer lacking rCopN protein as negative controls. Subcutaneous immunization of rCopN mixed with classical IC31[®] PBS (100nmol KLK/4nmol ODN1a) has previously been shown to elicit a strong T cell response and now serves as a positive control. Find detailed information in **Table 4**.

Immunizations were carried out on days 0, 14, 28 and spleens were taken 10 days after the 2nd and 12 days after the 3rd immunization. The respective IFN-γ ELISpot Data are shown in **Figure 5** and **Figure 6**.

In a second experiment shown in **Table 5** adjuvanticity of nanoparticulate IC31[®] His applied on patch was determined in comparison to nanoparticulate IC31[®] Tris. Additionally a group of mice was pretreated with sterile water and sandpaper, rCopN injected, but no patch applied with the aim to determine the immunogenic effects of patch pretreatment alone.

Immunizations took place on days 0, 14, 28 and an IFN-γ ELISpot was performed with spleen-cells on day 38, see **Figure 7**.

Group	Injectable	Route	Patch
1	rCopN (40µg)	i.d.	empty
2	rCopN (40µg)	i.d.	Classical IC31® Tris (100/4 nmol)
3	rCopN (40µg)	i.d.	Nano IC31® Tris (100/4 nmol)
4	Buffer	i.d.	Classical IC31® Tris (100/4 nmol)
5	Buffer	i.d.	Nano IC31® Tris (100/4 nmol)
6	rCopN (40µg) + Classical IC31® PBS (100/4 nmol)	s.c.	no patch treatment

Table 4: Experimental scheme, immunizations were carried out on day 0, 14 and 28 with 5 mice per group.

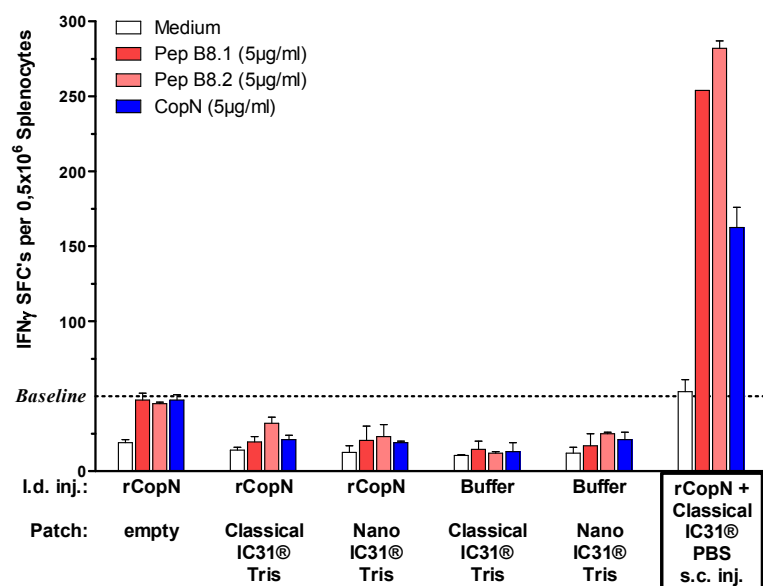


Figure 5: Adjuvanticity of IC31® applied on patch; groups of 5 mice were immunized on day 0, 14, 28 with rCopN injected s.c. and IC31® applied as wet patch upon injection site. Buffer control groups are included as well as a group immunized with rCopN subformulated with IC31® given s.c.. An IFN-γ ELISpot with splenocytes from 2 mice per group was carried out 10 days after the second immunization. T cells were stimulated with peptides B8.1. and B8.2, whole rCopN protein and Medium only.

A third experiment, shown in **Table 6**, followed that included nanoparticulate IC31® Tris/Sorbitol formulation, which contains an isotonic buffer that could potentially be

used as dry-patch. As this formulation hadn't been tested as injectable with CopN, a group of mice was immunized s.c. with nanoparticulate IC31® Tris/Sorbitol and another with classical IC31® Tris for comparison. Immunization were performed 3 times and an IFN-γ ELISpot was performed 12 days post 3rd immunization, see **Figure 8**.

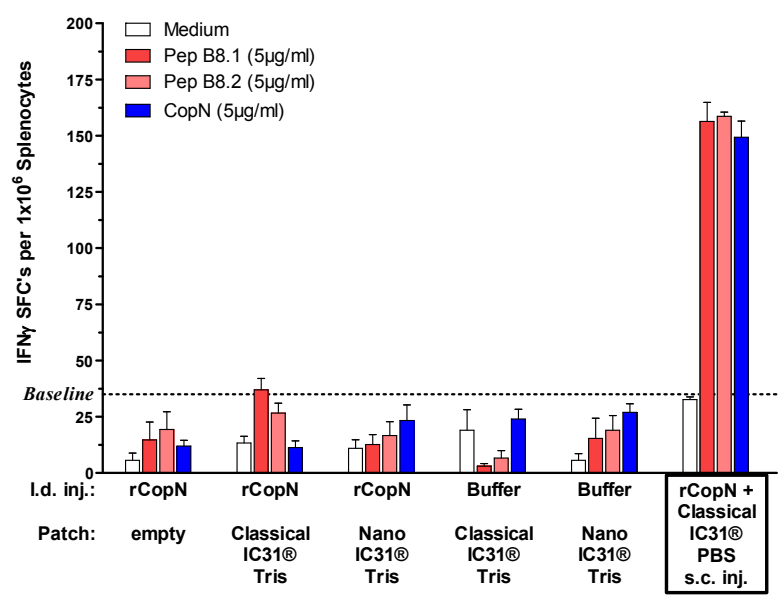


Figure 6: Adjuvanticity of IC31® applied on patch; immunizations were carried out on day 0, 14 and 28. 12 days after the third immunization an IFN-γ ELISpot assessed T cell responses of 3 mice per group.

Group	Injectable	Route	Patch
1	rCopN (40µg)	i.d.	empty
2	rCopN (40µg)	i.d.	sandpaper treatment only
3	rCopN (40µg)	i.d.	Nano IC31® Tris (100/4 nmol)
4	rCopN (40µg)	i.d.	Nano IC31® His (100/4 nmol)
5	Buffer	i.d.	empty
6	Buffer	i.d.	Nano IC31® Tris (100/4 nmol)
7	Buffer	i.d.	Nano IC31® His (100/4 nmol)
8	rCopN (40µg) + Classical IC31® PBS (100/4 nmol)	s.c.	no patch treatment

Table 5: Experimental scheme; immunizations took place on day 0, 14 and 28 with 5 mice per group.

Group	Injectable	Route	Patch
1	rCopN (40µg)	i.d.	empty
2	rCopN (40µg)	i.d.	Classical IC31® Tris (100/4 nmol)
3	rCopN (40µg)	i.d.	Nano IC31® Tris/Sorbitol (100/4 nmol)
4	Buffer	i.d.	Nano IC31® Tris/Sorbitol (100/4 nmol)
5	rCopN (40µg) + Classical IC31® PBS (100/4 nmol)	s.c.	no patch treatment
6	rCopN (40µg) + Nano IC31® Tris/Sorbitol (100/4 nmol)	s.c.	no patch treatment

Table 6: Experimental scheme; immunization were performed on day 0, 14 and 28 with 5 mice per group.

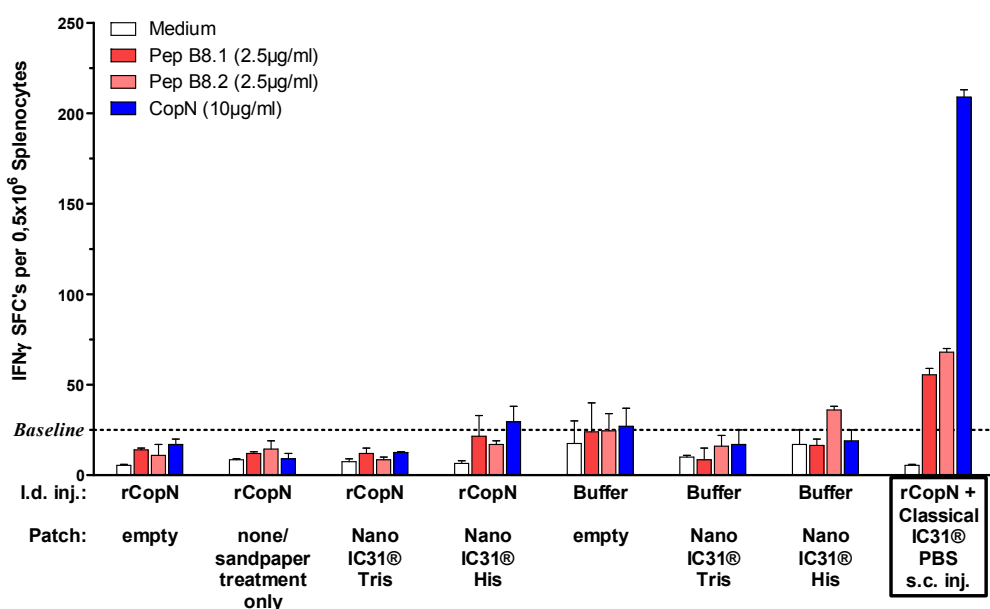


Figure 7: Adjuvanticity of IC31® applied on patch assessed via an IFN- γ ELISpot 10 days after the third immunization with spleen cells from 3 mice per group.

An adjuvant boost in regard of T cell activation could not be detected, when IC₃₁[®] in classical Tris, or nanoparticulate Tris, His and Tris/Sorbitol formulation was applied as wet-patch onto pretreated murine skin after 2 or even 3 consecutive immunizations.

No significantly different T cell immunogenic response could be observed, whether patch contained any IC₃₁[®]-formulation or left blank, rCopN or just buffer was injected and even whether patch was in fact applied or skin was pretreated without patch-application.

