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1 ABBREVIATIONS

APC	Antigen-presenting cell
BSA	Bovine serum albumin
CMIS	Common mucosal immune system
ConA	Concanavalin A
Cpn	Chlamydia pneumoniae
CT	Cholera Toxin
CTB	Cholera Toxin Subunit
CTL	Cytotoxic effector T lymphocyte(s)
DAB	3.3´-Diaminobenzidine
DAMP	Damage-associated molecular pattern
DC	Dendritic cells(s)
DMEM	Dulbecco´s modified Eagle medium
EDTA	Ethylendiamineteracetic acid
ELISpot assay	Enzyme-linked immunospot assay
FACS	Fluorescence-activated cell sorting
FasL	Fas Ligand
FCS	Fetal calf serum
FITC	Fluorescin Isothiocyanat
GEEC	GPI-Enriched Endocytic Compartments
GPI-AP	Glycosylphosphatidylinositol - anchor protein
HBc	Hepatitis B virus core
HBV	Hepatitis B Virus
HBVc	Hepatitis B Virus core
IC31®	Intercell´s adjuvant consisting of L-KLK and ODN1a
i.d.	intradermal
IFN	Interferon
IFN-γ	Interferon-gamma
IL	Interleukin
i.m.	intramuscular

i.p.	intraperitoneal
ISCOM	Immunostimulating complex
KLK	$KLK(L)_5KLK$ consisting of L-amino acids (L-KLK)
LN	Lymph nodes
LNC	Lymph node cells
LSM	Lymphocyte Separation Medium
M cells	Microfold cells
MACS	Magnet-assisted cell sorting
MALT	Mucosa-associated lymphoid tissues
MHC	Major histocompatibility complex
NALT	Nasopharynx-associates lymphoid tissue
NGF	Nerve growth factor
NOD	nucleotide oligomerization domain
ODN	Oligodeoxynucleotide(s)
ODN1a	Phosphodiester-substituted single-stranded ODN containing 13 deoxy (-iosine, -cytosine) motifs [o-$d(IC)_{13}$]
O/W	Oil-in-water emulsion
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline
PE	Phycoerythrine
PMA	Phorbol-12-myristate-13-acetete
PRR	Pattern-recognition receptor
QC-IV	Quality Control – In Vivo
SA-HPO	Streptavidin-horseradish peroxidase
s.c.	subcutaneous
SFC	Spot forming cells
SOP	Standard operation procedure
SPLCs	Spleen cell(s)
SVN	Survivin peptide
TAA	Tumor associates antigens
TCR	T cell receptor
T_H	T helper cell(s)

TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TLR	Toll-like receptor
TRP-$2_{180-188}$	Tyrosinase-related protein-$2_{180-188}$
W/O	Water-in-oil emulsion

2 ABSTRACT

Intercell AG has developed a novel bi-component adjuvant, called IC31®. It is a synthetic TLR9 agonist that has been shown to efficiently elicit dominant T_H1-type in a mixed T_H1/T_H2-type immune response.

This study at hand was designed to characterize and determine the ability of IC31® to induce potent $CD8^+$ T cell responses against clinically relevant cancer peptides upon systemic and mucosal administrations.

Therefore, six promising tumor-associated antigens were selected for testing their immunogenicity in combination with this novel adjuvant. Distinct Survivin and Tyrosinase-related protein 2 (TRP-2) derived peptides with defined human and mouse MHC class I molecule restricted $CD8^+$ epitopes, which makes them highly attractive as target for T cell based immune strategies against cancer, were used as antigens for immunization studies. The results of the screening for immunogenicity demonstrated the efficiency of the classical formulation of IC31® in supporting antigen-specific immune responses by specifically increasing IFN-γ production against SVN_{19-28}, SVN_{57-64} and TRP-$2_{180-188}$ peptides in central lymphoid organs.

Furthermore, differences on the potency of IC31® nano in terms of inducing $CD8^+$ T cell responses by the use of different immunization routes (subcutaneous, intramuscular, intradermal and intraperitoneal) were determined in adjuvanted SVN_{57-64} and TRP-$2_{180-188}$ vaccinated C57BL/6 mice. Groups of animals immunized with SVN_{57-64} or TRP-$2_{180-188}$ peptides and IC31® elicited higher IFN-γ production in comparison to groups vaccinated with antigen only after injection with all the above mentioned routes. Subcutaneous (s.c.) injection was superior to all other routes in terms of inducing antigen-specific responses *in vivo*. Both peptides formulated with the adjuvant were utilized for immunization studies but adjuvanted SVN_{57-64} failed in eliciting antigen-specific $CD8^+$ T cell responses. Therefore SVN_{19-28} was used as specific Survivin derived peptide for further experiments.

Parallel to this study, the efficacy and efficiency of IC31® nano in inducing cellular immune responses against SVN_{19-28} and TRP-$2_{180-188}$ peptides upon

mucosal (intranasal) as well as systemic applications (intradermal) was determined.

The data of the intradermal vaccination study revealed the high potency of IC31® in inducing systemic TRP-$2_{180-188}$ specific cytotoxic $CD8^+$ T responses in central lymphoid organs as well as at mucosal sites. Further, the data upon systemic (i.d. and s.c) application of the vaccine showed the induction of sustained long lasting immune responses by activation of memory $CD8^+$ T cell without the help of $CD4^+$ T cells in spleens and lymph nodes.

Furthermore, IC31® improved the induction of a high systemic T_H1 immune response *in vivo* upon systemic intradermal route vaccination using the second tumor-associated antigen candidate, the Survivin derived peptide epitope $SVN_{19-28.}$ The obtained data revealed that effector $CD8^+$ T cells were not polyfunctional because only IFN-γ and any other cytokine production was evaluated in central lymphoid organs and at mucosal sites.

While IC31® nano demonstrated the great potency to improve the cellular immune response upon systemic application, mucosal administration of the adjuvant never supported the induction of an antigen- specific $CD8^+$ T cell immune response against the cancer peptides tested. Therefore, intranasal administration seemed not to be a promising immunization route against cancer.

Altogether the results indicated the high efficiency of IC31® nano in eliciting a systemic $CD8^+$ T cell immune response in central lymphoid organs and at mucosal sites following intradermal vaccination. Additionally, TRP-$2_{180-188}$ and SVN_{19-28} are suitable candidates for the development of a vaccine against cancer.

3 ZUSAMMENFASSUNG

Intercell AG entwickelte ein aus zwei Komponenten bestehendes Adjuvant IC31®. Es ist ein synthetischer TLR9 Agonist, der eine dominierende T_H1 in einer gemischten T_H1/T_H2 Immunantwort auslöst.

Diese Studie wurde zur Charakterisierung und Bestimmung von einer IC31® induzierten $CD8^+$ T Zellen-Immunantwort gegen klinisch relevante Krebspeptide nach systemischer und mukosaler Applikation geplant. Dafür wurden sechs vielversprechende $CD8^+$ Epitope enthaltende Tumor- assoziierte Peptide ausgewählt, die des Weiteren hinsichtlich ihrer Immunität in Kombination mit IC31® getestet wurden. Survivin und Tyrosinase-related protein 2 (TRP-2) stammende Peptide besitzen definierte humane und murine MHC Klasse I zugehöriges $CD8^+$ Epitope und dienen als Target für eine T Zellen-basierende Immunstrategie gegen Krebs. Diese Peptide wurden als Antigene für die angewendeten Immunisierungsstudien verwendet. Die erhobenen Resultate zeigten das Potential von klassischem IC31®, eine erhöhte IFN-γ Produktion gegen SVN_{19-28}, SVN_{57-64} und TRP-$2_{180-188}$ in zentralen lymphoiden Organen zu induzieren.

Aufgrund der Ergebnisse der erhobenen Daten wurden folglich die Unterschiede in der Effizienz von IC31® nano im Bezug auf die Einleitung einer $CD8^+$ T Zellen-Immunantwort in Kombination mit zwei der vielversprechenden Peptide (SVN_{57-64} und TRP-$2_{180-188}$) bei der Verwendung unterschiedlicher Applikationsrouten (subkutan, intramuskulär, intradermal und intraperitoneal) untersucht. Alle angewendeten Routen lösten in SVN_{57-64} oder TRP-$2_{180-188}$ in Kombination mit IC31® immunisierten Tieren, höhere IFN-γ Produktionen im Vergleich zu Antigen allein immunisierte C57BL/6 Mäuse aus. In zentralen lymphoiden Organen induzierte subkutan (s.c.) Injektion im Vergleich zu allen anderen Routen die höchste Antigen-spezifische Immunantwort *in vivo*. Beide Peptide wurden für folgende Experimente verwendet, jedoch konnte keine weitere Antigen-spezifische $CD8^+$ T Zellen-Immunantwort im Bezug auf Adjuvant und SVN_{57-64} Applikation detektiert werden. Deshalb wurde für weitere Immunisierungsstudien SVN_{19-28} als spezifisches Survivin Peptide verwendet.

Aufgrund der vielversprechenden Ergebnisse der unterschiedlichen Immunisierungsrouten in C57BL/6 Mäusen, wurde der Fokus dieser Studie auf die Charakterisierung des Potenzials von IC31® nano, eine systemische SVN_{19-28} oder TRP-$2_{180-188}$ spezifische zelluläre Immunantwort mittels mukosaler (intranasal) sowie systemischer (intradermal) Applikationen auszulösen, gelegt.

Die erhobenen Resultate der intradermalen Impfstudien zeigten, dass IC31® aufgrund seiner hohen Effizienz, systemische TRP-$2_{180-188}$ spezifische cytotoxische $CD8^+$ T Zellen in zentralen lymphoiden Organen, sowie in mukosalen Bereichen aktiviert.

Zusätzlich wurde in dieser Studie das Potenzial von IC31® nano diesbezüglich des Auslösen einer langanhaltenden TRP-$2_{180-188}$ spezifischen $CD8^+$ T Zellen-Immunantwort nach systemischer (i.d. und s.c.) Applikation, mittels IFN-γ Produktion in zentralen lymphoiden Organen getestet. Die Daten demonstrierten, dass zentrale $CD8^+$ Gedächtniszellen ohne die Hilfe von $CD4^+$ Zellen in Milzen und Lymphknoten gebildet und nach *ex vivo* Restimulierung mit dem Antigen erneut aktiviert wurden.

Des Weiteren zeigte IC31® hinsichtlich des zweiten Krebspeptids, SVN_{19-28} die Induktion einer hohen systemischen T_H1 Immunantwort folglich systemischer intradermalen Impfung *in vivo*. Die erhobenen Daten weisen die Aktivierung von Effektor $CD8^+$ T Zellen auf, die jedoch aufgrund IFN-γ und keiner weitere Cytokinproduktion nicht polyfunktional in zentralen lymphoiden Organen sowie in mukosalen Bereichen sind.

Die erhobenen Daten dieser Studie zeigten, dass IC31® als systemisches aber nicht als mukosaler Adjuvant eine Antigen spezifische systemische $CD8^+$ T Zellen-Immunantwort in lymphoiden Organen, sowie in den Lungen auslöst. Die intranasale Applikation erwies sich als keine aussichtsreiche Immunisierungsroute.

Zusammengefasst zeigen die Ergebnisse einerseits das große Potential des Adjuvanten IC31® als Bestandteil dermaler Impfstoffe eine systemische $CD8^+$ T Zellen-Immunantwort in zentralen lymphoiden Organen sowie in mukosalen Bereichen gegen spezifische Krebspeptide auszulösen. Des Weiteren erweisen sich TRP-$2_{180-188}$ und SVN_{19-28} als bevorzugte Impfstoffkandidaten.

4 INTRODUCTION

4.1. Adjuvants

Adjuvants by definition are substances or molecular complexes that enhance, sustain and accelerate antigen-specific immune responses and therefore improve the efficiency of vaccines (1-4).

Vaccines based on some pathogens, like bacteria and viruse as well as vaccines based on proteins and peptides show insufficient immunogenicity when administered alone. Therefore the addition of an adjuvant is necessary to improve the evocation of protective and long lasting immune response (2, 5).

Effective adjuvants are on the one hand immunostimulatory agents (Toll-like receptor (TLR) ligands, cytokines, saponins and bacterial exotoxins) that stimulate immune responses (T-helper (T_H) 1 or T_H2 immune response), or on the other hand carriers (mineral salts, emulsions, liposomes, visosomes, immunostimulating complexes (ISCOM)) that present the antigen to the immune system, including the formation of a depot and controlled release of the antigen. Figure 1 summarizes the main types of adjuvants and their specific properties as immunostimulator and/or carrier (1-3).

Alum (mineral salt) and MF59^{TM} (a squalane oil-in-water emulsion) are adjuvants with different properties. Both are approved for human use and enhance induction of T_H2 immune response (2, 3). Cholera Toxin (CT) originally derives from *Vibrio cholera*. It is an enterotoxin that as effective adjuvant enhances mucosal and systemic T_H2 cell responses to co-injected antigens (4, 6). It is composed of a pentameric B subunit (CTB) that binds to GM-1 receptors, while subunit A (CTA) is responsible for toxicity. CTB has the capacity to act as efficient adjuvant by improving induction of an antigen-specific cytotoxic T lymphocyte (CTL) response via intranasal immunization route. The main problem for the use of CTB as adjuvant in humans is the high toxicity. This is not the case in mice and therefore it was used as an additional adjuvant control for intranasal immunization route during this study (2, 3, 7, 8) .

Alum and M59^{TM} are less attractive for vaccines against intracellular pathogens and cancer that require the induction of antigen-specific T cell immune

responses. Therefore, Intercell AG has developed an adjuvant, called IC31® that was designed to induce a potent and sustain cellular and humoral immune response in mice and humans (9-12).

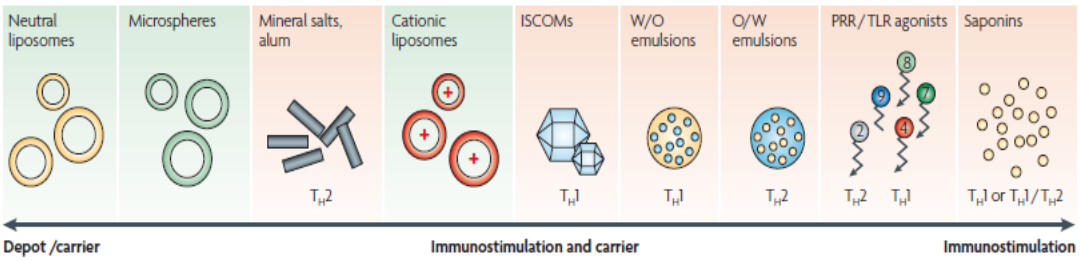


Figure 1: Main types of adjuvants. These are adjuvants that contain single or both properties (carrier and/or immunostimulator). Some compounds (red background) have the ability to direct the responses towards T helper (T_H) 1 or T_H2 immune response. (2)

4.1.1. IC31®

IC31® adjuvant is a potent immunostimulator inducing antigen-specific cellular and humoral immune responses without eliciting any pro-inflammatory side effects (10, 11, 13). The bi-component adjuvant is a mixture composed of a cationic peptide KLKLLLLLKLK (KLK_5KLK; KLK) and a single-stranded DNA phosphodiester, containing dimeric deoxy-Inosine/deoxy-Cytosin repeats (ODN1a; 5'ICICICICICICICICICICICIC-3'). These two components are combined in a molar ratio of 25:1 of KLK:ODN1a and generate in a synergistic way the immunostimulatory effect of IC31® (10, 14).

KLK is a synthetic derivate of a cationic antimicrobial peptide (CAMP) that mimics structural elements originally derived from sapecin B. (12, 15) The immunopotentiating effect of IC31® derives from KLK that has on the one hand the potency to induce antigen-specific type-2 T cell immune responses and on the other hand the capacity to promote the association of antigens to antigen presenting cells (APCs). This APC activation specifically leads to up-regulation of surface major histocompatibility complex (MHC) molecules and co-stimulation

molecules such as CD40, CD80 (B7.1) and CD86 (B7.2). The ability of KLK to activate APCs is more efficient in the presence of ODN1a (10, 13, 15, 16).