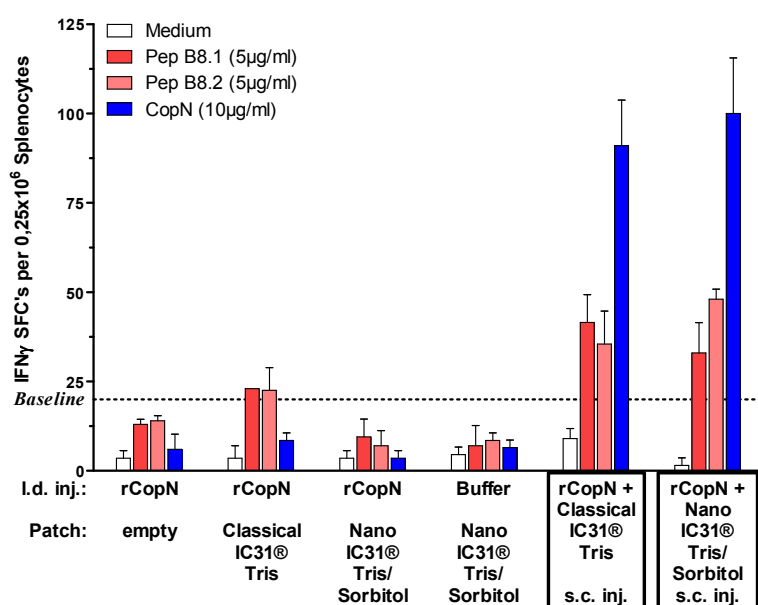


Figure 8: Adjuvanticity of IC₃₁[®] applied on patch; immunizations took place on days 0, 15, 29; IFN-γ ELISpot was performed 12 days after the third immunization with 3 mice per group.

Injection of classical IC₃₁[®] PBS and Tris co-formulated with CopN triggers a strong T cell activating response that could also be observed in equal extent with nanoparticulate IC₃₁[®] Tris/Sorbitol.

Based on that data, no single classical or nanoparticulate IC₃₁[®] formulation on patch could enhance a T cell immunogenic response upon injectable antigen, whereas co-formulated injectable nanoparticulate and of course classical IC₃₁[®] co-formulated with antigen responded in a strong and potent fashion.

At this early stage of a proof-of-concept study the question is raised, whether any technical issues including improper skin-pretreatment, patch-application, or even ELISpot assays and analysis are involved. Therefore LT should be included in further experiments as an adequate positive control.

2.3 Adjuvanticity of ETEC-Labile Toxin applied on patch

Enterotoxigenic Escherichia Coli labile toxin (LT) applied as patch has shown to be a strong immune-enhancing adjuvant inducing systemic and mucosal immune responses to co-administered antigens (24, 46, 47).

The following experiment sought to compare adjuvanticity of patch-applied LT, classical and nanoparticulate IC31® Tris upon injected rCopN based on T cell activation. In addition classical IC31® Tris and LT are compared as injectables with rCopN. The amount of LT applied as patch is 10µg, whereas only 0.1µg of LT are injected, as higher doses turned out to be toxigenic based on observations of Intercell Gaithersburg. Find detailed information in **Table 7**.

Group	Injectable	Route	Patch
1	rCopN (40µg)	i.d.	Classical IC31® Tris (100/4 nmol)
2	rCopN (40µg)	i.d.	Nano IC31® Tris (100/4 nmol)
3	rCopN (40µg)	i.d.	LT (10µg)
4	Buffer	i.d.	Classical IC31® Tris (100/4 nmol)
5	Buffer	i.d.	Nano IC31® Tris (100/4 nmol)
6	Buffer	i.d.	LT (10µg)
7	rCopN (40µg) + Classical IC31® Tris (100/4 nmol)	s.c.	no patch treatment
8	rCopN (40µg) + LT (0.1µg)	s.c.	no patch treatment

Table 7: Experimental scheme; days of immunization: 0, 15, 29 with 5 mice per group.

ETEC LT when applied as wet-patch exhibits a potent CTL activating response to co-injected CopN, comparable to co-formulated classical IC31® Tris or LT with rCopN and administered via the subcutaneous route.

Mice that received LT either via patch or injection show detectable T cell activation after stimulation with 1µg/ml of LT for 24h. The actual strength of T cell activation

cannot be directly compared to rCopN or peptide stimulated splenocytes, as LT has toxic effects and as a consequence the overall number of IFN- γ expressing T cells is decreased.

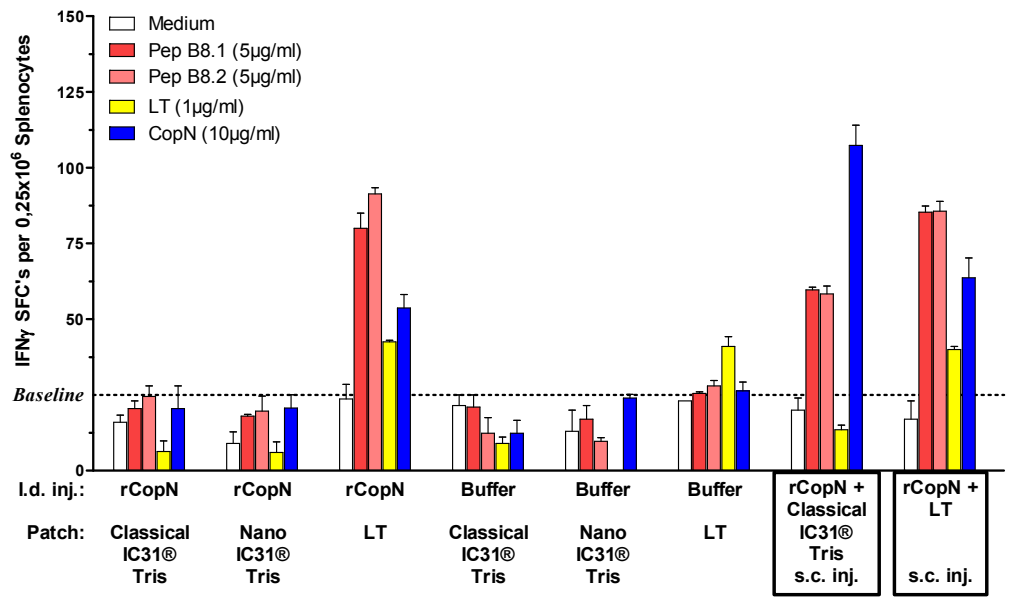


Figure 9: Adjuvanticity of ETEC labile toxin compared to IC31® applied on patch; the IFN- γ ELISpot was performed 12 days after the third immunization with 3 mice per group.

Thus LT proves to elicit potent adjuvant effects to injected rCopN, but LT also proves to be antigenic itself and therefore cannot be considered an ‘ideal adjuvant’.

This data suggests that patch application and immunological assays and read-outs were performed in a proper fashion, but the question remains, why IC31® administered via the transcutaneous route can’t potently adjuvant injected rCopN.

2.4 Adjuvanticity of single IC31[®] components applied on patch

As various classical and nanoparticulate IC31[®] formulations applied as wet-patch failed to enhance T cell responses to co-injected CopN, a next step is to analyze the adjuvanting potential of the single IC31[®] components. Especially KLK alone has shown to adjuvant a Th2-directed response against co-injected antigens by improving association of antigen and APC and by forming a depot at the injection site. (33)

For this purpose KLK or ODN1a at a concentration of 100nmol and 4nmol respectively diluted in dH₂O, and also nanoparticulate IC31[®] (100nmol/4nmol) and LT (10μg) are applied as wet-patch upon i.d. injected rCopN. Find experimental scheme in **Table 8**.

Group	Injectable	Route	Patch
1	rCopN (40μg)	i.d.	empty
2	rCopN (40μg)	i.d.	Nano IC31 [®] Tris (100/4 nmol)
3	rCopN (40μg)	i.d.	KLK (100 nmol)
4	rCopN (40μg)	i.d.	ODN1a (4 nmol)
5	rCopN (40μg)	i.d.	LT (10μg)
6	Buffer	i.d.	Nano IC31 [®] Tris (100/4 nmol)
7	Buffer	i.d.	KLK (100 nmol)
8	Buffer	i.d.	ODN1a (4 nmol)
9	Buffer	i.d.	LT (10μg)
10	rCopN (40μg) + Classical IC31 [®] Tris (100/4 nmol)	s.c.	no patch treatment
11	rCopN (40μg) + LT (10μg)	s.c.	no patch treatment

Table 8: Experimental scheme; immunizations were carried out on days 0, 22, 35 with 5 mice per group.

Figure 10 represents the IFN-γ ELISpot that was performed 12 days after 3 consecutive immunizations that took place on days 0, 22 and 35.

Apparently neither KLK, nor ODN1a applied as wet-patch following intradermal injection of rCopN could significantly elicit a T cell response.

This time administration of LT as wet-patch didn't result in a potent fashion as seen before, which most probably can be explained by variations in the methods.

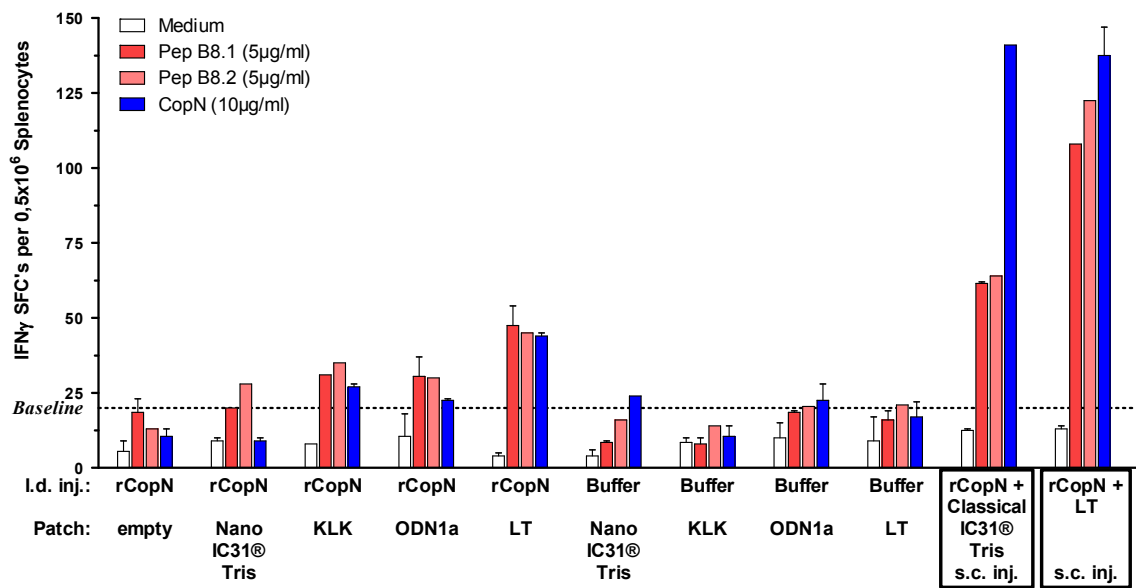


Figure 10: Adjuvanticity of single IC31® components applied on patch; IFN-γ ELISpot performed on 12 days after the 3 immunizations with 3 mice per group.

2.5 Adjuvanticity of rCopN co-formulations with IC31®, KLK or LT applied on patch

A potential lack of IC31® applied as wet-patch, which was intended to induce a potent CD8⁺ T cell response, could be the need for a co-formulation of IC31® with antigen. At this point it cannot be excluded that IC31® needs to be bound to CopN in order to elicit adjuvanting immunogenic effects.

In a subsequent experiment, groups of 5 mice were dorsally shaved and received rCopN and nanoparticulate IC31® via the intradermal route separated, by spacing the injection sites by about 1-2cm. Replacement of nanoparticulate IC31® or rCopN by corresponding buffers serve as negative controls and a co-formulation of nanoparticulate IC31® with rCopN injected i.d. as a positive control. Find all details in **Table 9**.

Group	Injectable 1	Injectable 2	Route
1	rCopN (40µg)	Nano IC31® Tris (100/4 nmol)	i.d. / i.d.
2	Buffer	Nano IC31® Tris (100/4 nmol)	i.d. / i.d.
3	rCopN (40µg)	Buffer	i.d. / i.d.
4	rCopN (40µg) + Nano IC31® Tris (100/4 nmol)		i.d.

Table 9: Experimental scheme; immunizations took place on day 0, 24 and 38 with 5 mice per group.

In the same experiment further groups of mice received rCopN mixed either with KLK, nanoparticulate IC31® Tris, or LT applied as wet-patch, as illustrated in **Table 10**. It should be remarked that there was no injection taking place.

Immunizations took place on days 0, 24, 38. **Figure 11** represents an IFN-γ ELISpot 10 days after the 2nd immunization, **Figure 12** shows IFN-γ ELISpot Data 10 days after third immunization.

Already after 2 consecutive immunizations, separated injection of rCopN and nanoparticulate IC31® Tris resulted in a potent T cell response based on IFN-γ expression, that appears to be similar to the response of rCopN co-formulated with nanoparticulate IC31® Tris.

Group	Injectable	Route	Patch
1			rCopN (40µg)
2			rCopN (40µg) + KLK (100 nmol)
3			rCopN (40µg) + Nano IC31 [®] Tris (100/4 nmol)
4			rCopN (40µg) + LT (10 µg)
5			KLK (100 nmol)
6			Nano IC31 [®] Tris (100/4 nmol)
7			LT (10 µg)
8			ODN1a (4 nmol)
9	rCopN (40µg) + Nano IC31 [®] Tris (100/4 nmol)	i.d.	no patch treatment

Table 10: Experimental scheme; immunizations were carried out on days 0, 24, 38.

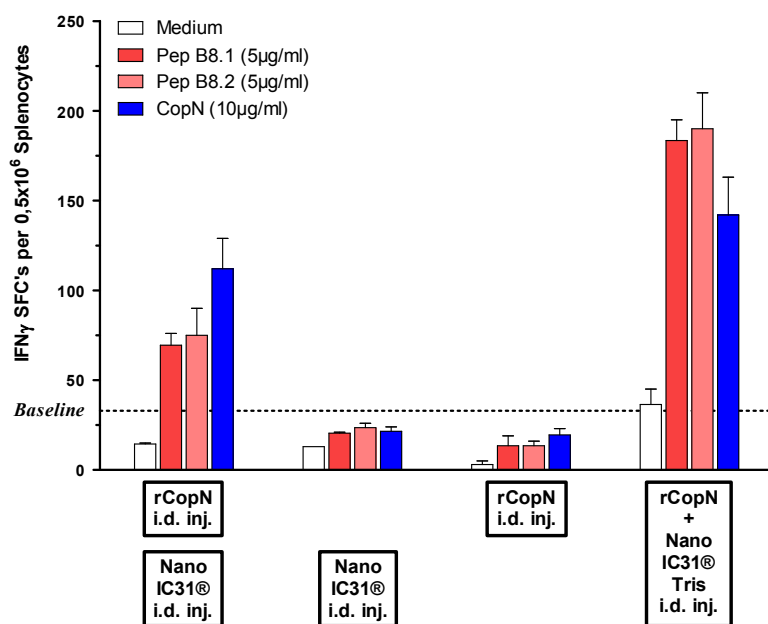


Figure 11: Adjuvanticity of IC31[®] injected spatially (~1-2cm) separated from antigen; IFN-γ ELISpot performed 10 days after the second immunization with 2 mice per group.

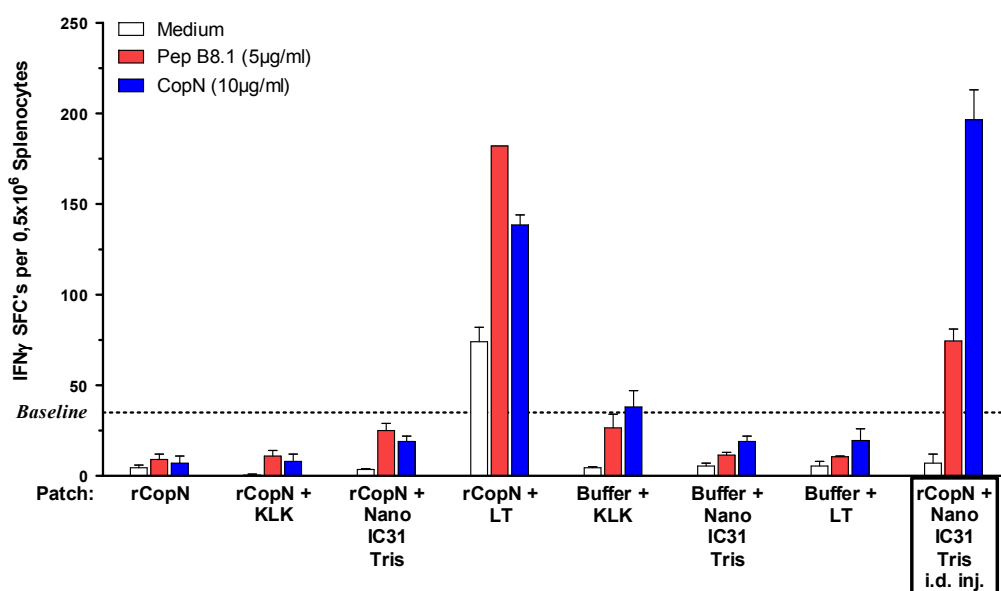


Figure 12: Adjuvanticity of rCopN co-formulations with IC31®, KLK or LT applied on patch; 10 days after the third immunization an IFN-γ ELISpot assay was performed with 3 mice per group.

Applying co-formulations of rCopN with nanoparticulate IC31® Tris or KLK as wet-patch doesn't exhibit a detectable splenocytic IFN-γ response, whereas rCopN co-formulated with LT leads to a potent T cell response equivalent to i.d. injected rCopN with nanoparticulate IC31®.

Stimulating splenocytes of rCopN with LT on patch immunized mice with medium lead to a high number of IFN-γ expressing T cells in **Figure 12**. Obviously the respective wells were unintentionally contaminated with stimulating agents like rCopN or ConA.

This data suggests that IC31® co-formulated with antigen and applied as wet-patch, cannot elicit a detectable T cell response. LT on the other hand mixed with antigen and applied as wet-patch has very potent adjuvant effects in terms of T cell responses. This implicates that rCopN has the potential to penetrate through pretreated murine skin and be taken up by APC's, followed by sufficient priming of naïve T cells.

So far IC31® has shown no potential as a T cell adjuvant applied as wet-patch.

2.6 Retention of IC31® on the patch

The patch-technology was developed based on applying pre-dominantly ETEC LT on patch. So far no tests have been performed analyzing IC31® compatibility with the wet-patch itself.

Thus a basic question had to be solved: Is the dose of IC31® applied on the patch in fact leaving the patch and being transferred to the skin? In a consequent study, carried out by the Adjuvant Formulation Department of Intercell Vienna, patch-gauzes in various conditions were analyzed via HPLC determining IC31® recovery.

For this purpose 24h after a regular immunization of a previously shown experiment (refer to **Table 5**) patches were removed and corresponding gauzes were isolated and transferred into individual sterile tubes. Additionally IC31® was applied to another set of patches that were kept for 24 hours gauze-side up in a humid chamber. Gauzes were isolated in the same procedure as previously described. 30 minutes prior to analysis IC31® was applied to another set of fresh patches and patch-gauzes were immediately removed and isolated.

Desorption of IC31® remaining on the patch was done by adding 500µL WFI+protease inhibitors/5mM EDTA onto the gauze and incubation for 1h at RT shaking. Values shown in **Table 11** and **Table 12** represent the recovery of IC31® relative to the applied amount (100nmol KLK / 4nmol ODN1a).

Hardly any IC31® could be detected after desorption of patch gauzes worn overnight on murine skin, irrespective of prior rCopN or buffer injection. Recovery rates of IC31® applied on patches left in wet chamber were about 1.5-2 fold decreased compared to gauzes with fresh applied IC31®.

This indicates that IC31® may be tightly bound by the gauze and can't be desorbed by the applied desorption method or IC31® is even absorbed by the rest of the patch.

Analysis should be regarded with respect, as gauzes kept in wet chamber were reported to be very wet and especially KLK analysis was highly impaired by mouse derived proteins that interfered with the method.

These results lead to further testings by Intercell's Formulation Team from Gaithersburg (data not shown) and it revealed that most IC31® remains on the patch. It is adsorbed to the adhesive film backing and to the gauze- and rayon-based matrices of the patches.

Based on these findings, the current wet-patch format doesn't seem to be an appropriate vehicle for transcutaneous delivery of IC31®.