Beside APC activation, KLK has the potential to facilitate the cytoplasmic delivery of ODN1a and antigen by an APC, such as Dendritic cells (DC) (10, 15, 17). Therefore, the cationic peptide assembles into a β-sheeted aggregate that enhances complex formation by entrapping the oligonucleotide (ODN1a) and the antigen. Moreover, the process of delivery is enhanced by the specifically association of the generated complex with the negatively charged lipid rafts on the DC surface. This interaction leads through an endocytotic uptake (mostly clathrin-dependent, caveolae-dependent or GEEC-pathway) and internalization of ODN1a and antigen into the DC cytosol. After delivery, the complex disassembles and ODN1a and the antigen are transported to their intracellular targets, while KLK use GPI-AP/GEEC recycling system to interact with the plasma membrane in the cell periphery (17, 18).

Furthermore, the cationic peptide is able to mediate the formation of a vaccine depot at the injection site to control and maintain a long-term antigen and adjuvant release (15).

The second component of IC31® is a non-CpG oligonucleotide (ODN1a) that represents its adjuvantive effects as a synthetic TLR9 agonist by signaling via TLR9/MyD88 signaling pathway. Thereby, the transcription factor NF-κB is activated and initiates antigen specific type 1 T cell immune response (9, 10).

Intercell AG has established two different formulations of IC31® which are constituted of the same single components (KLK and ODN1a), but differ in their particle size. The classical formulation of IC31® generates a 5-10µm complex. On the other hand the nano formulation of the bi-component adjuvant forms a particle with a size less than 200nm. The advantages of nanoparticulated adjuvantive systems are the improved uptake of vaccines by APCs and therefore the promotion of an effective immune response as well as the use for different therapeutic route vaccinations (19, 20).

The single components, KLK and ODN1a from the novel immunopotentiator IC31® enhances in combination with an immunogenic antigen, DC activation that results in a potent antigen-specific type 1 dominated mixed type

1/type 2 immune responses that are necessary for priming $CD4^+$ Th, cytotoxic T lymphocytes (CTL) and B cells (IgG1 and IgG2a) (5, 10, 16).

4.2. T cell activation

IC31® initiates an effective more type 1 than type 2 T cell immune response, including activation of naïve T cells which requires antigen uptake by an APC. APCs, such as DC start maturation after binding pathogen association molecular pattern (PAMP) to pattern recognition receptors (PRR), such as Toll-like receptors (TLR), nucleotide oligomerization domain (NOD)-like receptors and others. This APC activation results in combination with inflammatory cytokines (TNF and type I inerferones) in switching DCs from a inactivated and resting to an activated state that leads through up-regulation of MHC molecules, expression of co-stimulation molecules as well as expression and secretion of cytokines and chemokines. These facilities allow DCs to migrate from the thymus to the secondary lymphoid organs where they complete maturation and present the processed antigen to naïve T cells (10, 21, 22).

Antigen presentation on the cell surface of APCs and antigen recognition by $CD4^+$ and $CD8^+$ T cells is MHC molecule restricted. MHC molecules are subdivided into two classes, class I and class II, which mediate antigen presentation by using different antigen processing pathways. MHC class I present 8-10 amino acid long peptides derived from intracellular protein antigens to $CD8^+$ T cells, while MHC class II molecules presents processed peptides from extracellular protein antigens to $CD4^+$ cells. Furthermore, the antigen/MHC molecule complex is recognized by a surface αβ-T –cell receptor (TCR) on T cells. For a full T cell activation the association of co-stimulation molecules like CD80 (B7.1) and CD86 (B7.2) with CD28 on the surface of $CD8^+$ T cells and CD40 interaction with CD40L on the surface of $CD4^+$ T cells, are efficient. These interactions affirm fully functional T cell activation. CD80 (B7.1)/CD28 association provokes survival and proliferation of $CD8^+$ effector T cells, while CD40/CD40L interaction plays a role in enhancing and controlling B cell as well as $CD8^+$ T cells

activation (21, 22). Figure 2 summarizes the main types of effector T cells and their specific functions.

After T cell activation by APCs, distinct cytokine production enhances naïve T cell proliferation and differentiation. The activated T helper cells ($CD4^+$) proliferate and differentiate into effector cells and can be distinguished between T_H1 and T_H2 cells. While T_H1 cells secrete cytokines such as Interleukin (IL)-2, Interferon (IFN)-γ, Tumor necrosis factor (TNF)-α and granulocyte macrophage colony stimulating factor (GM-CSF) that promote the activation of macrophages and cytotoxic T cells as well as the production of opsonizing antibodies like IgG2a and IgG2b, for pathogen depletion. T_H2 express cytokines, such as IL-4, IL-5, IL,6, IL-10 and IL-13 that play key roles in humoral immunity by supporting differentiation and proliferation of naïve B cells as well as production of non-opsonizing antibodies (23-25).

$CD8^+$ T cells differentiate to cytotoxic T lymphocytes (CTL) that are effector cells which induce potent cytoloytic killing mechanisms against tumors and intracellular pathogens, including viruses, some bacteria and protozoa (26). CTLs are cytotoxic effector $CD8^+$ T cells and therefore antigen presentation by APCs is MHC class I restricted. CTL stimulation leads to cytokine production such as IFN-γ, IL-2 as well as TNF-α and TNF-β. These cytokines mediate increased macrophage activation, while IL-2 secretion and IL-2 receptor expression is elicited by presentation and recognition of an antigen/MHC complex. The cytokine IL-2 binds to IL-2 receptor with high affinity and triggers in an autocrine manner proliferation and differentiation of a naïve $CD8^+$ T cell into an effector cytotoxic T cell. In some instances $CD4^+$ T cells that are MHC class II restricted, secrete IL-2 in a paracrine manner and thereby supports $CD8^+$ effector T cell activation (24, 26). Therefore, $CD4^+$ T helper cells play a key role to generating and maintaining long-lasting humoral and cellular immune responses (25) .

CTL are effector cells that provoke efficient and rapid cell death of the target cell by the release of lytic components (perforine, granzymes) or direct cell-cell interaction (Fas ligand (FasL)). Perforine and granzymes are stored in cytotoxic granules that are transported to the surface of the target cells after CTL activation. Perforin, a pore-formin protein, perforates the cell membrane and

allows cytotoxic components and granzymes (serin proteases) to enter the target cell where it degrades DNA by initializing an enzyme cascade. Specifically, Granzyme B is one of the most efficiently granzymes and has the capacity to promote apoptotic cell death of the target cell by caspase activation (23, 27).

An alternative killing mechanism of CTLs is a cell-cell interaction (Fas-FasL). Fas receptor on the target cell and FasL on CTLs belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family and are the main components of this alternative cytotoxic pathway. The ligand, FasL stimulates after binding the Fas receptor on the infected target cell, a caspase cascade that furthermore elicit DNA degradation and apoptotic cell death of the target cell (24, 28).

Proliferation phase of antigen-specific cytolytic $CD8^+$ T cell response is followed by the formation of memory cells. Memory cells increase the protection of the host by qualitatively better and more rapid usage of effector function upon antigenic stimulation than naïve T cells. Memory cells derive from effector T cell and have protective capacity. They are subdivided into two subtypes based on their anatomical location: central memory cells (T_{CM}) and effector memory cells (T_{EM}). T_{CM} are located in secondary lymphoid organs, such as spleens, lymph nodes and blood that contain a great expansion potential. They contain CD62L and CCR7 as main markers in comparison to $CD62L^-CCR7^-$ T_{EM} cells that exhibit a rapid cytotoxic capacity. T_{EM} cells are specifically located in non-lymphoid tissues (e.g., liver, lung, intestine) and at mucosal sites (21, 29).

However, an effective type 1 T cell immune response includes the activation of CTL against intracellular pathogens as well as tumor-associated antigens (TAA) (10).

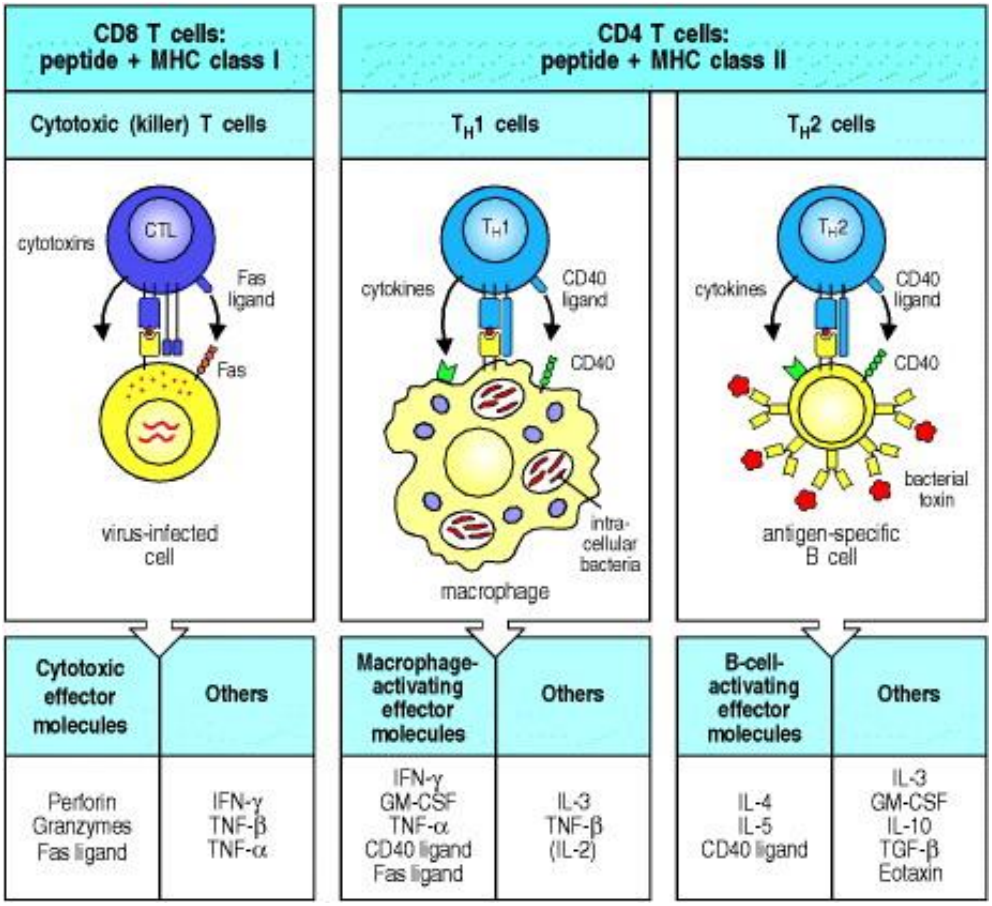


Figure 2: Main types and function of effector T cells. Adapted from Janeway, Tracers and Walport; Immunobiology: The Immune System in Health and Disease, Fifth Edition, Garland Science 2001.

4.3. Targets for T cell based immune strategies

The aim of cancer vaccine is to use tumor-associated antigens (TAA) as target and mediate an antigen-specific T cell immune response against cancer (30).

The largest number of CTL- responses has been identified for melanoma associated TAAs (31). One of these TAAs is the Tyrosinase-related protein-2 (TRP-2). It is a 519-amino-acid membrane protein including dopachrome tautomerase activity. While TRP-2 is involved in melanin synthesis and melanocyte differentiation in normal adult cells it is also widely expressed in melanoma patients (32, 33). This cancerogen expression of the protein results in

overcoming of self-tolerance and thereby in elicitingof a TRP-2 specific CTL anti-tumor immunity. TRP-$2_{180-188}$ is one specific $CD8^+$ epitopes of TRP-2. It is presented by MHC class I haplotypes (HLA-A*0201 and H-2 K^b) that is recognized by both human and murine CTLs and it is regarded as one of the most promising TAA candidates for an anti-tumor vaccine (30, 34).

Most of the identified $CD8^+$ epitope peptides are melanocyte specific and cannot be used for cancer vaccine against nonmelanocyte derived tumors. Therefore, Survivin (SVN) a small, 16.5-kDa protein and member of inhibitor of apoptosis family, is involved in cell cycle progression-, cell proliferation- and cell death pathways. It is characterized as a TAA that has the capacity to mediate anti-tumor CTL immune responses against solid tumors, such as lung, breast, prostate, colon, and pancreas cancer as well as hematopoietic malignancies. Therefore peptides of the protein represent a promising target for a cancer vaccine. Survivin is beside the appearance in different types of cancer also frequently expressed in normal adult cells. This leads to the overcome of self tolerance to elicit CTL anti-tumor immunity (35, 36). Survivin peptides are presented such as TRP-2 by mouse H-2 K^b and human HLA-A201 MHC class I haplotypes on DCs. Survivin is a protein that contains different $CD8^+$ epitopes, like SVN_{82-89} and SVN_{57-64} peptide that are homologous to human peptide and mediates specific CTL immune response. . A further specific $CD8^+$ epitope of the SVN protein is the SVN_{53-67} that generates both $CD4^+$ T helper and $CD8^+$ CTL immune response (37).

Both characterized tumor-derived proteins, TRP-2 and SVN contain defined human and murine MHC class I molecule restricted $CD8^+$ epitops that makes Survivin derived peptides and TRP-$2_{180-188}$ as target highly attractive for T cell based immune strategies against cancer (30, 36).

Furthermore, Hepatitis B Virus (HBV) induces acute and chronic hepatitis that results in the development of hepatocellular cancer. The nucleocapsid of HBV is a 27nm particle composed of multiple single polypeptides, called Hepatitis B virus core (HBc or HBVc). HBc can be used as a specific target for the immune system to regulate infection (38). HBc antigen generates spontaneously immunogenic particles that are presented by an APC-like B cell and elicit Th1 and CTL responses. The activated $CD4^+$ T helper cells help on the one hand in CTL

stimulation as well as in developing a long-term $CD8^+$ T cell memory response (39-41). Therefore it was used as an additional antigen to determine the long lasting immune response of $CD8^+$ T cells during this study.

4.4. Mucosal immune system and intranasal vaccination

The mucosal immune system includes the epithelial surface of the genitor-urinary, intestinal and upper and lower respiratory tract. The epithelium generates a physical barrier that forms the first line of immunological defense and thereby avoids pathogen invasion. However, epithelial cells have also the ability to recognize PAMPs by binding to PRRs, such as TLR. This results in DC and macrophages activation by cytokine and chemokine secretion as well as initiation of innate and antigen-specific adaptive immune responses (42, 43). Previous studies showed that mucosal DCs contain TLR9 in early endosomes that recognizes CpG region in bacterial and viral ssDNA as well as IC31® which contains a TLR9-mediated immune-enhancing activity. This capacity utilizes IC31® to act as systemic as well as mucosal adjuvant (4, 10, 44).

Anigen-specific mucosal immune responses are initiated by Microfold (M) cells that are found in the follicle-associated epithelium and are overlaid from mucosa-associated lymphoid tissues (MALT). M cells are responsible for antigen delivery by transepithelial vesicular transport (transcytosis) from the aerodigestive lumen to the Nasopharynx-associated lymphoid tissue (NALT) in the respiratory tract or Peyer´s patches in the gastrointestinal tract. Both, NALT and Peyer´s Patches are known as IgA inductive sites and are the main components of MALT (2, 20, 42). All immunocompetent cells (T cells, B cells, Th cells, CTLs and antigen presenting cells) that are required for the induction of an effective immune response, are concentrated in these specific lymphoid tissues. The antigen activated B and T cells migrate from the IgA inductive sites via lymphatic system and circulation through mucosal effector sites, such as lamina propria in respiratory-, intestinal- and the reproductive tract. The common mucosal immune system (CMIS) allows communication between organized mucosa-associated lymphoid tissues and effector sites. At the effector sites the induction of antigen-

specific mucosal antibody (IgA and IgG) responses as well as T_H1-cell- and CTL-dependent immune responses was determined in previous observations. IgA is the primary immunoglobulin (Ig) on mucosal surfaces and protects the host from pathogen invasion at these sites (4, 42, 43, 45, 46).

Studies have demonstrated that mucosal immune responses are most locally protective when induced by mucosal vaccine amplification (oral, intranasal (i.n.), intrarectal or intravaginal). By the use of oral or intranasal administration, mucosal vaccines are delivered safely and elicit strong antigen-specific mucosal and systemic immune responses. These vaccines avoid pathogen invasions at mucosal sites as well as expansion into the systemic circulation by inducing antigen-specific T_H1 and/or T_H2 immune responses (4, 43).

Intranasal route vaccination is a promising immunization strategy in terms of lymphoid tissue (NALT) in the nose that indicates in advance cell and humoral mediated antigen-specific immune responses. A further advantage of intranasal vaccine administration is the requirement of much smaller doses of antigens to induce mucosal and systemic immune responses (42).

4.5. Cutaneous immune system and intradermal vaccination

The skin is one of the largest organs of the mammalian body. It forms a physical barrier and thereby avoids pathogen invasions. Furthermore, the skin plays also an important role in eliciting antigen-specific adaptive immune responses that includes specific immune cells, such as mast cells, macrophages, specific dendritic cells (Langerhans cells and dermal dendritic cells) and T cells (47).

The skin is composed of two layers: the epidermis, that includes keratinocytes and Langerhans cells (LCs) that generates a physical barrier, and the dermis, which contains APCs and is necessary for thermoregulation, immune surveillance and blood supply for the epidermis (48, 49).

However, the epidermis contains a high density of specific DCs, called Langerhans cells (LC). These cells express different TLRs, especially TLR9 on the early endosomes of LCs. TLR9 plays an important role to guide the choice of

different adjuvants, like the TLR9 agonist IC31® (10, 50, 51). LCs are well characterized DCs in humans and mouse and differ phenotypiclly from antigen presenting cells in other tissues, as well as in terms of their function. LCs aims the induction of CTL and play an essential role for anti-tumor immunity *in vivo* (52).

LCs are immature DCs that are activated by antigen binding to specific PRRs. By capturing and antigen processing, maturation starts by expressing MHC class I or class II and co-stimulatory molecules beside down regulation of Langerin and E-cadherin expressions on the cell surface. The mature cells migrate by chemotxis to secondary lymph nodes where they efficiently present the antigen to T cells and initiate antigen-specific immune responses. Further, the maturation and migration of LCs to the paracortical region (T cell zone) of lymph nodes is stimulated by secretion of cytokines such as IL-1 β and TNF-α (50, 51, 53-55).

Further advantages of the skin are the easy accessibility and high immunogenicity which makes it attractive for intradermal vaccine application. Intradermal route is a simple, rapid and safe vaccination strategy (54).

5. RESULTS

5.1. Initial screening for immunogenicity of antigenic peptides formulated with IC31®

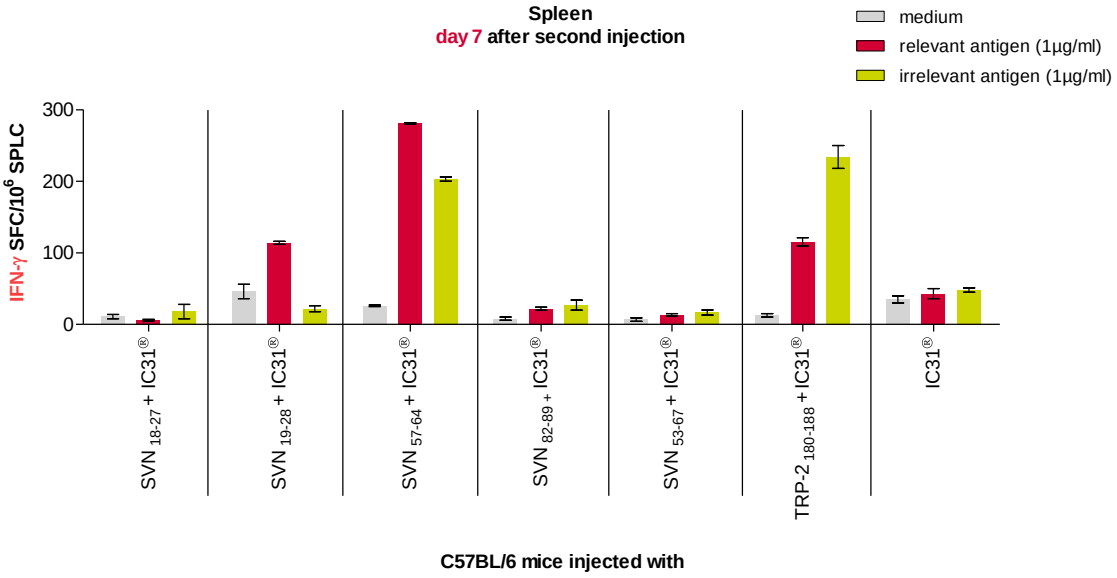
A reasonable argument for an initial screening for immunogenicity of tumor-associated antigens with IC31® was the limited information on the efficacy and efficiency of this novel adjuvant in eliciting potent $CD8^+$ T cell responses against clinically relevant antigens. Therefore, this study was designed to determine the immunogenicity of IC31® adjuvanted Survivin and Tyrosinase-related protein-2 derived peptides. Six promising tumor-derived peptides were selected based on their defined human and murine MHC class I molecule restricted $CD8^+$ epitope that makes them highly attractive as target for cytotoxic T cells (CTLs) responses (30, 34, 37).

Therefore, an initial screening was set up using tumor-associated antigens, namely SVN_{18-27}, SVN_{19-28}, SVN_{57-64}, SVN_{82-89}, SVN_{53-67} and TRP-$2_{180-188}$, were tested IFN-γ ELIspot assay was used to observe differences in antigen-specific T cell responses induced by those antigens in combination with IC31®.

The obtained IFN-γ ELIspot data indicated that mice vaccinated with SVN_{19-28} (IATFKNWPFL), SVN_{57-64} (CFFCFKEL) and TRP-$2_{180-188}$ (SVYDFFVWL) and adjuvanted in IC31® , elicited significant $CD8^+$ T cell responses in spleens (Figure 3A) and lymph nodes (iliac) (Figure 3B) in comparison to the negative control group, animals injected with IC31® alone.

In summary, the obtained data indicate that SVN_{19-28}, SVN_{57-64} and TRP-$2_{180-188}$ when immunized with classical IC31® were highly immunogenic in central lymphoid organs in comparison to all other adjuvanted Survivin derived peptides. Different irrelevant peptides where further used to avoid cross-presentation.

A



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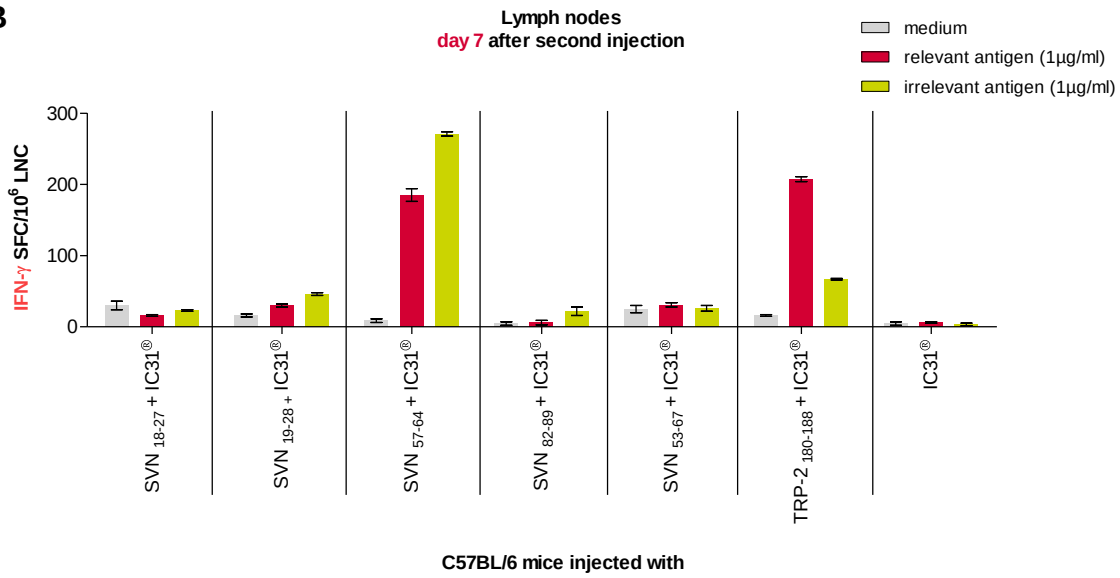


Figure 3. Analysis of CD8$^+$ T cell response upon 2nd s.c. co-injection of distinct tumor-derived peptides with classical IC31® by IFN-γ ELIspot assay. C57BL/6 mice were immunized on day 0 and 14 s.c. (100µl, tail base) with different antigens (60µg) plus classical IC31® (a mixture of 100nmole KLK and 4nmole ODN1a in 10mM Tris/ 135mM NaCl buffer). For control purpose, mice were injected with classical IC31® only. **(A)** Spleens and **(B)** lymph nodes (iliac) were harvested on day 7 after second immunization. Cells from each group were plated in triplicates at

$5x10^5$ cells/well and *ex vivo* restimulated with the specific relevant antigen (SVN_{18-27}, SVN_{19-28}, SVN_{57-64}, SVN_{82-89}, SVN_{53-67} and TRP-$2_{180-188}$; 1µg/ml) and with an irrelevant antigen (TRP-$2_{180-188}$ for Survivn peptides and SVN_{57-64} for TRP-$2_{180-188}$; 1µg/ml). Medium was used as negative control. IFN-γ production in spleen and lymph node cells was determined 24 hours after restimulation in an ELIspot assay. The results are shown as number of spot forming cells (SFC)/$1x10^6$ total cells ± SD.

5.2. Determination of $CD8^+$ T cell mediated immune responses in central lymphoid organs after immunization with TRP-$2_{180-188}$ and IC31® in mice vaccinated by different injection routes

The obtained results from the screening for immunogenicity of different tumor-derived peptides revealed that TRP-$2_{180-188}$, SVN_{57-64} and SVN_{19-28}, in combination with the classical formulation of IC31® where highly immunogenic. To investigate differences on the efficacy and efficiency of IC31® nano in the induction of a potent antigen-specific $CD8^+$ T cell response in spleens and draining lymph nodes, different injection routes and the nano formulation of IC31® were used. IC31® nano is composed of the same single components as the classical formulation (L-KLK and ODN1a), but the mean particles forms a size of >200nm. For all following experiments the nano formulation of IC31® was used.

To obtain $CD8^+$ T cell responses in central lymphoid organs, C57BL/6 mice were vaccinated three times with TRP-$2_{180-188}$ formulated with IC31® by Bernhard Roeder of the Quality Control – In Vivo (QC-IV) department, at two-week intervals (d0 and 14 and 28), subcutaneously (s.c.) at the tail base, intramuscularly (i.m.) at the hind limbs, intradermally at the tail base and intraperitoneally (i.p.). For control purpose, mice were immunized with antigen only. On day 12 after second and third immunization, spleens and draining lymph nodes were harvested by Bernhard Roeder and single cell suspensions were prepared. IFN-γ ELIspot assay and Intracellular Cytokine Stainings were performed as described in Material and Methods.

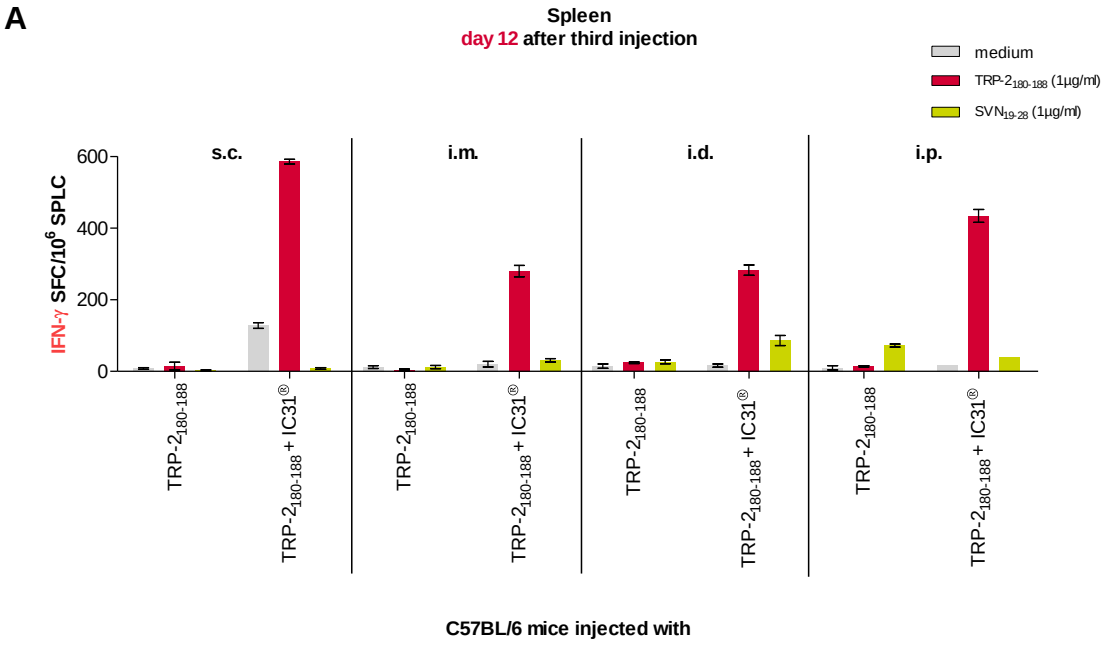
The ELIspot data indicate that all different TRP-$2_{180-188}$ and IC31® route vaccinated mice, elicited high $CD8^+$ T cell responses in central lymphoid organs in comparison to peptide alone vaccinated mice. After the third immunization all antigen plus adjuvant immunized mice produced a significant higher amount of IFN-γ in spleens (Figure 4A) and draining lymph nodes (Figure 4B) than after the second injection (Figure 5).

Regarding the results of i.m. vaccinated mice, which were twice immunized intramuscularly and once intradermally, no significant IFN-γ production

was observed in inguinal lymph nodes, although a $CD8^+$ T cell response was observed in spleens on day 12 after third injection. These IFN-γ ELIspot data indicate that mixed vaccination routes did not effectively induce an immune response in draining lymph nodes.

Figure 5 compares IFN-γ production in spleens (Figure 5A) and lymph nodes (Figure 5B) of TRP-$2_{180\text{-}188}$ and IC31® vaccinated C57BL/6 mice by the use of different injection routes over 42 days. In central lymphoid organs, s.c. injection was superior to all other routes in terms of inducing antigen-specific responses *in vivo*. The IFN-γ production from splenocytes in ELIspot was comparable with the Intracellular Cytokine Staining (data not shown).

In summary, using different routes of vaccination with TRP-$2_{180\text{-}188}$ and IC31®, induced a strong antigen-specific T cell response after the third immunization especially the s.c. injected groups.