Patch contents	Condition	KLK recovery (%)	ODN1a recovery (%)
empty	o/n on mouse skin upon rCopN i.d. inj.	0 / 0 / 0	0 / 0 / 0
Nano IC31® Tris	o/n on mouse skin upon rCopN i.d. inj.	<10/ 0 /<10	<10/ 0 /<10
Nano IC31® His	o/n on mouse skin upon rCopN i.d. inj.	<10/ 16 / 0	<10/ 14 / 0
empty	o/n on mouse skin upon buffer i.d. inj.	0 / 0 / 0	0 / 0 / 0
Nano IC31® Tris	o/n on mouse skin upon buffer i.d. inj.	0 /<10/<10	0 /<10/ 0
Nano IC31® His	o/n on mouse skin upon buffer i.d. inj.	0 /<10 / -	0 /<10 / -
Classical IC31® Tris	o/n on mouse skin, no injection	0 / 0 /<10	0 / 0 /<10
Nano IC31® Tris	o/n on mouse skin, no injection	<10/<10/<10	0 /<10/<10

Table 11: Relative retention of classical and nanoparticulate IC31® (100nmol KLK/4nmol ODN1a), applied on patches remaining o/n on murine skin and detected by HPLC-analysis. (n=3)

Patch contents	Condition	KLK recovery (%)	ODN1a recovery (%)
Nano IC31® Tris	o/n in humid chamber	20 / 34 / 24	29 / 30 / 22
Nano IC31® His	o/n in humid chamber	36 / 35 / 32	47 / 34 / 33
Nano IC31® Tris	fresh applied	41 / 46 / 38	56 / 39 / 37
Nano IC31® His	fresh applied	58 / 56 / 57	85 / 58 / 57

Table 12: Relative retention of nanoparticulate IC31® (100nmol KLK/4nmol ODN1a), applied 30min prior analysis or that remained o/n in a humid chamber, detected by HPLC-analysis. (n=3)

2.7 Adjuvanticity of IC31[®], Single IC31[®] components and LT rubbed on skin

As IC31[®] applied on patch repeatedly failed to induce a T cell response and as there is indication of incompatibility between IC31[®] and the patch, a next approach aimed at evaluating patch-free topical application of IC31[®].

In this new approach the mouse skin is pre-treated with water and sandpaper and antigen is injected i.d. in the same manner as before, but instead of applying adjuvant onto the patch, it is directly applied onto the pre-treated skin around the injection site and rubbed into the skin with the tip of a sterile Eppendorf-tube. The adjuvant-moistened area should cover about 2cm².

In a first pilot experiment, as portrayed in **Table 13**, mice received rCopN injected i.d. and classical IC31[®] Tris, Nano IC31[®] Tris, KLK, ODN1a and LT topically. One group of mice received rCopN co-formulated with Nano IC31[®] Tris topically, but no injection was taking place.

Group	Injectable	Route	Rubbed
1	rCopN (40µg)	i.d.	Buffer
2	rCopN (40µg)	i.d.	Classical IC31 [®] Tris (100/4 nmol)
3	rCopN (40µg)	i.d.	Nano IC31 [®] Tris (100/4 nmol)
4	rCopN (40µg)	i.d.	KLK (100 nmol)
5	rCopN (40µg)	i.d.	ODN1a (4 nmol)
6	rCopN (40µg)	i.d.	LT (10µg)
7	rCopN + Nano IC31 [®] Tris (100/4 nmol)		
8	Buffer	i.d.	Nano IC31 [®] Tris (100/4 nmol)
9	Buffer	i.d.	LT
10	rCopN (40µg) + Classical IC31 [®] Tris (100/4 nmol)	s.c.	No skin treatment

Table 13: Experimental scheme; immunizations were carried out on days 0, 14, 45 with 5 mice per group.

Immunizations were performed on day 0, 14 and 45. **Figure 13** shows data from an IFN- γ ELISpot with spleens carried out 11 days after second immunization and **Figure 14**

represents an IFN- γ ELISpot with spleens performed 9 days after the third immunization.

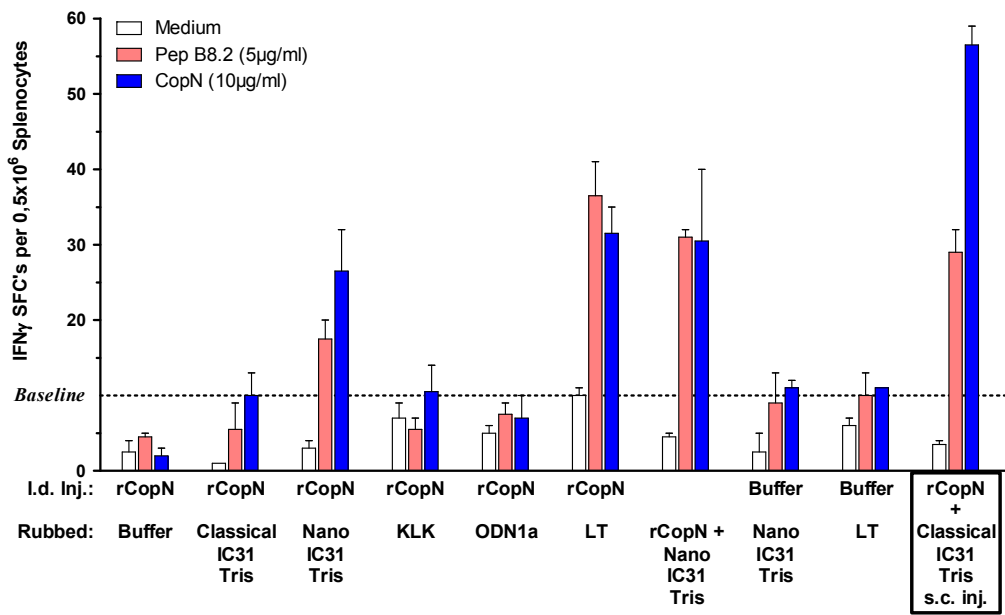


Figure 13: Adjuvanticity of IC31[®], single IC31[®] components and LT rubbed on skin; Data shows an IFN- γ ELISpot performed 11 days after 2 consecutive immunizations with splenocytes from 2 mice per group.

After two consecutive immunizations skin rubbing of nanoparticulate IC31[®] Tris resulted in moderate adjuvant activity for the induction of anti-CopN CD8⁺ T cell responses. KLK or ODN1a rubbed on skin fail to induce a detectable response. Adjuvant activity of nanoparticle IC31[®] Tris is slightly enhanced when it is rubbed on skin as a co-formulation with rCopN. LT rubbed on skin results in an equal response to skin-rubbed IC31[®] co-formulated with rCopN and is comparable to IC31[®] given by s.c. injection.

The adjuvant activity of skin-rubbed classical or nanoparticulate IC31[®] Tris for the induction of anti-CopN CD8⁺ T cell responses does not further increase after the third dose of injectable rCopN. The same applies for skin-rubbed rCopN co-formulated with nanoparticulate IC31[®] Tris, even though s.c. injection of co-formulated IC31[®] with rCopN lead to an overall 2-3fold higher response.

A follow-on experiment investigated the adjuvant activity of nanoparticulate IC31[®] Tris rubbed on skin upon i.d. injection of rCopN and as a co-formulation with rCopN, without prior injection, as displayed in **Table 14**.

Immunizations were performed on days 0, 14 and 29. **Figure 15** shows an IFN- γ ELISpot Data 9 days after the third immunization.

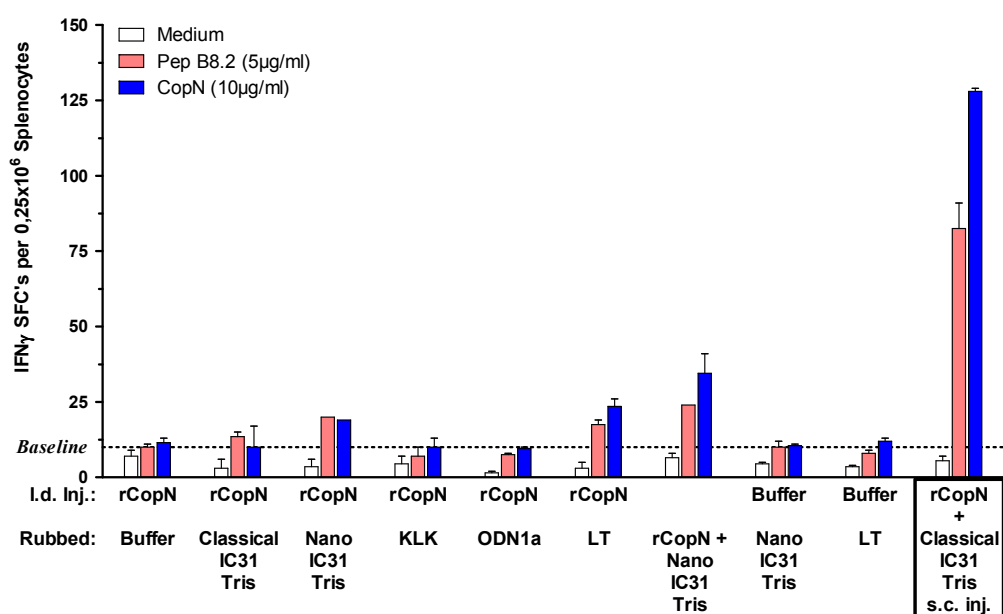


Figure 14: Adjuvanticity of IC31®, single IC31® components and LT rubbed on skin; The IFN-γ ELISpot assay was performed 9 days after the third immunization with 2 mice per group.

Group	Injectable	Route	Rubbed
1	rCopN (40μg)	i.d.	Buffer
2	rCopN (40μg)	i.d.	Nano IC31® Tris (100/4 nmol)
3	rCopN (40μg)	i.d.	LT (10μg)
4			rCopN + Nano IC31® Tris (100/4 nmol)
5	Buffer	i.d.	Nano IC31® Tris (100/4 nmol)
6	rCopN (40μg) + Classical IC31® Tris (100/4 nmol)	s.c.	No skin treatment
7	Buffer	i.d.	LT
8	rCopN (40μg) + LT (0.1 μg)	s.c.	No skin treatment

Table 14: Experimental scheme, immunizations were performed on days 0, 14, 29 with 5 mice per group.