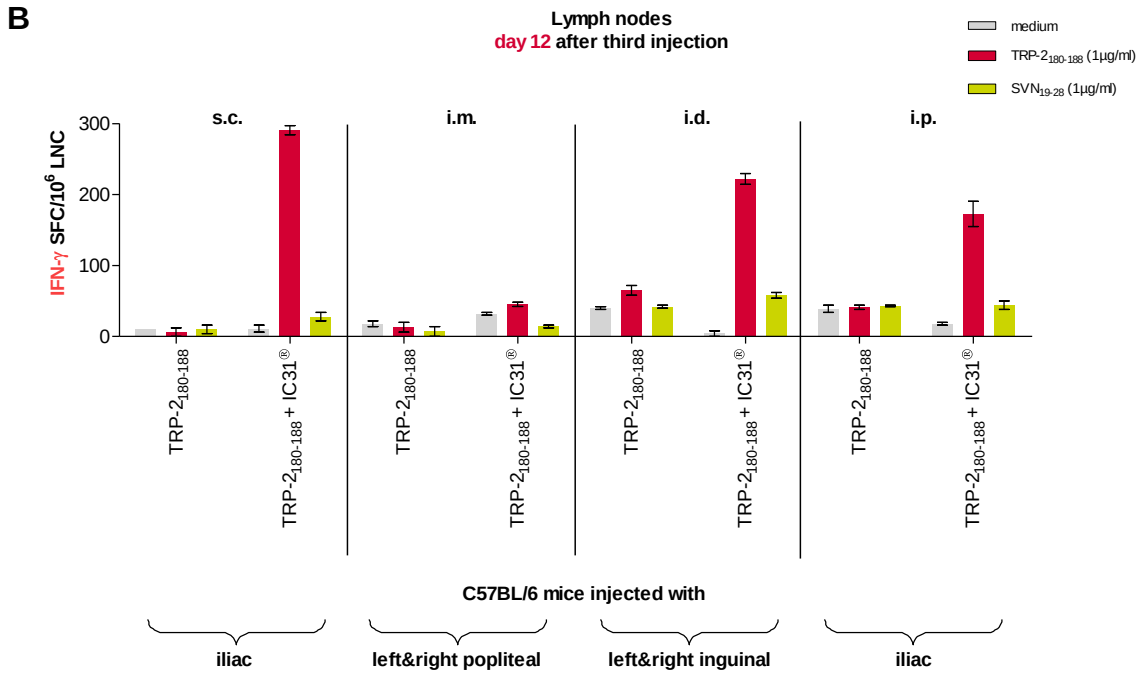


Figure 4. IFN-γ ELISpot analysis of CD8$^+$ T cell responses upon immunization with TRP-2$_{180-188}$ and IC31® in central lymphoid organs. C57BL/6 mice were immunized on day 0, 14 and 28, s.c. (100µl; tail base), i.m. (50µl/hind limb), i.d. (2x50µl, tail base) and i.p. (100µl) with TRP-2$_{180-188}$ (60µg) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer). For control purpose, mice were injected with antigen only. **(A)** Spleens and **(B)** draining lymph nodes were harvested on day 12 after third immunization. Cells from each group were plated in triplicates at 2.5x10^5 cells/well for spleens and at 5x10^5 cells/well for draining lymph nodes and *ex vivo* restimulated with the relevant antigen (TRP-2$_{180-188}$; 1µg/ml; red) and with an irrelevant antigen (SVN$_{19-28}$; 1µg/ml; green). Medium was used as negative control. IFN-γ producing in spleen and draining lymph node cells was determined 24 hours after restimulation in an ELIspot assay. The results are shown as number of spot forming cells (SFC)/1x10^6 total cells ± SD.

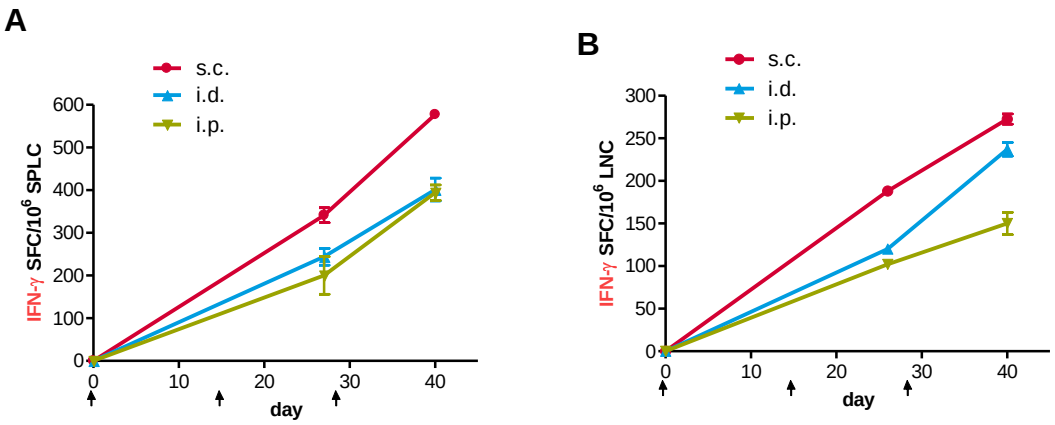


Figure 5. Injection route comparison of IFN-γ production in lymphocytes of vaccinated mice. C57BL6 mice were injected three times on day 0, 14 and 28, s.c., i.d. and i.p. with TRP-$2_{180\text{-}188}$ (60µg/mouse) plus IC31®. On day 26 and 42 organs were removed and cells harvested. IFN-γ production on ELIspot in spleen **(A)** and draining lymph node cells **(B)** was determined. The results are shown as the number of spot forming cells (SFC)/1x10^6 total cells ± SD. The arrows indicate immunization time points.

5.3. Identification of splenic $CD8^+$ T cell meditated immune responses in mice vaccinated with SVN_{57-64} and IC31® vaccinated mice by the use of different injection routes

The data from the screening for immunogenicity of different tumor-derived peptides revealed that not only TRP-$2_{180-188}$, but SVN_{57-64} in combination with classical IC31® induced strong $CD8^+$ T cell responses in central lymphoid organs. In the following experiment different injection routes as well as the nano formulation of IC31® were used to determine differences in the potential of IC31® to induce antigen-specific T cell immune responses in spleens and draining lymph nodes.

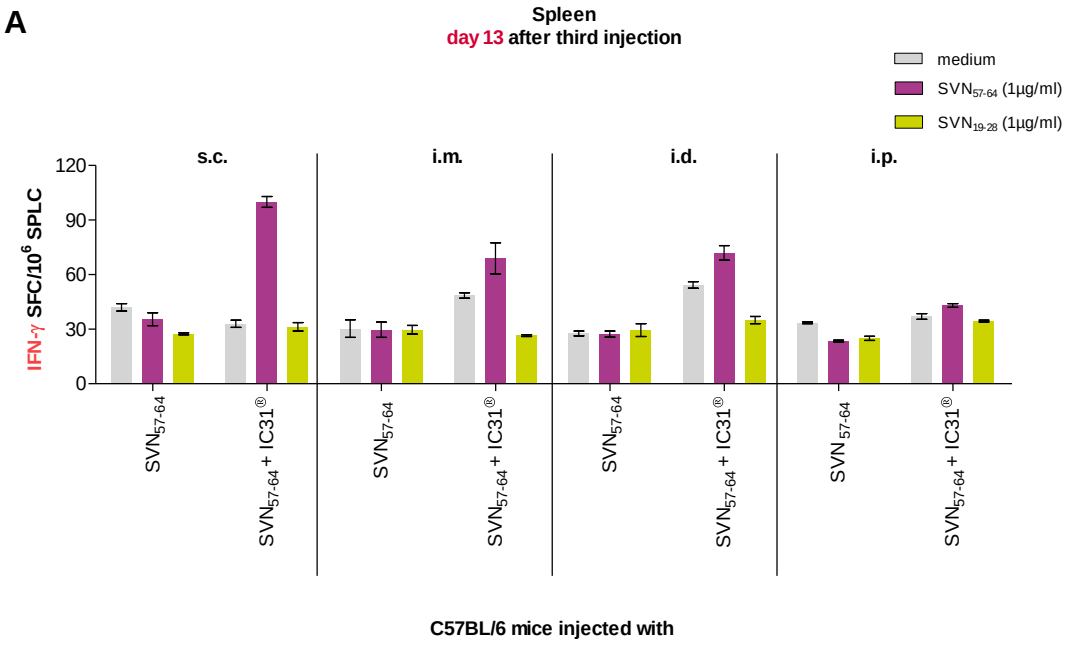
The same experimental set up as described in 5.2. was used. Bernhard Roeder immunized C57BL/6 mice with SVN_{57-64} and IC31® by the use of different injection routes (s.c., i.m., i.d. and i.p.) at three time points in a two-week interval (d0, 15 and 30). For control purpose, mice were immunized with antigen only.

On day 15 after second and day 13 after third immunization, spleens and draining lymph nodes were harvested by Bernhard Roeder of the QC-IV department and single cell suspensions were prepared. IFN-γ ELIspot assay and Intracellular Cytokine Staining were performed as described in Material and Methods.

The ELIspot data indicate that after two immunizations of IC31® adjuvanted SVN_{57-64} did not induce a significant immune response in central lymphoid organs. On day 13 after third injection all different route vaccinated mice with antigen and IC31®, induced a weak T cell immune response in spleen cells (Figure 6A) in comparison to mice vaccinated with antigen alone. In the lymph nodes no antigen-specific $CD8^+$ T cell response was observed (data not shown).

Figure 6B compares IFN-γ production in spleen cells of SVN_{57-64} plus IC31 vaccinated mice by the use of different injection routes over 43 day. S.c. injection induced the strongest T cell response compared with all other injection routes. The IFN-γ production from splenocytes in ELIspot was comparable with the Intracellular Cytokine Staining (data not shown).

In summary, s.c. injections demonstrated to be the best route for the *in vivo* induction of splenic antigen-specific T cell responses. In following experiments these results were not reproducible and therefore SVN_{19-28} was used as Survivin derived peptide for further analysis.



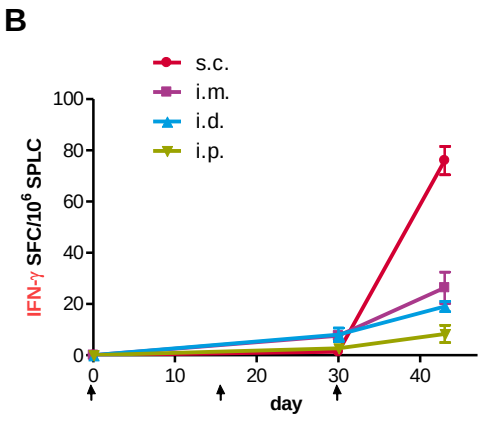


Figure 6. IFN-γ ELISpot analysis of $CD8^+$ T cell responses upon immunization with SVN_{57-64} and IC31® in spleens. C57BL/6 mice were immunized on day 0, day 15 and day 30, s.c. in the tail base (100µl), i.m. (50µl/hind limb), i.d. (2x50µl, tail base) and i.p. (100µl) with SVN_{57-64} (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris

buffer). For control purpose, mice were injected with antigen only. **(A)** Spleens were harvested on day 13 after third injection. Cells from each group were plated in triplicates at $1x10^6$ cells/well for spleens and at $5x10^5$ cells/well for lymph nodes and *ex vivo* restimulated with the relevant antigen (SVN_{57-64}; 1µg/ml; purple) and with an irrelevant antigen (SVN_{19-28}; 1µg/ml; green). Medium was used as negative control. IFN-γ producing in spleen and draining lymph node cells was determined 24 hours after restimulation in an ELIspot assay. The results are shown as number of spot forming cells (SFC)/$1x10^6$ total cells ± SD. **(B)** IFN-γ production on ELIspot in spleen cells was determined over 43 days. The results are shown as the number of spot forming cells (SFC)/$1x10^6$ total cells ± SD. The arrows indicate immunization time points.

5.4. Determination of systemic $CD8^+$ T cell immune responses in central lymphoid organs and lungs induced by tumor-derived peptides adjuvanted with IC31® upon intradermal and intranasal route vaccination

Previous studies showed that by the use of intranasal and intradermal administration, vaccines elicit strong antigen-specific mucosal and systemic antigen-specific T_H1 and/or T_H2 immune responses (4, 43).

Therefore, the focus of this study was to determine and characterize the efficiency of IC31® nano in improving the induction of a $CD8^+$ T cell immune response as systemic and mucosal adjuvant against specific tumor-derived peptides, like TRP-$2_{180-188}$ and SVN_{19-18} upon intradermal and intranasal application.

An experimental setup with three immunizations in bi-weekly intervals was used. C57BL/6 mice were intrademally (tail base) and intranasally immunized with antigen plus IC31®, performed by Bernhard Roeder of the QC-IV department. For control purpose, mice were injected with antigen and IC31® only. For intranasal administration Isofluran was used as anesthesia. To exclude errors during intranasal administration, an adjuvant control group plus antigen was included. Cholera Toxin B Subunit (CTB) is a potent adjuvant, often inducing even non-specific stimulation, and in combination with the antigen, it induced a potent immune response (7).

As positive control for Flow cytometry analysis and FlowCytomix™, Phorbol-12-myristate-13-acetete (PMA)/Ionomycin and Concanavalin A (ConA) were used. PMA is on the one hand a tumor promoter that activates Kinase C in the presence of a physiological intracellular concentration of calcium. Ionomycin raises the calcium concentration and stimulates in combination with PMA intracellular cytokine production (56). ConA is on the other hand a mitogen that is known to stimulate T cells (57).

5.4.1. Induction of TRP-$2_{180-188}$ / IC31® $CD8^+$ T cell immune responses in spleens and draining lymph nodes by the use of intradermal and intranasal immunization

To investigate a systemic $CD8^+$ T cell response of adjuvanted TRP-$2_{180-188}$ vaccinated mice *in vivo*, C57BL/6 mice were immunized at three time points in a two week interval (day 0, 13 and 28) intradermally and intranasally by Bernhard Roeder of the QC-IV department. For control purpose, mice were injected with peptide and IC31® only. Due to the vaccine formulation aspects, the amount of IC31® used for the two injection routes differed as followed: 100/4 IC31® for intradermal immunization and 20/0.8 IC31® for intranasal administration. Spleens and draining lymph nodes were harvested by Bernhard Roeder on day 14 after second and third injection. Cell suspensions were prepared and IFN-γ ELIspot, Intracellular Cytokine Staining, Granzyme B Staining and FlowCytomix™ were performed as described in Materials and Methods.

By ELIspot assay IFN-γ production was evaluated in spleens (Figure 7A) and inguinal lymph nodes (Figure 7B) of TRP-$2_{180-188}$ adjuvanted in IC31® intrademally vaccinated mice over a period of 42 days. After the third injection a significant increase of $CD8^+$ T cell immune response was determined by an increasing IFN-γ production in central lymphoid organs. Intranasally administered groups did not elicit any T cell immune responses in spleen and broncheo-tracheal lymph node cells, except the adjuvant control group, antigen plus CTB (data not shown).

Regarding to Intracellular Cytokine Staining (Figure 8A), a high increase of double positive cytokine producing $CD8^+$ T cells in spleens (IFN-γ/IL-2, IFN-γ/TNF-α and TNF-α/IL-2 expression) were observed in TRP-$2_{180-188}$ plus IC31® intradermal vaccinated mice on day 14 after third injection in comparison to respective control groups. The cells were *ex vivo* restimulated with the relevant peptide TRP-$2_{180-188}$ and the irrelevant peptide SVN_{19-28} as respective control. Table 1 summarizes the obtained percentage of double positive cytokine producing $CD8^+$ T cells in spleens after third intradermal immunizations by Flow cytometry analysis.

A significant up-regulation of Granzyme B in spleens was observed upon third immunization in comparison to the control groups, antigen or IC31® only (Figure 8B). The cells were *ex vivo* restimulated with the relevant peptide TRP-$2_{180-188}$ and the irrelevant peptide SVN_{19-28} as respective control. These data reveal that TRP-$2_{180-188}$ in combination with IC31® significantly activated cytotoxic T cells in spleens after three intradermal immunizations.

For cytokine production profiling, splenocytes were plated and *ex vivo* restimulated with ConA as positive control, complete medium as negative control, TRP-$2_{180-188}$ as relevant peptide and SVN_{19-28} as irrelevant peptide. After 48 hours supernatants were taken and the cytokine production profile was performed using a FlowCytomix™ kit as described in Materials and Methods.

TRP-$2_{180-188}$ adjuvanted in IC31® vaccinated mice induced a significant high IFN-γ expression and a slight increase in IL-2, IL-6, IL-10, TNF-α and GM-CSF production after three intradermal immunizations (Table 2). IL-1α, IL-4, IL-5 and IL-17were produced under the detection limit. The obtained IFN-γ and TNF-α expression data confirm the results obtained with flow cytometry analysis. The intranasally immunized mice did not produce any of these ten cytokines, except the adjuvant control group, antigen plus CTB, which elicited IL-2, IL-6, IL-10, IL-17, GM-CSF, INF-γ and TNF-α cytokine production (data not shown).

In conclusion, the presented data indicate strong antigen-specific $CD8^+$ T cell responses in central lymphoid organs upon third intradermal injection of TRP-$2_{180-188}$ adjuvanted in IC31® vaccinated mice.