Even after three consecutive immunizations no significant adjuvant activity of nanoparticulate IC31® Tris either rubbed into skin upon i.d. injection of rCopN, or co-

formulated with rCopN and rubbed into skin could be detected in terms of T cell responses.

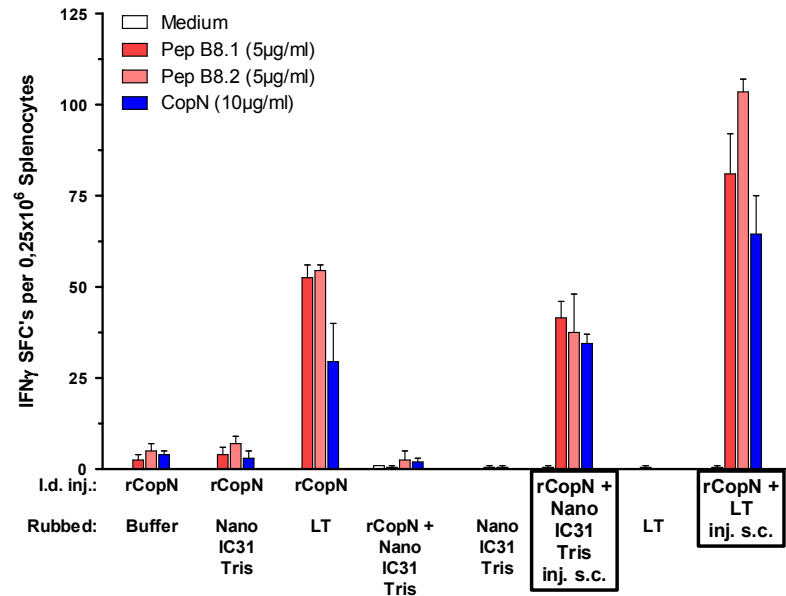


Figure 15: Adjuvanticity of IC₃₁[®], Single IC₃₁[®] components and LT rubbed on skin; T cell responses were assessed 9 days after 3 consecutive immunizations via an IFN-γ ELISpot with 3 mice per group.

LT rubbed into skin upon injection of rCopN lead to a potent response, comparable to rCopN injected s.c. co-formulated with nanoparticulate IC₃₁[®] Tris. Interestingly LT in a co-formulation with rCopN lead to an even stronger CD8⁺ T cell response.

Obviously results of the pilot experiment couldn't be reproduced, which could be explained by variations in the method.

Data of other experiments performed at Intercell with the Japanese Encephalitis virus demonstrated the induction of protective PRNT₅₀ titers with skin rubbed IC₃₁[®] (data not shown) support the first experiments' data.

Based on these findings IC₃₁[®] has T cell adjuvant activity, when applied topically and rubbed into skin after skin pre-treatment. The impact is even stronger when IC₃₁[®] is co-formulated with antigen and equivalent to LT rubbed on skin after injection of rCopN. Still s.c. injection of IC₃₁[®] finally lead to an 2-3 fold increased response.

2.8 Antibody-response to intranasally, patch-applied and skin-rubbed IC31®

So far only IFN- γ responses were assessed via IFN- γ ELISpots, neglecting B cell activity. Therefore sera of immunized mice taken 9 to 12 days after the third immunization of each experiment were analyzed for presence of anti-CopN IgG antibodies in an ELISA assay.

Figure 16 shows ELISA Data of the first experiment, where several IC31® formulations were compared to each other given in co-formulation with rCopN via the intranasal route, displayed in **Table 3**. It can be observed, that all IC31® formulations induce high anti-CopN IgG titers. IC31® or CopN given alone can't elicit a detectable anti-CopN antibody response.

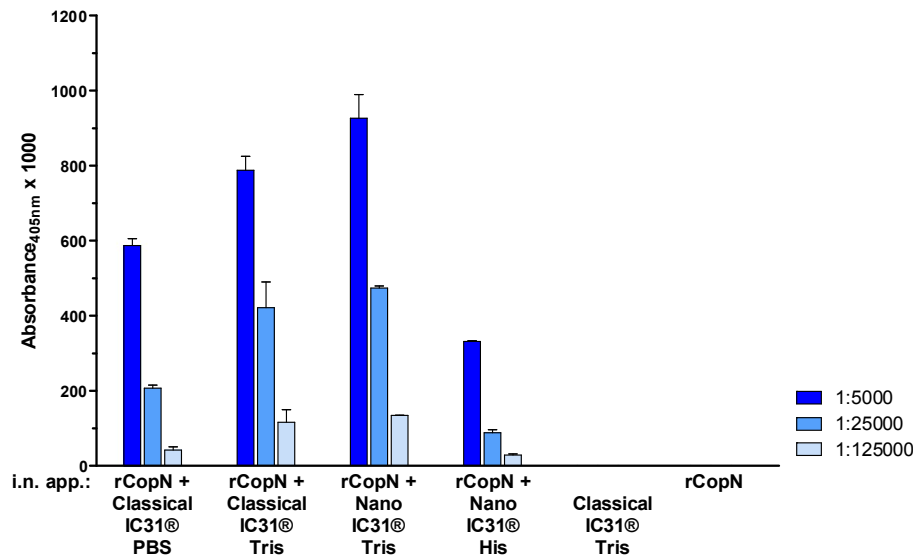


Figure 16: Antibody-response to intranasally applied IC31® with CopN; anti-CopN total IgG ELISA with pooled sera from terminal bleeds of 3 mice taken 10 days after the third immunization.

It's unclear why the two IC31® formulations in a Tris buffer give slightly higher titers. As no further measurements were performed this might be due to variations in the ELISA assay.

Next, sera taken 12 days after the third immunization of the first experiment involving patch application, corresponding to **Table 4** were analyzed for total IgG antibodies against CopN. ELISA data is shown in **Figure 17**.

Interestingly injection of CopN and application of empty patch elicits detectable anti-CopN total IgG titers. Titers are increased when IC31® either in classical Tris or Nano Tris formulation are applied on patch.

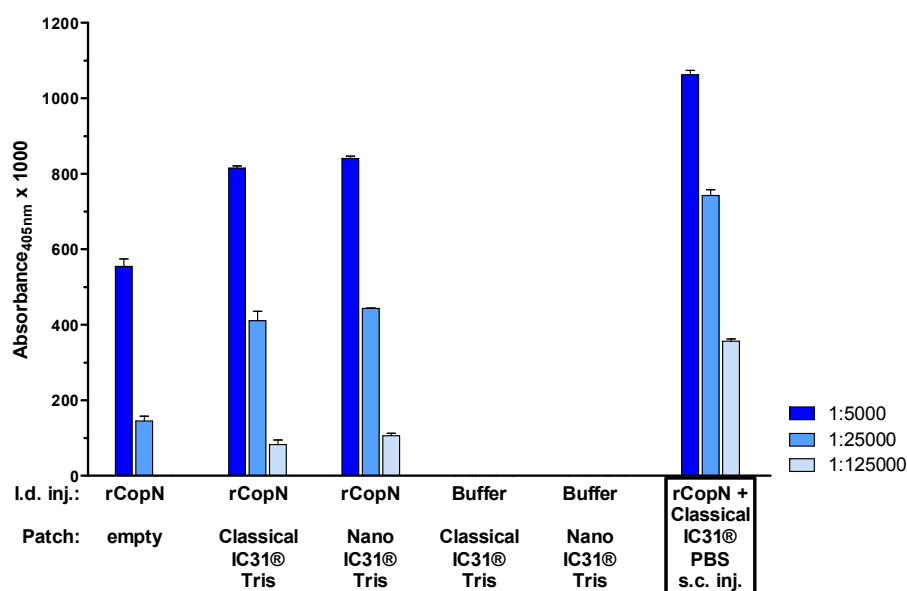


Figure 17: Antibody-response to patch-applied IC₃₁[®] upon co-injection of CopN; 12 days after three immunizations an anti-CopN total IgG ELISA was performed with pooled sera from 3 mice.

First apparently CopN seems to have intrinsic B cell activating epitopes. Skin-pretreatment and patch application might contribute. Another reason are low amounts of LPS in the CopN stock preparations used for immunizations that could potentially have weak adjuvant effects.

Increased titers as a result of IC₃₁[®] application on the patch can be explained by the fact that the limited amount of IC₃₁[®] that leaked through the patch was sufficient to activate B cells, but was insufficient to trigger a detectable T cell response. Still CopN in co-formulation with IC₃₁[®] injected subcutaneously elicited the strongest B cell response.

Figure 18 displays data of an ELISA assay performed with terminal bleed mice, corresponding to the experiment shown in **Table 10**. In this experiment co-formulations of CopN with either IC₃₁[®], KLK or LT were applied on patch without prior injection.

Even when CopN without adjuvant was applied topically via a patch, it could induce weak anti-CopN IgG titers. An equivalent response is observed in the first group, where KLK was added as an adjuvant, which therefore doesn't seem to exhibit any adjuvant activity in terms of B cell responses. In fact nanoparticulate IC₃₁[®] in Tris buffer slightly increases antibody titers, which again can be explained by traces of IC₃₁[®] leaking from patch to the skin.

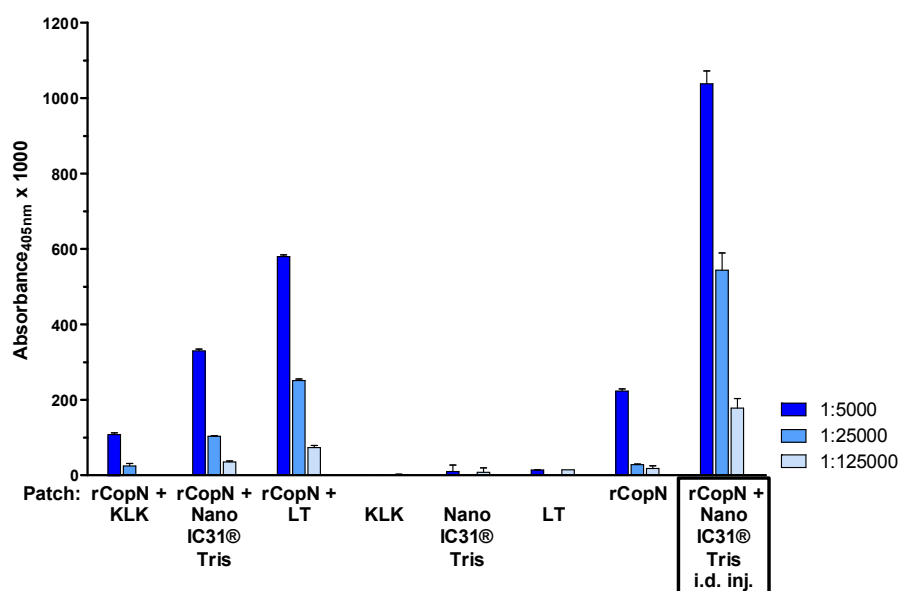


Figure 18: Antibody-response to patch-applied IC₃₁[®] co-formulated with CopN; the figure shows an anti-CopN total IgG ELISA with pooled sera from 3 mice taken 10 days after three consecutive immunizations.

Co-formulation of CopN with IC₃₁[®] applied as wet-patch resulted in weaker antibody titers relative to the injectable control, when compared to the previous experiment with injected CopN and patch-applied IC₃₁[®]. This supports the theory that CopN alone is most relevant for B cell activation and the response is of course stronger when the full dosis of CopN is directly injected.

Finally sera taken from mice, which received adjuvant through rubbing into skin, were analyzed for total anti-CopN IgG antibodies. For experimental setup see **Table 13**, results are displayed in **Figure 19**.

Irrelevant which agent was rubbed into skin, i.d. injection of CopN is the dominant factor developing moderate anti-CopN antibody titers. When CopN is applied topically titers drop to weak, but still detectable levels. It is unclear why skin-rubbing of LT doesn't result in increased antibody titers as before. As this was the only ELISA performed, variations are possible.

Comprising the ELISA data we can assume that IC₃₁[®] has weak, but detectable adjuvant effects in terms of B cell responses, when applied as wet-patch onto murine skin upon injection of or in co-formulation with rCopN. This adjuvant effect arises most probably through traces of IC₃₁[®] delivered through the patch sufficient to induce a B cell, but no T cell response.

The most important factor for B cell activation relies on CopN, which activates B cells via B cell activating epitopes or traces of LPS in the recombinant CopN stocks used for immunizations. B cell activation is more potent when CopN is injected instead of being

applied topically. Rubbing of IC31® or LT into skin has no detectable adjuvant effect in terms of T cell responses, considering data from a single experiment respectively.

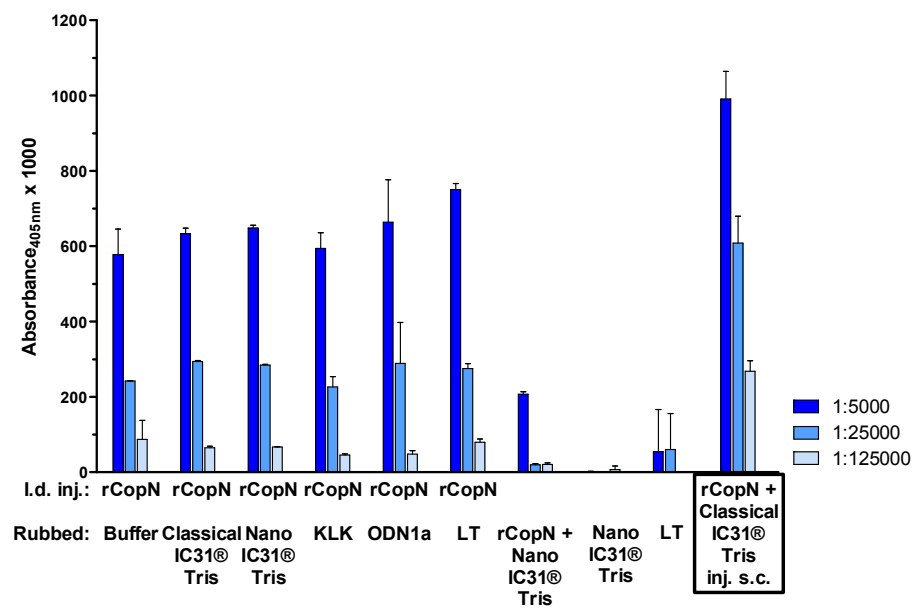


Figure 19: Antibody-response to IC31® rubbed into skin; anti-CopN total IgG antibody titers were measured in an ELISA assay with pooled sera from 3 mice taken 9 days after the three consecutive immunizations.

3 Discussion

3.1 General

I could show that IC₃₁[®] in classical and nanoparticulate buffer formulations co-administered with antigen via the intranasal route exhibits potent CTL activation.

My data confirmed strong CD8⁺ T cell responses caused by immunizations of classical and nanoparticulate IC₃₁[®] in co-formulation with CopN antigen injected subcutaneously, shown as control in multiple experiments.

IC₃₁[®] applied as wet-patch in various formulations and even as single components could not elicit any detectable CD8⁺ T cell responses to co-injected antigen in multiple experiments. Even co-formulation of IC₃₁[®] with antigen on patch failed to induce noticeable CTL responses. ELISA data on the other hand demonstrate weak adjuvant effects of patch-applied IC₃₁[®], which can be explained by small amounts of IC₃₁[®] penetrating the skin, sufficient to induce a B cell-directed response. But it should be mentioned that the most important factor for B cell activation relies on the antigen CopN itself, which activates B cells via B cell activating epitopes or traces of LPS in the recombinant CopN stocks used for immunizations.

To confirm proper patch-application, Escherichia Coli labile toxin (LT) was applied as wet-patch to injected antigen and proved to exhibit potent adjuvant effects in terms of CD8⁺ T cell responses. The adjuvant effect was even higher, when LT was injected.

Tests of the Adjuvant Formulation Department Team of Intercell Vienna and further findings of Intercell Team in Gaithersburg support the fact that most IC₃₁[®] is adsorbed to the adhesive film backing and the gauze and rayon-based matrices of the patches.

A new approach sought to circumvent this problem by applying IC₃₁[®] straight on top of skin including prior skin treatment equivalent to patch-pretreatment and rubbing the adjuvant solution directly into the skin around the spot of antigen injection.

In a first experiment ELISpot data after two consecutive immunizations demonstrate a moderate response of skin-rubbed nanoparticulate IC₃₁[®] Tris in terms of CD8⁺ T cell responses. The response is even stronger, when nanoparticulate IC₃₁[®] Tris is co-formulated with antigen and rubbed. The T cell responses are equivalent to injected IC₃₁[®] with antigen.

Interestingly this response didn't further increase after the third immunization in contrast to IC₃₁[®] injected in co-formulation with antigen.

Prior results couldn't be reproduced in a partly repeat of the previous experiment. Skin-rubbing of IC31® didn't elicit a measurable CTL response in this case. Errors in the methods or destructed agents for immunization or analysis can't be precluded.

Unshown data of different experiments performed at Intercell with Japanese Encephalitis Virus revealed induction of protective PRNT₅₀ titers with skin rubbed IC31®, which supports evidence for the first experiments' data.

3.2 Problems involving the rubbed-into-skin approach

Applying IC31® directly on top of skin without cover is a raw approach, susceptible to significant variations, as the spot of adjuvant rubbed on skin is exposed to the environment. It cannot be excluded that some mice lick foreign or their own backs, which would result in oral application. We have to keep in mind that the spot of adjuvant application received harsh pre-treatment, involving wettening and sandpaper strokes, resulting in a quite high irritation and increased sensitivity of the skin. Contact with dirt may trigger an immune response by itself.

Another practical problem is the act of rubbing into the skin. I mentioned earlier that about 1-2cm² of skin is covered with rubbed adjuvant. Depending on the topology of the skin including wrinkles the space of dispersing the adjuvant can vary, resulting in a less site-specific concentrated adjuvant.

3.3 What are potential reasons for a lack of IC31® adjuvanticity applied on skin?

First, it is possible that a non-adjuvanting dose of IC31® is delivered to the skin.

Second, in case of wet-patches most IC31® seems to be adsorbed to the adhesive film backing and to the gauze- and rayon-based matrices of patches, considering data from Gaithersburg.

Third, IC31® is bound or degraded by mouse proteins migrating to the patch, as indicated by data from Adjuvant Formulation Department.

Fourth, the dose of IC31® that contacts the skin remains on the skin and is not breaking the skin barrier.

In order to overcome the mentioned problems I suggest carrying out further experiments in first place and then seek for new alternative strategies for topical immunization.

3.4 Suggestions for further experiments

As additional experiments I would recommend performing dose-dependency studies with IC₃₁[®] rubbed into skin. Previous experiments sought to compare 100/4 nmol KLK/ODN1a applied on skin as wet-patch or rubbed into the skin versus the same dose injected subcutaneously. It should be noted that in using a patch system, IC₃₁[®] is applied on the gauze of the patch and is therefore only slowly and definitely not entirely released to the skin. Furthermore the skin is a barrier including sheets of dead keratinocytes, which reduces the effective dose of IC₃₁[®] reaching deeper strata and finally targeting antigen presenting cells. Considering rubbed-into-skin application water evaporation may lead to loss or inactivation of IC₃₁[®].

Now compensating any loss of IC₃₁[®] I would apply higher doses of IC₃₁[®] both as patch, or rubbed into skin as titrations and see if comparable potent adjuvant effects can be exhibited.

3.5 Alternative delivery strategies

First, I would suggest to develop new optimized patches with different materials, that do not adsorb IC₃₁[®]. As mentioned earlier dose-dependency studies via titrations should be considered when testing IC₃₁[®] with these new patch variants.

Following a strategy of applying IC₃₁[®] directly on skin, liposomes pose an attractive alternative, constituting an already successful vehicle for drug delivery across the skin. Although recently it became apparent that in most cases conventional liposomes are unable to reach deeper strata of the skin. (17) Intensive research lead to development of novel lipid carriers, namely transferosomes and ethosomes. Traditional liposomes consist of phospholipids and cholesterol building artificial vesicles able to encapsulate the drug for delivery and also protect against the environment. (15)

Transferosomes are multi-component ultradeformable vesicular carriers optimized for delivery through permeability barriers like non-occluded skin. The main difference to liposomes is the addition of surfactants, which results in higher membrane elasticity and hydrophilicity ensuring penetration along a gradient into the water-rich skin. (48) Insulin-loaded transferosomes were successfully transferred through intact human skin in comparable amounts to subcutaneous application. (49)

Ethosomes, developed in 2000 by Touitou et al. (16), consist principally of phospholipids, ethanol and water and have shown to deliver various drugs in vivo and in vitro through the skin, as reviewed by Elsayed et al.. (17)

Formulation of Nanoparticulate IC₃₁[®] in those novel ultra-deformable transfersomes or ethosomes could potentially be a great transcutaneous delivery strategy perhaps superior to a patch-dependent system.