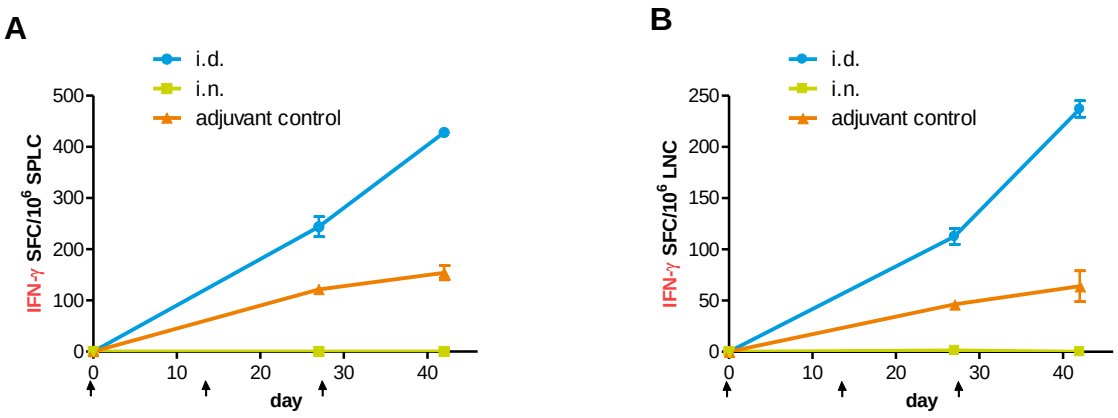
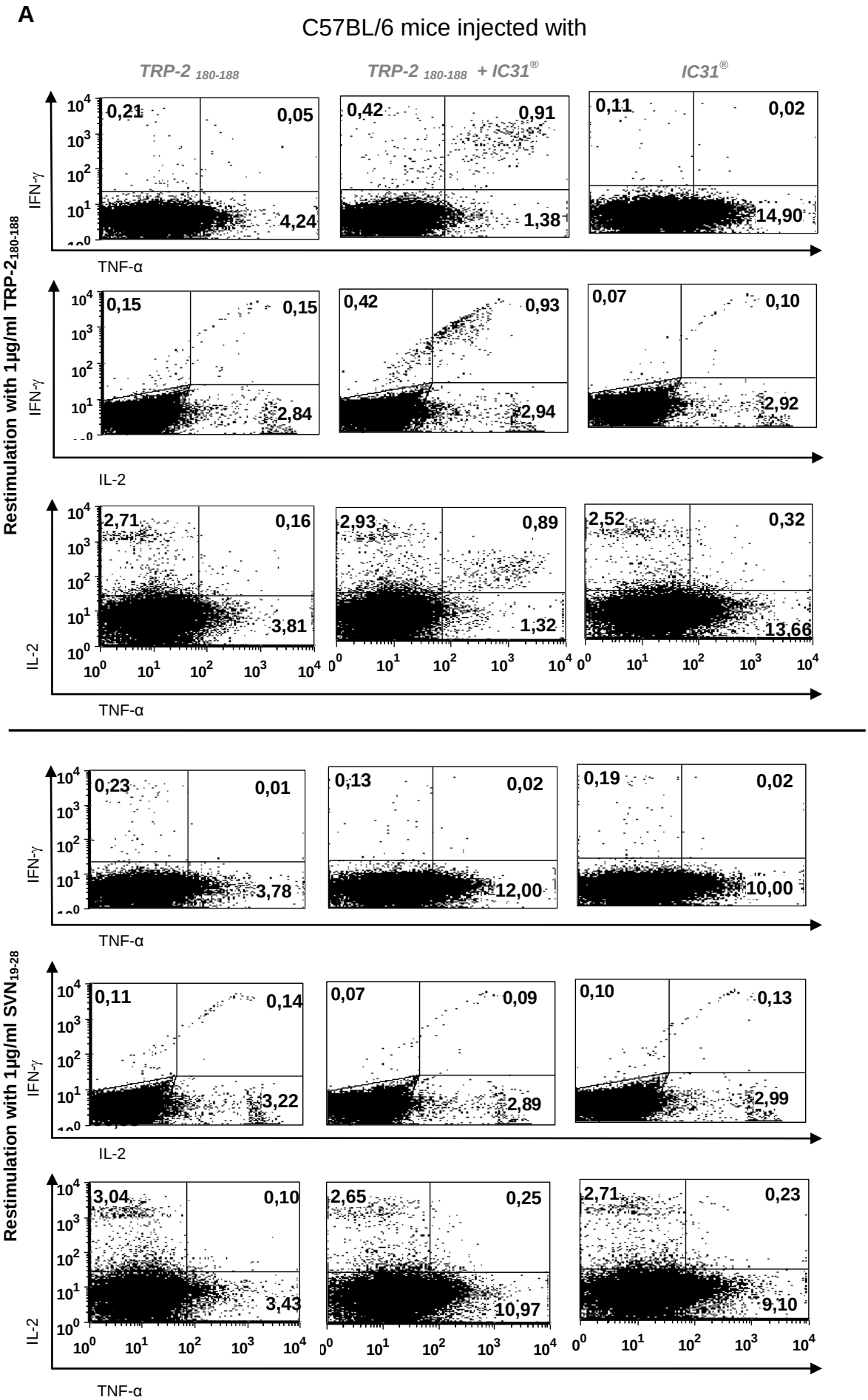


Figure 7. Analysis of IFN-γ production in spleens and lymph nodes upon intrademal and intranasal immunization of TRP-$2_{180-188}$ with IC31® over 42 days by ELIspot assay. C57BL/6 mice were immunized on day 0, day 13 and day 28 i.d. (2x50µl) and i.n. (40µl) immunized with TRP-$2_{180-188}$ (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer). For i.n. control purpose mice were immunized with TRP-$2_{180-188}$ (60µg/mouse) plus CTB (1µg/mouse; adjuvant control). **(A)** Spleens and **(B)** inguinal lymph nodes were harvested on day 14 after second and third immunization. Cells from each group were plated in triplicates at $5x10^5$ cell/well for splenocytes and at $1x10^6$ cells/well for draining lymph nodes and were *ex vivo* restimulated with the relevant antigen (TRP-$2_{180-188}$; 1µg/ml) and an irrelevant antigen (SVN_{19-28}; 1µg/ml). IFN-γ producing in spleen and draining lymph node cells was determined 24 hours after restimulation in an ELIspot assay. IFN-γ production is shown over a period of 42 days. The results are shown as number of spot forming cells (SFC)/$1x10^6$ total cells ± SD. The arrows indicate the immunization time points.

	Stimuli	Immunization	IFN-γ/IL-2	IFN-γ/TNF-α	TNF-α/IL-2
Relevant peptide	TRP-2 $_{180-188}$	Antigen + IC31®	0.9	0.9	0.9
		Antigen	0.1	0.0	0.1
		IC31	0.1	0.0	0.3
Irrelevant peptide	SVN_{19-28}	Antigen + IC31®	0.1	0.0	0.2
		Antigen	0.1	0.0	0.1
		IC31	0.1	0.0	0.2

Table 1: Summary: Antigen in combination with IC31® induced up-regulation of cytokine producing double positive $CD8^+$ T cells following intradermal immunization and *ex vivo* restimulation (relevant antigen: TRP-$2_{180-188}$ and irrelevant peptide: SVN_{19-28}); numbers are shown as percentage of double-positive cells in comparison to the respective control groups. Data were obtained by Flow cytometry analysis.

A


C57BL/6 mice injected with
TRP-2 180-188
TRP-2 180-188 + IC31®
IC31®
Restimulation with 1µg/ml TRP-2180-188
Restimulation with 1µg/ml SVN19-28
IFN-γ
TNF-α
IL-2
0,21
0,05
4,24
0,42
0,91
1,38
0,11
0,02
14,90
0,15
0,15
2,84
0,42
0,93
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2,71
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0,89
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B

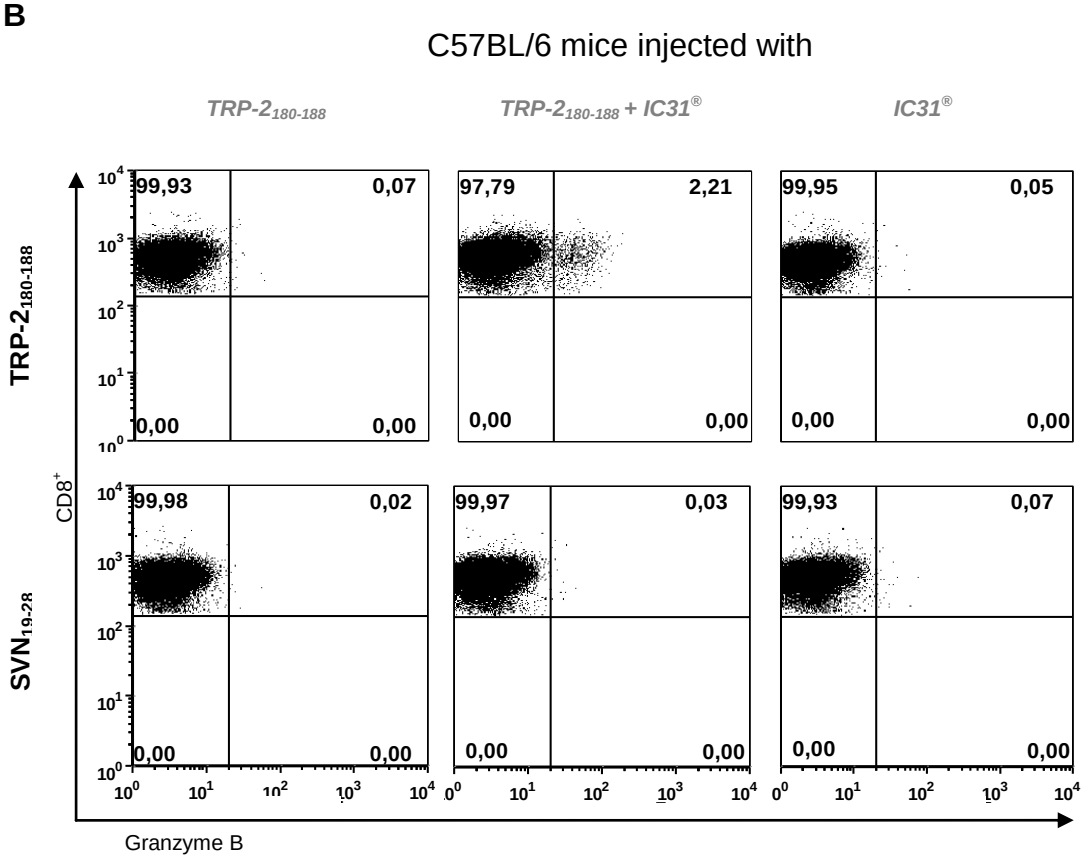


Figure 8. Flow cytometry analysis of cytotoxic CD8$^+$ T cells in spleen of TRP-2$_{180-188}$ and IC31®. C57BL/6 mice were immunized on day 0, day 13 and day 28, i.d. (2x50µl) with TRP-2$_{180-188}$ (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer). For control purpose, mice were injected with antigen and IC31® only. Spleens were harvested on day 14 after third immunization. Cells of each group were plated in triplicates at 1x10^6 cells/well and *ex vivo* restimulated with the relevant antigen (TRP-2$_{180-188}$; 1µg/ml; upper blots) and with an irrelevant antigen (SVN$_{19-28}$; 1µg/ml; lower blots). **(A)** Intracellular Cytokine Staining was performed to evaluate the percentage of cytokine producing splenic double positive CD8$^+$ T cells in mice i.d. vaccinated. **(B)** Number of Granzyme B producing CD8$^+$ T cells in spleens of i.d. immunized mice were determined. Stained cells were gated on the CD8$^+$ cells. 500.000 events per sample were acquired.

CYTOKINES	STIMULI	TRP-$2_{180-188}$	TRP-$2_{180-188}$ + IC31®	IC31®
IFN-γ	ConA	>27764,92	18280,66	19989,89
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**2414,88**	<d.l.
	SVN_{19-28}	<d.l.	<d.l.	<d.l.
TNF-α	ConA	224,95	228,1	218,74
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**9,4**	<d.l.
	SVN_{19-28}	<d.l.	<d.l.	2,3
GM-CSF	ConA	398,97	502,66	397,5
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**37,19**	23,36
	SVN_{19-28}	<d.l.	<d.l.	15,92
IL-6	ConA	771,3	614,69	795,31
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**114,77**	<d.l.
	SVN_{19-28}	<d.l.	<d.l.	<d.l.
IL-2	ConA	239,78	409,6	329,87
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**119,17**	<d.l.
	SVN_{19-28}	<d.l.	<d.l.	<d.l.
IL-10	ConA	>27764,92	18280,66	19989,89
	Medium	<d.l.	<d.l.	<d.l.
	TRP-$2_{180-188}$	<d.l.	**2414,88**	<d.l.
	SVN_{19-28}	<d.l.	<d.l.	<d.l.

Table 2. FlowCytomix™ cytokine profile of splenic lymphocytes after third intradermal immunizations of antigen plus IC31®. For control purpose, mice were injected with antigen or IC31® only. *Ex vivo* restimulation for 48hrs (positive control: ConA; negative control: complete medium; relevant antigen: TRP-$2_{180-188}$ (1µg/ml) and irrelevant antigen SVN_{19-28} (1µg/ml)); numbers are shown as pg/ml cytokine in the supernatants harvested. All other cytokines were below detection limit. (<d.l. = below detection limit).

5.4.2. FlowCytomix™ determination of specific CD8+ T cell immune responses in lungs of mice intradermally and intranasally vaccinated with TRP-2$_{180-188}$ and IC31® adjuvanted.

In parallel to IFN-γ ELIspot assay and Flow cytometry analysis, cell suspensions of lungs without the use of Lymphocyte Separation Medium, was prepared as described in Materials and Methods. To use only CD8$^+$ T cells for further analysis, cell suspensions were sorted for CD8$^+$ T cells by the use of magnetic cell sorting (MACS) as described in Material and Methods. The purity of CD8$^+$ T cells was on an average of 98% as determined by Flow cytometry analysis (data not shown).

Cells were plated for FlowCytomix™. To present antigen to purified CD8$^+$ T cells, Antigen Presenting Cells (APCs) from spleens of 3 naïve C57BL/6 mice were added (1:2). Cells were 48 hours restimulated with ConA as positive control, complete medium as negative control, TRP-2$_{180-188}$ as relevant peptide and SVN$_{19-28}$ as irrelevant peptide. After two days the supernatants were taken and FlowCytomix™ was performed as described in Materials and Methods.

The obtained results reveal that after the second intradermal immunization of TRP-2$_{180-188}$ and IC31®, IFN-γ was upregulated by CD8$^+$ T cells in lungs in comparison to a slight production of TNF-α, IL-6, IL-10, IL-2 and GM-CSF. A moderate increase of all these mentioned cytokines was determined after the third immunization (Table 3). IL-4, IL-5, IL-17 and IL-1α were produced under detection limit in the lungs after the third immunization of antigen in combination with IC31®.

The intranasally vaccinated mice did not induce any production of these ten cytokines, except for the adjuvant control group, antigen plus CTB (data not shown).

In conclusion, the data reveal the induction of CD8$^+$ T response in central lymphoid organs and at mucosal sites of TRP-2$_{180-188}$ and IC31® vaccinated mice.

CYTOKINES	STIMULI	TRP-$2_{180-188}$	TRP-$2_{180-188}$ + IC31®	IC31®
IFN-γ	ConA	<d.l.	<d.l.	>10403,84
	Medium	41,56	37,79	47,25
	TRP-$2_{180-188}$	301,02	**>23880,41**	249,93
	SVN_{19-28}	196,59	67,46	71,51
TNF-α	ConA	139,48	115,83	31,94
	Medium	14,54	<d.l.	2,3
	TRP-$2_{180-188}$	24,8	87,74	4,14
	SVN_{19-28}	9,4	<d.l.	<d.l.
GM-CSF	ConA	424,82	718,02	168,8
	Medium	46,16	26,5	57,89
	TRP-$2_{180-188}$	48,24	**296,93**	54,15
	SVN_{19-28}	25,75	25,75	28,6
IL-6	ConA	1117,72	1496,57	1351,4
	Medium	109,9	350,3	697,47
	TRP-$2_{180-188}$	185,97	**851,72**	722,33
	SVN_{19-28}	115,99	399,14	725,13
IL-2	ConA	5597,76	1845,87	361,11
	Medium	<d.l.	<d.l.	19,69
	TRP-$2_{180-188}$	3,52	**151,02**	3,52
	SVN_{19-28}	<d.l.	<d.l.	17,56
IL-10	ConA	<d.l.	<d.l.	<d.l.
	Medium	<d.l.	<d.l.	88,69
	TRP-$2_{180-188}$	6,66	**154,15**	25,62
	SVN_{19-28}	<d.l.	18,13	24,07

Table 3. FlowCytomix™ cytokine profile of mucosal lymphocytes after third intradermal immunizations of antigen plus IC31®. For control purpose, mice were injected with antigen or IC31® only. *Ex vivo* restimulation for 48hrs (positive control: ConA; negative control: complete medium; relevant antigen: TRP-$2_{180-188}$ (1µg/ml) and irrelevant antigen SVN_{19-28} (1µg/ml)); numbers are shown as pg/ml cytokine in the supernatants harvested. All other cytokines were below detection limit. (<d.l. = below detection limit).

5.4.3. Determination of cellular immune response of SVN_{19-28} with IC31® intradermally and intranasally vaccinated mice by using IFN-γ ELIspot assay

A reasonable argument for changing the Survivn derived peptide, SVN_{57-64} to SVN_{19-28}, was that only in one experiment the induction of an antigen-specific $CD8^+$ T cell immune response in combination with IC31® nano was observed after third immunization. These data were never reproducible and in following immunization studies SVN_{57-64} and the adjuvant failed in eliciting an immune response.

However, to determine systemic antigen-specific $CD8^+$ T cell responses with the nano formulation of IC31® in central lymphoid organs upon intradermally and intranasally vaccination, C57BL/6 mice were at three time points in a bi-weekly interval (day 0, 13 and 29) intradermally and intranasally immunized with SVN_{19-28} and IC31® by Bernhard Roeder of the QC-IV department. For control purpose, mice were injected with peptide and adjuvant only. Due to the vaccine formulation aspects, the amount of IC31® used for the two injection routes differed as followed: 100/4 IC31® for intradermal immunization and 20/0.8 IC31® for intranasal administration. Spleens and draining lymph nodes were harvested on day 14 after second and day 13 after third injection by Bernhard Roeder. Cell suspensions were prepared and IFN-γ ELIspot, Granzyme B Staining and FlowCytomix™ were performed as described in Materials and Methods.

Figure 9 shows IFN-γ production in spleens and inguinal lymph nodes of SVN_{19-28} adjuvanted with IC31® vaccinated mice over 42 days obtained by ELIspot assay. After the third injection, a moderate increase of $CD8^+$ T cell response was elicited in spleens (Figure 9A) and lymph nodes (Figure 9B) by IFN-γ expression. Intranasal administration did not induce any immune responses central lymphoid organs, except in the control group, antigen plus CTB, where again a weak immune response was determined (data not shown).

Regarding Granzyme B Staining a slight Granzyme B production was observed on day 13 after third intradermal immunizations in spleen cells (Figure

10). These data reveal that SVN_{19-28} in combination with IC31® activated cytotoxic T cells (CTLs) in spleens after three interdermal immunizations.

In summary, the obtained data indicate that SVN_{19-28} in combination with IC31® indicate an induction of a systemic CTLs in central lymphoid organs after third intradermal immunizations, but the $CD8^+$ T cells were not polyfuncional, because only IFN-γ production was determined.

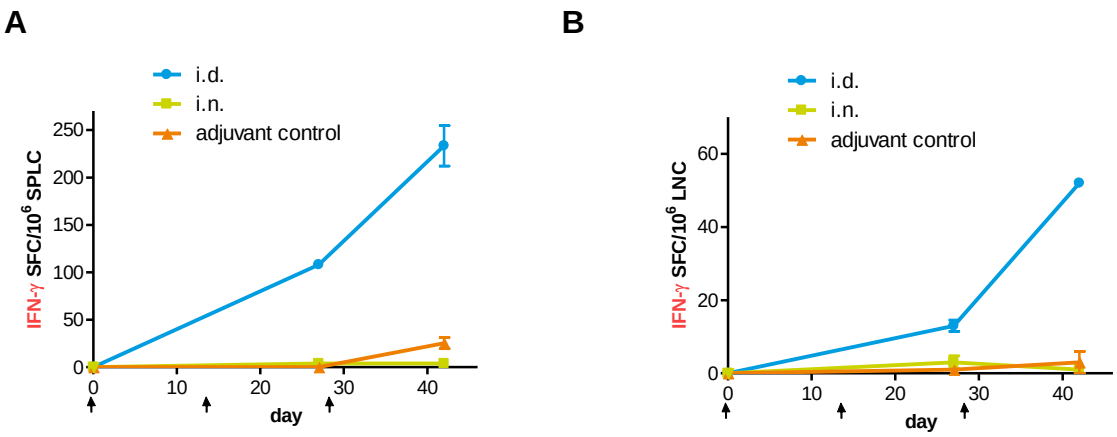


Figure 9. Analysis of IFN-γ production in spleens and lymph nodes after intradermal immunization of SVN_{19-28} and IC31® by IFN-γ ELIspot assay. C57BL/6 mice were immunized on day 0, day 13 and day 28, i.d. (2x50µl) with SVN_{19-28} (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer). **(A)** Spleens and **(B)** lymph nodes were harvested on day 14 after second and day 13 after third immunization. Cells from each group were plated in triplicated at $5x10^5$ cells/well for spleens and at $1x10^6$ cells/well for lymph nodes and were *ex vivo* restimulated with the relevant antigen (SVN_{19-28}; 1µg/ml) and with an irrelevant antigen ($TRP\text{-}2_{180-188}$; 1µg/ml). Medium was used as negative control. IFN-γ production in spleen and lymph node cells was determined 24 hours after restimulation in an ELIspot assay. The results are shown as number of spot forming cells (SFC)/$1x10^6$ total cells ± SD.

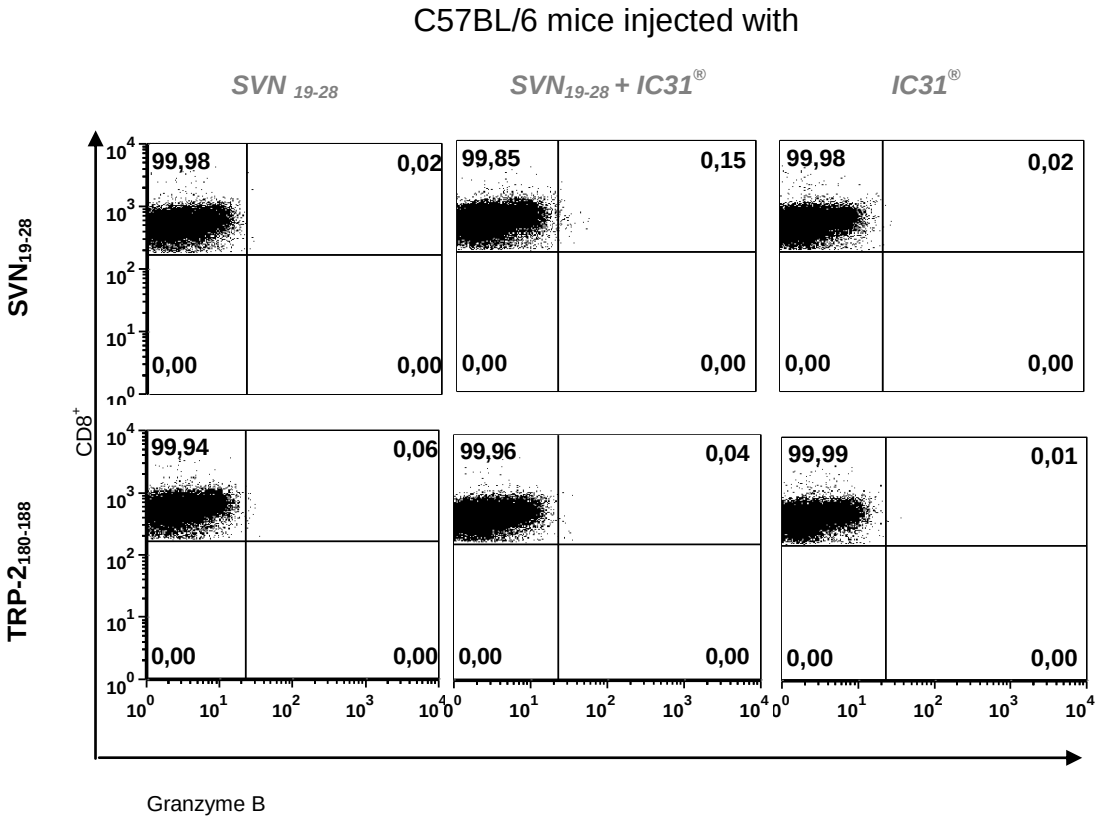


Figure 10. Flow cytometry analysis of Granzyme B producing cytotoxic CD8$^+$ T cells in spleen of SVN$_{19-28}$ adjuvanted in IC31® intradermal vaccinated mice. C57BL/6 mice were immunized on day 0, day 13 and day 28 i.d. (2x50µl) with SVN$_{19-28}$ (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer). For control purpose, mice were injected with antigen and IC31® only. Spleens were harvested on day 13 after third immunization. Cells were plated in triplicates at 1x10^6 cells/well and *ex vivo* restimulated with the relevant antigen (SVN$_{19-28}$; 1µg/ml) and with an irrelevant antigen (TRP-2$_{180-188}$; 1µg/ml). Number of Granzyme B producing CD8$^+$ T cells in spleens of i.d. immunized mice were determined. Stained cells were gated on the CD8$^+$ cells. 500.000 events per sample were acquired.

5.4.4. Determination of cytokine production in lungs and spleens of SVN_{19-28} and IC31® intradermally and intranasally vaccinated mice by using FlowCytomix™

In parallel to IFN-γ ELIspot and Flow cytometry analysis, FlowCytomix™ was performed to characterize antigen-specific systemic $CD8^+$ T cell responses by cytokine production in spleens as well as at mucosal sites.

Single cell suspensions from both organs were prepared as described in Materials and Methods. Additionally, lungs were purified without the use of LSM. The lung cells were sorted for $CD8^+$ T cells by the use of MACS sort as described in Materials and Methods. The purity of $CD8^+$ T cells was on an average of 99% as determined by Flow cytometry analysis (data not shown).

Spleen and purified $CD8^+$ T cells from lungs were plated for FlowCytomix™. Cells were *ex vivo* restimulated with ConA as positive control, complete medium as negative control, SVN_{19-28} as relevant antigen and $TRP\text{-}2_{180-188}$ as irrelevant antigen. After 48 hours supernatants were taken and FlowCytomix™ was performed as described in Materials and Methods.

The obtained data reveal high IFN-γ production in spleens of SVN_{19-28} adjuvanted in IC31® vaccinated mice and a slight increase of IL-6 and IL-10 upon intradermal immunization (Table 4). IL-4, IL-5, IL-17, IL-1α, GM-CSF, TNF-α and IL-2 were produced under detection limit.

At mucosal sites of intradermally SVN_{19-28} and IC31® vaccinated mice high amounts of IFN-γ (Table 5) were produced. IL-4, IL-5, IL-17, IL-1α, GM-CSF, TNF-α, IL-6, IL-10 and IL-2 were expressed under detection limit.

Intranasally vaccinated mice did not induce any production of these cytokines in spleens and lungs, except the adjuvant control group, antigen plus CTB (data not shown).

In conclusion, the obtained results indicate the highly efficiency of IC31® in inducing a systemic antigen specific immune response in central lymphoid organs and lungs. But these data suggest that the elicited $CD8^+$ T cells were not polyfunctional, because only IFN-γ and any other cytokine was evaluated by FlowCytomix™.

CYTOKINES	STIMULI	SVN_{19-28}	SVN_{19-28} + IC31®	IC31®
IFN-γ	ConA	12208,99	13972,9	13814,37
	Medium	<d.l.	<d.l.	<d.l.
	SVN_{19-28}	<d.l.	**326,35**	<d.l.
	$TRP\text{-}2_{180-188}$	<d.l.	<d.l.	<d.l.

Table 4. FlowCytomix™ cytokine profile of splenic lymphocytes after third intradermal immunizations of antigen plus IC31®. For control purpose, mice were injected with antigen and IC31® only. *Ex vivo* restimulation for 48hrs (positive control: ConA; negative control: complete medium; relevant antigen: SVN_{19-28} (1µg/ml) and irrelevant antigen $TRP\text{-}2_{180-188}$ (1µg/ml)); numbers are shown as pg/ml cytokine in the supernatants harvested. All other cytokines were below detection limit. (<d.l. = below detection limit).

CYTOKINES	STIMULI	SVN_{19-28}	SVN_{19-28} + IC31®	IC31®
IFN-γ	ConA	>26298,45	15689,68	18337,55
	Medium	<d.l.	3,52	<d.l.
	SVN_{19-28}	445,09	**1195,11**	71,51
	$TRP\text{-}2_{180-188}$	<d.l.	112,19	<d.l.

Table 5. FlowCytomix™ cytokine profile of mucosal lymphocytes after third intradermal immunizations of antigen plus IC31®. For control purpose, mice were injected with antigen and IC31® only. *Ex vivo* restimulation for 48hrs (positive control: ConA; negative control: complete medium; relevant antigen: SVN_{19-28} (1µg/ml) and irrelevant antigen $TRP\text{-}2_{180-188}$ (1µg/ml)); numbers are shown as pg/ml cytokine in the supernatants harvested. All other cytokines were below detection limit. (<d.l. = below detection limit).

5.5. Determination of long lasting immune responses in spleens and lymph node by using IFN-γ ELIspot assay

The last question arising from these results was, if the observed specific antigen immune responses in combination with the nano formulation of IC31® maintain a long lasting immune response. To investigate this, the $CD8^+$ T cell responses of antigen plus adjuvant vaccinated mice were analyzed at a later time point according to three immunization. Due to the long lasting aspect, the effect of a $CD4^+$ Helper peptide, Hepatitis B Core (HBc or HBVc) peptide, was included.

For this purpose, an experiment was set up, again consisting of three immunization time points (day 0, 14 and 28). C57BL/6 mice were injected bi-weekly subcutaneously, intradermally and intranasally by Bernhard Roeder, with TRP-$2_{180-188}$ in combination with IC31® or TRP-$2_{180-188}$ plus IC31® and inclusive HBc, to determine differences in the $CD8^+$ T cell responses within spleens and draining lymph nodes. For control purpose, mice were immunized with antigen and IC31® only, as well as with peptide plus HBc. Spleens and draining lymph nodes were harvested on day 11 after second, and day 12 and 70 after third injection by Berhard Roeder. IFN-γ ELIspot assay and Intracellular Cytokine Staining were performed as described in Materials and Methods. The number of spot forming cells in the IFN-γ ELIspot assay was in the following described experiment lower than in all other described experiments, because the batches of TRP-$2_{180-188}$ peptide were mixed.

The IFN-γ ELIspot data indicate that after three immunizations the observed $CD8^+$ T cell responses in draining lymph nodes (Figure 11A) and spleens (Figure 11) increased in mice immunized s.c. and i.d. with TRP-$2_{180-188}$ plus IC31® as well as with TRP-$2_{180-188}$ plus IC31® and HBc, in comparison to results after two injections (data not shown) and respective control groups. The intranasally administered mice did not induce any IFN-γ production at all time points.

The obtained results indicate that the Helper peptide (HBc) induced not a significant higher antigen-specific immune response in central lymphoid organs in combination with the results from mice, vaccinated with TRP-$2_{180-188}$ and IC31®.