4 Materials and Methods

4.1 Vaccination ingredients

Antigen

Chlamydial outer protein N, CopN (Chlamydia Pneumoniae CWL029 Low Calcium Response E, AAD18473.1) used for all immunizations as model antigen was synthesized in-house at Intercell AG Vienna including a N-terminal 6xHis Tag dissolved in 50mM Tris/HCl buffer at pH8.0 with 150mM NaCl stored at -80° Celsius. For immunizations and ELISpot assays CopN stock vials were kept on ice 20 minutes prior to use.

Adjuvants

IC₃₁ is composed of the peptide KLKL₅KLK, KLK (synthesized by Bachem AG) and the phosphodiester-backboned oligodeoxynucleotide ODN1a (deoxy-inosine – deoxycytosine: [dIdC]₁₃). Both components are mixed in a ratio of 25:1 (KLK:ODN1a), buffered in PBS, Tris, His and Tris/Sorbitol buffers, listed in detail in **Table 1** and stored at 4°C. Optimal doses for intranasal administration are 20nmol KLK and 0.4nmol ODN1a, whereas injection or topical application requires 100nmol KLK and 4nmol ODN1a.

LT stocks were received from Intercell USA Team in Gaithersburg, buffered in PBS (Gibco) and stored at 4°C.

4.2 Mode of Vaccination

Intranasal immunization

Mice were anesthetized with Isoflurane (Baxter) and immunized with 40µl per dose of vaccine pipetted into the mouse nostrils.

Subcutaneous immunization

Mice received tail-based injections of 100µl volumes per dose, without prior anesthesia.

Patch immunization

The first step involved hair removal with an electric shaver in the center of the back covering a space of about 10cm². Anesthesia was performed by intraperitoneal injection of a mixture of ketamine (Ketamidol, Richter Pharma) and xylazine (Rompun, Bayer) with doses between 100-150µl. Anesthetized animals were dorsally wetted with water-soaked gauze and then stroke 10 times with an emery paper. If required, intradermal injection took place at the center of the shaved, wetted and disrupted dorsal skin. As a final step the vaccine or adjuvant was now pipetted on the 1-1.3cm² patch gauze, which is fixed in the center of an adhesive plastic overlay. Applicable volumes are 30µl-45µl

depending on gauze size. The adhesive patch was then placed in middle of the back or rather right above the intradermal injection site. Removal of patches took place 24h after application.

Intradermal immunization

Prior to immunization mice were dorsally shaved and 50µl doses were injected into the skin.

4.3 Mice

6-8 week old naïve female BALB/c (H-2^d) mice were ordered from Charles River laboratories, Germany and kept in a pathogen-free environment in the Intercell Vienna in-house animal facility with free food and water access. The maximum experimentally accepted age was 12 weeks. All animal experiments were conducted under the Austrian law.

4.4 Cell isolation and preparation

Animals were sacrificed by cervical dislocation, spleens were isolated and stored pooled in groups in tubes filled with RPMI 1640 (Invitrogen) on ice. Spleens were mechanically dispersed via straining against a 70µm strainer. Red blood cells were lysed by resuspension in Red Blood Cell Lysing Buffer (Sigma-Aldrich) for 2 minutes. After centrifugation TCM, T cell Medium (RPMI 1640 by Invitrogen, 10% heat-inactivated Fetal Calf Serum by PAA, 20mM HEPES by Invitrogen, 1mM Sodium Pyruvate by PAA, 0.1mM Non-essential Amino Acids by PAA, 2mM L-glutamine by Invitrogen, 50µM β-mercaptoethanol by Invitrogen and 1% Penicillin/Streptomycin by Invitrogen) was added to the cell pellets. Viable cell number was determined by Trypan Blue Exclusion (Invitrogen) staining.

4.5 Bleedings

Terminal bleedings were performed at the end of each experiment prior to organ removal. Mice were anesthetized with Isoflurane (Baxter) followed by left eye removal and blood collection via Vacutainer® Blood Collection Tubes (Becton Dickinson). After centrifugation tubes were stored at -20°C.

4.6 Immunological Assays

IFN- γ ELISpot

96-well Multiscreen HA Filtration Plates (MAHAS4510, Millipore) were pre-wetted with 100 μ l of PBS (Gibco) per well. Anti-mouse IFN- γ Antibody (Becton Dickinson) was diluted in coating buffer (0.1M NaHCO₃ in dH₂O, pH 9.2-9.4) and added to wells in a concentration of 3 μ g/mL. Coating took place overnight at 4°C, or 2 hours at 37°C. Blocking was performed by adding 1% BSA (Sigma-Aldrich) in PBS (Gibco) to the wells for 1 hour at 37°C.

Wells were filled in duplicates with 0.25-1x10⁶ splenocytes derived from the single cell suspension protocol. Supplementary RCAS (Becton Dickinson) was added at a final concentration of 1% in TCM. Stimuli diluted in TCM were added to the corresponding wells. As a positive control (not shown in data) ConA (Sigma) was added as a distinct stimulus. Millipore plates were kept at 37°C in a humid chamber for 16-24 hours.

Washing steps were performed by flushing wells 3 times over 15 minutes with washing buffer (PBS, Gibco and 0.1% Tween20, Sigma) at room-temperature. Biotin Anti-mouse IFN- γ Antibody (Becton Dickinson) was added to wells at 1 μ g/mL final concentration in PBS (Gibco) and incubation took place for 2 hours at 37°C in a humid chamber. Another washing cycle was performed equivalent to the one described before.

Streptavidin-Horseradish Peroxidase (Roche) at a final concentration of 0.1U/mL in PBS (Gibco) was added to the wells and incubated for 30min at 37°C. A 15 minutes washing cycle followed.

Finally a substrate solution (100mM Tris-HCl, 0.8mg/mL Diaminobenzidine and NiCl₂, all by Sigma-Aldrich) was sterile filtrated and approximately 0.15% H₂O₂ (Sigma-Aldrich) was added immediately prior to application to the washed wells at room-temperature.

After 5 minutes the reaction was ended by washing wells with cold tap water. After at least 2 hours of drying, plates were analyzed in a Bioreader 5000 (Bio-Sys) and illustrated by Graphpad Prism 5 with error bars according to the standard error of the mean, SEM of the duplicates.

Total IgG ELISA

96well MaxiSorp™ Low Protein Binding (NUNC) plates were coated with 0.31 μ g/mL rCopN in PBS (Gibco) over night at 4°C. Wells were then filled with blocking buffer (PBS, Gibco and 2% BSA, Sigma-Aldrich) incubating for 1.5 hours at 37°C. Sera were added in duplicates in serial dilutions of 1:1000 – 1:25000 in dilution buffer (PBS, Gibco and 0.1% BSA, Sigma-Aldrich) and incubated for 1.5 hours at 37°C. After washing 3 times with PBS (Gibco) including 0.05% Tween20 (Sigma-Aldrich) an Polyclonal Rabbit Anti-Mouse Ig/HRP conjugation antibody (Dako Cytomation) diluted 1:1000 in

PBS (Gibco) was applied to the wells and incubated for 1 hour at 37°C. After another washing step ABTS Liquid Substrate (Sigma-Aldrich) was added to the wells and incubated for 20 minutes in the dark prior to reading.

A Tecan Sunrise microplate reader analyzed absorbance at 405nm. Results were calculated and illustrated by Graphpad Prism 5, while error bars reflecting the SEM of the duplicates.

5 References

1. Nicolas JF, Guy B. 2008. Intradermal, epidermal and transcutaneous vaccination: from immunology to clinical practice. *Expert Rev Vaccines* 7: 1201-14
2. Giudice EL, Campbell JD. 2006. Needle-free vaccine delivery. *Adv Drug Deliv Rev* 58: 68-89
3. Dahlan A, Alpar HO, Stickings P, Sesardic D, Murdan S. 2009. Transcutaneous immunisation assisted by low-frequency ultrasound. *Int J Pharm* 368: 123-8
4. Mitragotri S, Kost J. 2004. Low-frequency sonophoresis: a review. *Adv Drug Deliv Rev* 56: 589-601
5. Ng KY. 2004. Enhancing transdermal drug transport with low-frequency ultrasound. *Drug Discov Today* 9: 913
6. Ogura M, Paliwal S, Mitragotri S. 2008. Low-frequency sonophoresis: current status and future prospects. *Adv Drug Deliv Rev* 60: 1218-23
7. Smith NB. 2007. Perspectives on transdermal ultrasound mediated drug delivery. *Int J Nanomedicine* 2: 585-94
8. Denet AR, Vanbever R, Preat V. 2004. Skin electroporation for transdermal and topical delivery. *Adv Drug Deliv Rev* 56: 659-74
9. Lerner EN, van Zanten EH, Stewart GR. 2004. Enhanced delivery of octreotide to the brain via transnasal iontophoretic administration. *J Drug Target* 12: 273-80
10. Panchagnula R, Pillai O, Nair VB, Ramarao P. 2000. Transdermal iontophoresis revisited. *Curr Opin Chem Biol* 4: 468-73
11. Coulman SA, Barrow D, Anstey A, Gateley C, Morrissey A, Wilke N, Allender C, Brain K, Birchall JC. 2006. Minimally invasive cutaneous delivery of macromolecules and plasmid DNA via microneedles. *Curr Drug Deliv* 3: 65-75
12. Kim YC, Quan FS, Yoo DG, Compans RW, Kang SM, Prausnitz MR. 2009. Improved influenza vaccination in the skin using vaccine coated microneedles. *Vaccine* 27: 6932-8
13. McAllister DV, Allen MG, Prausnitz MR. 2000. Microfabricated microneedles for gene and drug delivery. *Annu Rev Biomed Eng* 2: 289-313
14. Sivamani RK, Liepmann D, Maibach HI. 2007. Microneedles and transdermal applications. *Expert Opin Drug Deliv* 4: 19-25
15. Karande P, Mitragotri S. 2009. Enhancement of transdermal drug delivery via synergistic action of chemicals. *Biochim Biophys Acta* 1788: 2362-73
16. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. 2000. Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. *J Control Release* 65: 403-18