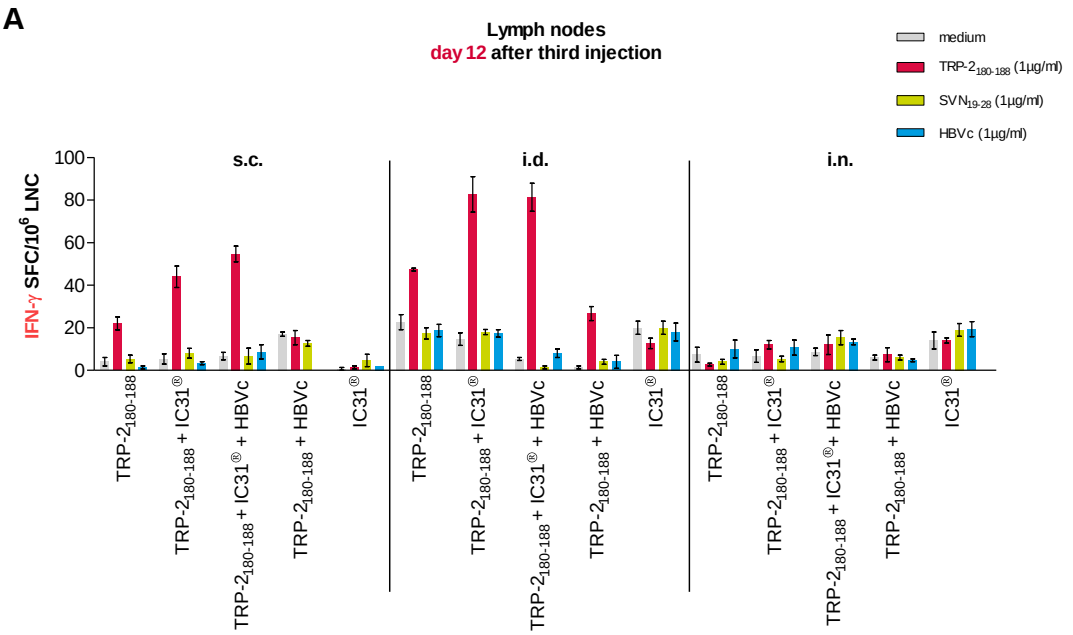
The results of IFN-γ ELIspot assay were comparable with the Intracellular Cytokine Staining (data not shown).

Figure 12 focus on intranasal and intradermal vaccination and indicate significant $CD8^+$ T cell responses in spleens (Figure 12A and C) and draining lymph nodes (Figure 12B+D) of TRP-$2_{180-188}$ plus IC31® (Figure 12A+B) as well as TRP-$2_{180-188}$ plus IC31® and HBc (Figure 12C+D) immunized mice over 98 days. These data reveal that long lasting memory $CD8^+$ T cells were activated in spleens and inguinal lymph nodes on day 70 after third intradermally vaccination of TRP-$2_{180-188}$ in combination with IC31® . The $CD4^+$ Helper peptide did not induce any significant increase of $CD8^+$ T cell responses in splenocytes and lymph node cells over a longer period of time.

The intranasal administered mice did not elicit any T cell response in primary lymphoid organs at any time point. The ELIspot data matched with the results of Flow cytometry analysis.

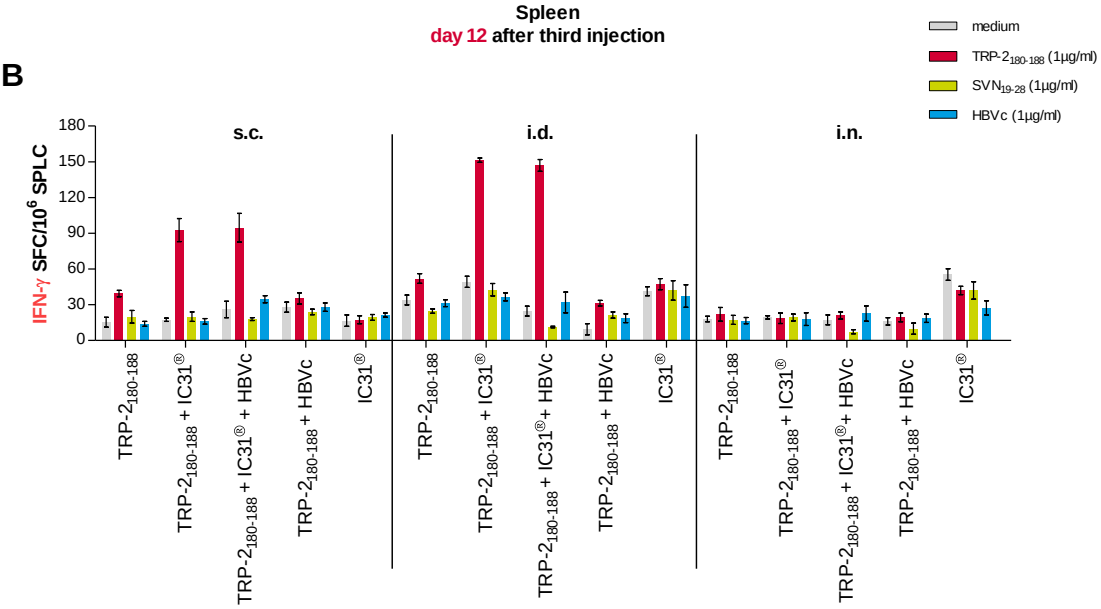
In conclusion, the obtained data indicate that the potency of IC31® nano in inducing a strong TRP-$2_{180-188}$ specific $CD8^+$ T cell responses in central lymphoid organs upon third intradermal immunizations. The vaccination of antigen plus adjuvant could elicit the formation of a depot that in terms is necessary for the induction of a long lasting immune response. The $CD4^+$ Helper peptide had no significant increasing effect on induction of a $CD8^+$ T cell response in primary lymphoid organs.

A



C57BL/6 mice injected with

B



C57BL/6 mice injected with

Figure 11. Analysis of antigen-specific CD8$^+$ T cell immune response upon 3rd immunization by IFN-γ ELIspot assay. C57BL/6 mice were immunized on day 0, day 14 and day 28, s.c. (100µl, tail base), i.d. (2x50µl) and i.n. (40µl) with TRP-2$_{180-188}$ (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer) as well as the CD4$^+$ helper peptide HBc. For control purpose, mice were injected with antigen and IC31® only. **(A)** Draining lymph nodes and **(B)** spleens were harvested on day 12 after third immunization. Cells of each group were plated in triplicates at 5x10^5 cells/well for spleens and at 1x10^6 cells/well for lymph nodes and *ex vivo* restimulated with the relevant antigen (TRP-2$_{180-188}$; 1µg/ml; red), with an irrelevant antigen (SVN$_{19-28}$; 1µg/ml; green) and CD4$^+$ helper peptide (HBc; 1µg/ml; blue). Medium was used as negative control. IFN-γ production in spleen and lymph node cells was determined 24 hours after restimulation in an ELIspot assay. **(C)** The same procedure was done with spleens on d70 after third immunization. The results are shown as number of spot forming cells (SFC)/1x10^6 total cells ± SD.

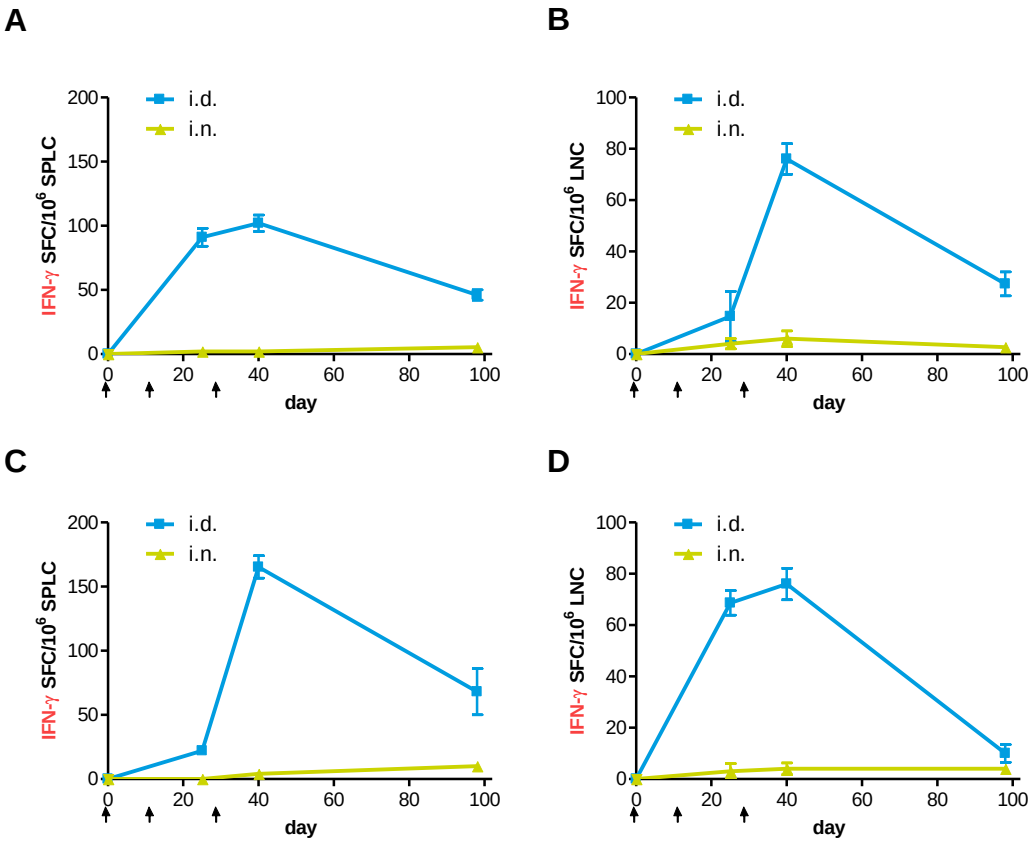


Figure 12. Analysis of IFN-γ production after intradermal and intranasal vaccination in spleens and draining lymph nodes over 98 days. C57BL/6 mice were immunized on day 14 and day 28, i.d. (2x50µl) and i.n. (40µl) with TRP-$2_{180-188}$ (60µg/mouse) plus IC31® nano (a mixture of 100nmole KLK and 4nmole ODN1a in 2.5mM Tris buffer) as well as the $CD4^+$ helper peptide HBc.intradermally. IFN-γ production in spleen **(A+C)** and lymph node **(B+D)** cells was determined 24 hours after restimulation in an ELIspot assay. **(A)** and **(B)** shows IFN-γproduction after immunization of TRP-$2_{180-188}$ adjuvanted with IC31®. **(C)** and **(D)** indicate IFN-γ production in mice, vaccinated with TRP-$2_{180-188}$, HBc as well as IC31®. The results are shown as number of spot forming cells (SFC)/1x10^6 total cells ± SD. Arrows indicate the immunization time points.

6. DISCUSSION

Recent identifications on the property of IC31® in improving an antigen-specific $CD8^+$ T cell immune response prompted us to further investigate the potential effects of the novel bi-component adjuvant in inducing a CTL response against cancer. Therefore, distinct TRP-2 and Survivin derived peptides, which are important tumor-associated antigens with restricted expression, are excellent candidates for antitumor vaccines (10, 34, 37).

Six promising clinical relevant peptides, namely SVN_{18-27}, SVN_{19-28}, SVN_{57-64}, SVN_{82-89}, SVN_{53-67} and TRP-$2_{180-188}$, were selected on the basis of eliciting CTL responses. A screening for detecting immunogenicity of the peptides as well as the adjuvantive effect of classical IC31® in terms of improving the induction of antigen-specific $CD8^+$ T cell immune responses was setup. Previous studies showed the great potency of classical IC31® in inducing potent T_H1 T cell immune responses and cytotoxic T cell activity when co-injected with immunogenic peptides or proteins (18). The data obtained during this study supported the notion that IC31® strongly enhanced the immunogenicity of SVN_{19-28}, SVN_{57-64} and TRP-$2_{180-188}$ characterized by the induction of type 1 cellular immune responses evaluated by IFN-γ production in spleens and lymph nodes. Altogether TRP-$2_{180-188}$, SVN_{57-64} and SVN_{19-28} seemed to be preferable vaccine candidates.

Further, SVN_{57-64} and TRP-$2_{180-188}$ as well as different immunization routes (s.c., i.d., i.m. and i.p.) were used to determine differences on the immunostimulatory effect of IC31® nano. This study was designed to get more information on the adjuvantive potency of IC31® nano in terms of enhancing the induction of antigen-specific $CD8^+$ T cell responses. The advantages of the development of nanoparticulated adjuvantive systems are the improved uptake of antigens by APCs and therefore the promotion of an effective immune response as well as the use for different beneficial therapeutic route vaccinations (19, 20). The obtained data of this immunization studies revealed the high efficiency of IC31® nano in eliciting an antigen-specific $CD8^+$ T cell immune response by IFN-γ production in central lymphoid organs. In general, s.c. and i.d. route vaccination were more

efficient in inducing higher antigen-specific $CD8^+$ T cell responses upon co-injection of the antigens and the adjuvant than other immunization routes (i.p. and i.m.). These efficiency derives from the high densities of Langerhans cells (LCs) that are present in the skin and enhances as specific DCs the induction of an immune response (58).

In more detail, IC31® supported the induction of a TRP-$2_{180-188}$ (SVYDFFVWL) peptide-specific $CD8^+$ T cell immune response in C57BL/6 mice. IFN-γ production increased in spleens and lymph nodes after the third vaccination and was twice as high as after second immunization except i.m. route vaccinated animals. The increased immune response may be explained by the fact that after the second immunization a number of peptide-specific T cells were already primed by IC31® adjuvanted TRP-$2_{180-188}$ that strongly were enhanced and largely expanded once again after the third immunization of the vaccine. The results of the i.m. immunized animals revealed that the use of mixed immunization routes (i.m. and i.d.) affected IFN-γ production in lymph nodes neglecting the induction of $CD8^+$ T cells.

However, the presented data showed a limited efficiency of IC31® nano in terms of inducing SVN_{57-64} specific IFN-γ production in central lymphoid organs. Three immunizations were necessary to elicit a $CD8^+$ T cell immune response. The fact that immunization studies in C57BL/6 mice assessed that SVN_{57-64} was not sufficiently immunogenic and failed to elicit immune responses in combination with IC31®, SVN_{19-28} was used for further immunization studies. SVN_{19-28} is a Survivin derived peptide which showed in the screening a strong immune response in terms of IFN-γ production in combination with the adjuvant. The lacking of $CD8^+$ T cell responses towards the adjuvanted $CD8^+$ epitope, SVN_{57-64} (CFFCFKEL) could be explained by two cysteines in the amino acid sequence that may have induced the formation of a disulfide bond in contact with oxygen. Consequently this could lead through an conformational change of the peptide (59). A suboptimal binding of the peptide through IC31® could influence the antigen presentation to APCs as well as induction of antigen-specific $CD8^+$ T cell immune responses.

Previous studies showed that by the use of intranasal and intradermal administration, vaccines elicit strong antigen-specific mucosal and systemic antigen-specific T_H1 and/or T_H2 immune responses (4, 43). Therefore the efficiency of IC31® in supporting induction of tumor-derived peptide specific immune responses as mucosal and systemic adjuvant was evaluated by immunization studies of TRP-$2_{180-188}$ or SVN_{19-28} adjuvanted in co-injected with IC31® elicited strong systemic $CD8^+$ T cell immune response in central lymphoid organs and at mucosal sites upon intradermal vaccination. These data imply the activation of specific murine and human DCs in the skin, (Langerhans cells, LCs) that express different TLRs, especially TLR9 which play an important role to guide the choice of the TLR9 agonist IC31® (10, 50, 51). LCs aim the induction of CTL and are essential for anti-tumor immunity *in vivo* and therefore intradermal route vaccination is a promising immunization route candidate (52).

Further, three i.d. immunizations of the adjuvanted peptides were necessary to get the best antigen-specific immune response. The fact that the highest immune response was achieved upon three vaccinations was already observed in previous studies (personal communication Karin Riedl).

In more detail, the data obtained from TRP-$2_{180-188}$ in combination with IC31® vaccinate mice, revealed the induction of a high systemic $CD8^+$ T cell response in terms of IFN-γ production in central lymphoid organs and at mucosal sites when delivered via the intradermal route. The induction of a strong systemic $CD8^+$ T cell immune response was further supported by FlowCytomix™ data where upon *in vitro* restimulation of spleen and lung cells, which were derived from C57BL/6 mice on day 14 after third i.d. immunization indicated beside the improved IFN-γ production, a substantial increase of IL-2, IL-6, IL-10, GM-CSF and TNF-α even more in the group immunized with IC31® adjuvanted TRP-$2_{180-188}$. Adjuvant- and peptide alone vaccinated mice did not show these highly cytokine production. Regarding specific cytokine expression, such as IL-2 that was characterized as a potent T cell growth factor, provide in combination with the proinflammatory IFN-γ and TNF-α, a set of cytokines that defines a vaccine-elicited cytolytic T cell response against specific antigens (21, 23) Concerning the pronounced production of IL-6 and IL-10 in cancer, various studies demonstrated

the importance of both cytokines, beside others to regulate tumor growth or antitumor immune responses. IL-6 on the one hand is involved in enhancing immunological responses while IL-10 influence the magnitude of $CD8^+$ T cell responses by eliciting regulatory T cells (23, 60). Further, GM-CSF upregulation in spleens and lungs could be explained by previous data that imply the involvement in immunological response enhancement as well as an effect on the maturation of LCs (55).