17. Elsayed MM, Abdallah OY, Naggat VF, Khalafallah NM. 2007. Lipid vesicles for skin delivery of drugs: reviewing three decades of research. *Int J Pharm* 332: 1-16
18. Gupta PN, Mishra V, Rawat A, Dubey P, Mahor S, Jain S, Chatterji DP, Vyas SP. 2005. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. *Int J Pharm* 293: 73-82
19. Prausnitz MR, Langer R. 2008. Transdermal drug delivery. *Nat Biotechnol* 26: 1261-8
20. Cevc G. 2003. Transdermal drug delivery of insulin with ultradeformable carriers. *Clin Pharmacokinet* 42: 461-74
21. Benson HA. 2006. Transfersomes for transdermal drug delivery. *Expert Opin Drug Deliv* 3: 727-37
22. Rai K, Gupta Y, Jain A, Jain SK. 2008. Transfersomes: self-optimizing carriers for bioactives. *PDA J Pharm Sci Technol* 62: 362-79
23. Jain S, Jain P, Umamaheshwari RB, Jain NK. 2003. Transfersomes--a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation. *Drug Dev Ind Pharm* 29: 1013-26
24. Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR. 2000. Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* 6: 1403-6
25. McKenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, Glenn GM. 2007. Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. *Vaccine* 25: 3684-91
26. Frolov VG, Seid RC, Jr., Odutayo O, Al-Khalili M, Yu J, Frolova OY, Vu H, Butler BA, Look JL, Ellingsworth LR, Glenn GM. 2008. Transcutaneous delivery and thermostability of a dry trivalent inactivated influenza vaccine patch. *Influenza Other Respi Viruses* 2: 53-60
27. Frerichs DM, Ellingsworth LR, Frech SA, Flyer DC, Villar CP, Yu J, Glenn GM. 2008. Controlled, single-step, stratum corneum disruption as a pretreatment for immunization via a patch. *Vaccine* 26: 2782-7
28. Frech SA, Dupont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, Okhuysen PC, Guerrero NH, Martinez-Sandoval FG, Melendez-Romero JH, Jiang ZD, Asturias EJ, Halpern J, Torres OR, Hoffman AS, Villar CP, Kassem RN, Flyer DC, Andersen BH, Kazempour K, Breisch SA, Glenn GM. 2008. Use of a patch containing heat-labile toxin from *Escherichia coli* against travellers' diarrhoea: a phase II, randomised, double-blind, placebo-controlled field trial. *Lancet* 371: 2019-25
29. Aguilar JC, Rodriguez EG. 2007. Vaccine adjuvants revisited. *Vaccine* 25: 3752-62
30. Tritto E, Mosca F, De Gregorio E. 2009. Mechanism of action of licensed vaccine adjuvants. *Vaccine* 27: 3331-4

31. Guy B. 2007. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol* 5: 505-17
32. Aichinger MC, Ortbauer M, Reipert S, Zauner W, Bogner P, Froschauer E, Nowikovsky K, Lingnau K, von Gabain A, Schweyen R, Henics T. 2008. Unique membrane-interacting properties of the immunostimulatory cationic peptide KLKL(5)KLK (KLK). *Cell Biol Int* 32: 1449-58
33. Fritz JH, Brunner S, Birnstiel ML, Buschle M, Gabain A, Mattner F, Zauner W. 2004. The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a TH2-type immune response to co-injected antigens. *Vaccine* 22: 3274-84
34. Krieg AM. 2007. Antiinfective applications of toll-like receptor 9 agonists. *Proc Am Thorac Soc* 4: 289-94
35. Lingnau K, Egyed A, Schellack C, Mattner F, Buschle M, Schmidt W. 2002. Poly-L-arginine synergizes with oligodeoxynucleotides containing CpG-motifs (CpG-ODN) for enhanced and prolonged immune responses and prevents the CpG-ODN-induced systemic release of pro-inflammatory cytokines. *Vaccine* 20: 3498-508
36. Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I. 2009. IC₃₁, a two-component novel adjuvant mixed with a conjugate vaccine enhances protective immunity against pneumococcal disease in neonatal mice. *Scand J Immunol* 69: 194-202
37. Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, Wagner H. 1997. Bacterial DNA causes septic shock. *Nature* 386: 336-7
38. Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, Swatosch G, Zauner W, Kast C, Akira S, von Gabain A, Buschle M, Lingnau K. 2006. IC₃₁, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. *Vaccine* 24: 5461-72
39. Lingnau K, Riedl K, von Gabain A. 2007. IC₃₁ and IC₃₀, novel types of vaccine adjuvant based on peptide delivery systems. *Expert Rev Vaccines* 6: 741-6
40. Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, Lingnau K, von Gabain A, Andersen CS, Korsholm KS, Andersen P. 2006. Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC₃₁. *Vaccine* 24: 5452-60
41. van Dissel JT, Arend SM, Prins C, Bang P, Tingskov PN, Lingnau K, Nouta J, Klein MR, Rosenkrands I, Ottenhoff TH, Kromann I, Doherty TM, Andersen P. 2010. Ag85B-ESAT-6 adjuvanted with IC₃₁ promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. *Vaccine* 28: 3571-81
42. Riedl K, Riedl R, von Gabain A, Nagy E, Lingnau K. 2008. The novel adjuvant IC₃₁ strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice. *Vaccine* 26: 3461-8
43. Tammiruusu A, Haveri A, Pascolo S, Lahesmaa R, Stevanovic S, Rammensee HG, Sarvas M, Puolakkainen M, Vuola JM. 2005. Clearance of Chlamydia pneumoniae infection in H-2 class I human leucocyte antigen-A2.1 monochain transgenic mice. *Scand J Immunol* 62: 131-9

44. Wizen B, Nystrom-Asklin J, Cortes C, Tinnereim A. 2008. Role of CD8(+)T cells in the host response to Chlamydia. *Microbes Infect* 10: 1420-30
45. Tammiruusu A, Penttila T, Lahesmaa R, Sarvas M, Puolakkainen M, Vuola JM. 2007. Intranasal administration of chlamydial outer protein N (CopN) induces protection against pulmonary Chlamydia pneumoniae infection in a mouse model. *Vaccine* 25: 283-90
46. Guereña-Burgueno F, Hall ER, Taylor DN, Cassels FJ, Scott DA, Wolf MK, Roberts ZJ, Nesterova GV, Alving CR, Glenn GM. 2002. Safety and immunogenicity of a prototype enterotoxigenic Escherichia coli vaccine administered transcutaneously. *Infect Immun* 70: 1874-80
47. Yu J, Cassels F, Scharton-Kersten T, Hammond SA, Hartman A, Angov E, Cortes B, Alving C, Glenn G. 2002. Transcutaneous immunization using colonization factor and heat-labile enterotoxin induces correlates of protective immunity for enterotoxigenic Escherichia coli. *Infect Immun* 70: 1056-68
48. Mozafari MR, MyLibrary Ltd. 2007. Nanomaterials and nanosystems for biomedical applications. pp. xvii, 159 p. Dordrecht, The Netherlands: Springer
49. Cevc G, Gebauer D, Stieber J, Schatzlein A, Blume G. 1998. Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochim Biophys Acta* 1368: 201-15

CURRICULUM VITAE

CHRISTIAN SCHENK

Born on the 19th of March, 1987 in Vienna
Austrian citizen
Bennogasse 22 / 16
1080 Vienna, Austria
+43 - (0) - 650 555 2002
cn.schenk@gmail.com



EDUCATION

1994 - 1997	Elementary school Zeltgasse, Vienna
1997 - 2005	High school BGRG8 Albertgasse, Vienna Graduation with distinction
2005 - 2010	Molecular Biology at the University of Vienna (10th semester) Focus on Immunology/Microbiology, Developmental Biology, Business in Biotech
2006 - present	Business Administration at the Vienna University of Economics and Business Administration (minor activity)
2010 - present	Study course for Medical Representative at the Danube University Krems

EMPLOYMENT

- 07/2005** **Internship at the Molecular Oncology, Vienna General Hospital (AKH), Vienna**
Department of Obstetrics & Gynecology, Group of Molecular Oncology
Research: Univ.-Prof. Dr. phil. Robert Zeillinger
- 07/2007 - 06/2008** **Internship at the Research Institute of Molecular Pathology (IMP), Vienna**
Cell polarity establishment during development in *C. elegans*
Research: Carrie Cowan, PhD
- 07/2008** **Internship at Life Science Austria Vienna Region (LISA VR), Vienna**
Research on life sciences in international biotechnology clusters
- 03/2009 - 11/2009** **Intercell AG, Vienna**
Diploma Thesis on the Tcell research project investigating the immunopotential of IC31® as a Vaccine Enhancement Patch
Research: O. Univ.-Prof. Dr. Alexander von Gabain, Eszter Nagy, PhD, Benjamin Wizel, PhD
- 05/2010 – 06/2010** **Internship at ERP Fund, Vienna**
Due-diligence: technology research, market analysis, patent examination and financial plan assessments for pre-seed and seed financing

PUBLICATION **Cortical domain correction repositions the polarity boundary to match the cytokinesis furrow in *C. elegans* embryos**
Research Institute of Molecular Pathology
Schenk C, Bringmann H, Hyman AA, Cowan CR / May, 2010 / Development

SKILLS **Excellent German and English in writing and speech, basics in French, superb computer, software and internet skills**

ACTIVITIES **Treasurer of the Molecular Biological Society (MBS)**
A social network of young life scientists focusing on intellectual scientific exchange and social interaction

Symposia registrations:

Semmering Vaccine Symposium 2009 “*Vaccines in the light of immune therapy and therapeutic antibodies*” (2009)

International Symposium “*100 years Poliovirus from Discovery to Eradication*” (2009)

Passionate member of several dance ball opening committees, including the Vienna Philharmonic Ball 2010, the Ball of Industry and Technology 2009 & 2010 & 2011, the Moscow Ball 2010 and the Theresianisten Picknick 2010.