Additionally, the induction of an antigen specific CTL response was further supported by Granzyme B production in spleens of intradermal route vaccination C57BL/6 mice. These data indicated the potency of IC31® in strongly enhancing antigen-specific CTLs which deliver cytotoxic granules to the surface of the target cell and promote rapid and efficient cell death (27). Altogether, these data supported the TRP-$2_{180\text{-}188}$ specific cytotoxic $CD8^+$ T cell immune response-inducing effect of IC31®.

Regarding the data of the Survivin derived peptide, $SVN_{19\text{-}28}$ in combination with the adjuvant, revealed the induction of a high systemic $CD8^+$ T cell response evaluated by IFN-γ production in central lymphoid organs and at mucosal sites following intradermal application. The results indicated that together with IFN-γ production IC31® improved the generation of a $SVN_{19\text{-}28}$-specific CTL response obtained by the upregulation of Granzyme B in spleen cells of C57BL/6 mice. The data observed in this study demonstrate the enhancement of a systemic immune response by IFN-γ production in $SVN_{19\text{-}28}$ adjuvanted in IC31® animals. Nevertheless, the $CD8^+$ T cells produced were not polyfunctional as any other cytokine than IFN-γ were detected by FlowCytomix™ and Flow cytometry analysis. Altogether these data revealed the $SVN_{19\text{-}28}$-specific CTL response-inducing effect of IC31® upon intradermal administration.

Interestingly, both peptides adjuvanted in IC31® were not sufficiently immunogenic and failed in eliciting an antigen specific systemic $CD8^+$ T cell immune response in combination with IC31® in central lymphoid organs as well as at mucosal sites when delivered via intranasal route. Intranasal immunization studies of the tumor-derived peptides in combination with CTB as mucosal adjuvant led to the activation of an antigen-specific T_H1 immune response

obtained by IFN-γ production. CTB has the capacity to act as efficient adjuvant and carrier for antigen-specific cytotoxic T lymphocyte (CTL) induction and shows adjuvantiv effects via the nasal immunization route. Therefore it was used as an additional adjuvant control during this study (7, 8).

Previous data showed the adjuvantive effect of IC31® in local as well as systemic compartments following intranasal application in combination with the *Chlamydia pneumoniae* (*Cpn)* protein, CopN (unpublished data of Dominic Aschenbrenner). An explanation for the lacking tumor-derived peptide specific $CD8^+$ T cell immune response upon intranasal administration could be a suboptimal binding of the peptides through IC31®. The single component KLK usually entrap the antigen and ODN1a and therefore facilitates an efficient uptake in association of antigen to APCs and formation of a vaccine depot at the injection site (18). It could be that the antigen is weakly bound to the bi-component adjuvant and not encapsulated. Consequently, the adjuvantive effect of IC31® may not support the induction of an antigen-specific $CD8^+$ T cell immune response and additionally the adjuvant may be unable to form a depot at the injection site.

An alternative explanation for failing the induction of a peptide specific immune response upon intranasal route vaccination could be that another delivery system, such as liposomes are necessary to deliver the antigen instead of the cationic peptide KLK. Studies reported that liposomes in complex with another TLR9 agonist, namely CpG greatly enhanced immune responses after intranasal administration and therefore it could be used for further intranasal immunization studies in combination with ODN1a and tumor-derived peptides (61).

Altogether these data revealed that intranasal application of IC31® did not induce any antigen-specific CTL response and it seems that IC31® in comparison to CTB is not a promising mucosal adjuvant in enhancing a $CD8^+$ T cell immune response against tumor-derived peptides.

The last question which arose was if IC31® nano supported the generation of a long lasting TRP-$2_{180\text{-}188}$ specific immune response and, whether if there is any difference in regard of adding a $CD4^+$ Helper peptide, called HBc. In brief, we tested the ability of the adjuvant and the peptide, in a three injections protocol were able to induce a long lasting immune response on day 70 after the third

immunization. The data obtained during this study revealed that IC31® had the potency to induce a significant number of IFN-γ producing memory cells in spleens and lymph nodes of C57BL/6 mice upon intradermal and subcutaneous application. HBc did not improved the generation of memory cells by activating $CD4^+$ T cells. The data for the sustained long lasting $CD8^+$ T cell response could be explained by the fact that the immunostimulatory component KLK of IC31® has the efficiency to form a depot at the injection site and therefore control a sustained long-term antigen and adjuvant release (15).

In summary, the encouraging results obtained for distinct tumor-derived peptides such as adjuvanted SVN_{19-28} and TRP-$2_{180-188}$ revealed that both peptides are promising vaccine candidates. Furthermore, these data demonstrate the high potency of IC31® nano in supporting the induction of a long lasting systemic $CD8^+$ T cell immune response as systemic adjuvant in central lymphoid organs and at mucosal sites. Intradermal route vaccination and IC31® as systemic adjuvant showed efficacy in inducing significant tumor-derived peptide specific immune responses without eliciting any toxic effects. Intranasal application and IC31® as mucosal adjuvant implicated any induction of antigen specific immune responses against tumor-derived peptides.

7. MATERIALS AND METHODS

7.1. Materials

7.1.1. Reagents

Aqua Distillate	Ampuwa
Biotinylated anti-mouse IFN-γ developing antibody	BD Pharmingen™ (0.5mg/ml stock conc.)
Biotinylated anti-mouse IL-4 developing antibody	BD Pharmingen™ (0.5mg/ml stock conc.)
BSA	PAA- Laboratories
Calcium Ionophore	Sigma (stock $7.5x10^{-5}$M)
CD4 (L3T4) microbeads, mouse	Miltenyi Biotec (10µl/10^7 cells working conc.)
ConA	Sigma (stock conc. 10mg/ml)
Cytoperm/Cytofix	BD Pharmingen™
DAB	Sigma
DMEM	PAA-Laboratories
DMSO	Applichem
Fc (anti-mouse CD16/31 blocks Fc binding)	BD Pharmingen™
Fetal Bovine Serum (FBS)	PAA-Laboratories
Gentamicin	PAA-Laboratories
Golgi-Plug	BD Pharmingen™
30% H_2O_2	Sigma – Aldrich
L-Glutamin	PAA-Laboratories
LSM	PAA-Laboratories
β-Mercaptoethanol	GIBCO
Mouse Th1/Th2 10plex Kit (Flow Cytomix)	eBioscience
NaCl (0.9%)	B. Braun
NaN_3	Sigma
$NiCl_2$	Sigma
Non-Essential amino acids	PAA-Laboratories

Pan T Cell isolation kit	Miltenyi Biotec
Paraformaldehyde	Sigma
1x PBS	PAA-Laboratories
10x PBS	GIBCO
Perm/Wash (10x)	BD Pharmingen™
Purified rat anti-mouse IFN-γ coating antibody	BD Pharmingen™ (0.5mg/ml stock conc.)
Purified rat anti-mouse IL-4 coating antibody	BD Pharmingen™ (0.5mg/ml stock conc.)
Red Blood Cell Lysis Buffer	Sigma
SA-HPO	Roche
Sodium Pyruvate	PAA-Laboratories
Trypan Blue (0.4%)	GIBCO
Tween 20	Sigma-Aldrich

7.1.2. Labeled antibodies for Flow Cytometry

Table 6. Flow cytometry antibodies used for Flow cytometry analysis

Antigen specifity	Clone	Isotype	Fluorochrome	Stock conc. mg/ml	Working conc. µg/1x10e6 cells	Source
7AAD			PerCP		1	eBio*
CD4	RM4-5	rIgG2a AF488	FITC	0.5	1	BD**
CD8a (Ly-2)	53-6.7	rat IgG2a	Per-CP	0.2	2	BD
CD8a (Ly-2)	53-6.7	rat IgG2a	APC	0.2	1	BD
CD8a (Ly-2)	53-6.7	rat IgG1	PE	0.2	1	BD
IFN-γ	XMG1.2	rat IgG1	AF488	0.2	2	BD
IL-2		rat IgG2b	PE	0.2	2	BD
TNF-α		rat IgG1	APC	0.2	2	BD
Granzyme B		rat IgG2b	PE	0.2	1	eBio
rat IgG1			PE	0.2	2	BD
rat IgG2a			PerCP	0.2	2	BD
rat IgG2a	eBR2a		AF488	0.2	1	eBio
rat IgG2a			APC	0.2	1	BD
rIgG2b			PE	0.2	2	BD
rat IgG2b	eB149/10H5		PE	0.2	2	eBio

**eBio: eBioscience*

***BD: Becton-Dickinson Pharmingen ™*

7.1.3. Media

For the isolation and in vitro incubation of splenocytes, lymph node or lung cells, complete medium was used. Complete medium consisted of DMEM, 5% FBS, 5ml sodium pyruvate, 5ml L-glutamine, 5ml non essential amino acids and additional 500 µl β-mercaptoethanol.

For Flow cytometry analysis complete medium without 5% FBS was used.

7.1.4. Mice

Female C57BL/6 mice ($H2^b$) were procured from Charles River laboratories (Germany) at an age of 6-8 weeks. All animal work was performed according to Austrian law.

7.1.5. Adjuvant

IC31® is a mixture of synthetic cationic poly-amino acid containing lysine and leucine [KLKL5KLK – (COOH)] and ODN1a (phosphodiester backbone ODN, oligo-(dIdC)13) which were purchased from Bachem (KLK) and Transgenomic (ODN1a). The single components of IC31® (nano) were mixed in 2.5mM Tris (pH = 7) at a molar ratio of 2000nmol KLK / 80nmol ODN1a and was stored at room temperature. The same single components were used for classical IC31®, but they were mixed in 110mM Tris/135mM NaCl buffer. The classical IC31® was used only once for the screening for immunogenicity of tumor-derived antigenic peptides. The nano formulation of IC31® in 2.5mM Tris buffer was used in all other experiments.

In two experiments Cholera Toxin B Subunit (CTB) was used as adjuvant control for intranasal vaccination (1µg/mouse).

7.1.6. Peptides

The peptides were synthesized by Think Peptides (USA). They were dissolved in DMSO and stored at -20°C until use.

HBc	TPPAYRPPNAPIL
SVN_{18-27}	RISTFKNWPK
SVN_{19-28}	IATFKNWPFL
SVN_{53-67}	DLAQCFFCFKELEGW
SVN_{57-64}	CFFCFKEL
SVN_{82-89}	SGCAFLSV
$TRP\text{-}2_{180-188}$	SVYDFFVWL

7.2. Vaccination of mice

All in-vivo work was done by Berhard Roeder of the Quality Control – In Vivo (QC-IV) department.

The amount of antigen delivered to each mouse was 60µg. The volume for subcutaneous and intra dermal injection was 100µl at the tail base. Intramuscular (i.m.) immunization was given two times 50µl at the hind limbs and for intraperitoneal (i.p.) the volume for vaccination was 100µl.

The volume for intranasal vaccination was 40µl per mouse. For intranasal administration Isofluran was used as anesthesia. Immunizations were performed on day 0, 14 and 28 (±1-2 days).

7.3. Methods

All methods were performed according to the respective SOPs used at Intercell AG.

7.3.1. Preparation of single cell suspensions of murine spleens and lymph nodes

Bernhard Roeder of the QC-IV department sacrificed the mice by cervical dislocation. Spleens and draining lymph nodes were harvested and pooled from all mice of the same experimental group. Single cell suspensions of spleens or draining lymph nodes were prepared by grinding through a cell strainer into a petri dish by using the plunger of a syringe. The plunger and the cell strainer were washed with complete medium and the cell suspension was transferred into a 15ml Falcon tube. After centrifugation (1200rpm, 10minutes) supernatant was removed by aspirating. For lysis of erythrocytes in spleen cell suspension, red blood cell lysis buffer was used (1ml/spleen). After 2 minutes the reaction was stopped by adding complete medium. Single cell suspensions were again centrifuged (1200rpm, 10 minutes) and supernatants were removed by aspirating. For the lymph nodes lysis of erythrocytes was not necessary. Cells were resuspended in complete medium. The cell number was determined by trypan blue exclusion staining via hemocytometer method.

7.3.2. Preparation of single cell suspension of lungs with Lymphocyte Separation Medium

Bernhard Roeder of the QC-IV department sacrificed the mice by cervical dislocation. Lungs were collected and pooled from all mice of the same experimental group into a 50ml Falcon tube with 25ml complete medium. Single cell suspensions of the lungs were prepare with a homogenizer and passed through a 100-µm pore size mesh cell strainers (BD Biosciences). In a new 50ml

Falcon tube, 20ml Lymphocyte Separation Medium (LSM) were prepared and carefully overlaid with the cell suspension. To create a gradient, cell suspension was centrifuged (2000rpm; 20minutes; without brake).

In advance, Lung mononuclear cells (LMNC) ring was carefully removed and complete medium was added. Cells were again centrifuged (2000rpm; 10 minutes; with brake) and supernatant was aspired. The pellet was resuspended in complete medium and centrifuged (1500rpm; 5 minutes). Supernatant was aspired. For lysis of erythrocytes in cell suspension red blood cell lysis buffer was used (1ml). After 1 minute the reaction was stopped by adding 20ml complete medium. Single cell suspensions were again centrifuged (1500rpm, 5 minutes) and supernatants were removed by aspirating. Cell pellet was resuspended in 5ml complete medium. The cell number was determined by trypan blue exclusion staining via hemocytometer method.

7.3.3. Preparation of single cell suspension of lungs without Lymphocyte Separation Medium

Bernhard Roeder of the QC-IV department sacrificed the mice by cervical dislocation. Lungs were collected and pooled from all mice of the same experimental group into a 50ml Falcon tube with 25ml complete medium. Single cell suspensions of the lungs were prepare with a homogenizer and passed through a 100-µm pore size mesh cell strainers (BD Biosciences). Cells were centrifuged (1500rpm, 5 minutes; with brake) and supernatant was aspired. The pellet was resuspended in complete medium and centrifuged (1500rpm, 5 minutes). Supernatant was aspired. For lysis of erythrocytes in cell suspension red blood cell lysis buffer was used (5ml). After 1 minute the reaction was stopped by adding complete medium. Single cell suspensions were centrifuged (1500rpm, 5 minutes) and supernatants were removed by aspirating. Cell pellet was resupended in 5ml complete medium and passed through a 100-µm pore size mesh cell strainers again. The cell number was determined by trypan blue exclusion staining via hemocytometer method. Cells observed to be too small were excluded from the counting.

7.3.4. MACS cell sorting

In advance to the preparation of cell suspensions of lungs without the use of LSM, the cell numbers were determined by using hemocytometer method. Cells were centrifuged (300g, 10minutes). Supernatantes were aspired and cells were resuspended in 500µl ice cold MACS buffer and 20µl of Biotin Antibody Cocktail (T Pan Isolation Kit). More MACS buffer and Biotin-Antibody Cocktail were used than described in the protocol of Miltenyi Biotec, because the pellets were bigger than expected. Cell suspensions were incubated for 10 minutes at 4°C. The reaction was stopped by adding MACS buffer. 40µl of Anti-Biotin MicroBeads were added. Cells were incubated for 15minutes at 4°C. Cells were washed with 2ml buffer and centrifuged (300g, 10minutes). In the meanwhile the cold columns were pre-rinsed with ice cold MACS buffer. Supernatants were aspired and pellets were resupended in 50µl MACS buffer. Columns were loaded and four times washed with buffer. To separate $CD8^+$ cells and $CD4^+$ cells, 10µl per 10^7 total cells of CD4 (L3T4) microbeads were added after centrifuged the cell suspension (300g, 10minutes). Cells were incubated for 15minutes at 4°C. Cells were washed with 5ml MACS buffer. In the meanwhile the columns were prepared and again pre-rinsed with ice cold buffer. Cell pellets were resuspended in 1ml of ice cold buffer and columns were loaded. $CD8^+$ cells were collected in a tube and counted with hemocytometer method. An aliquot of each sample was stained with $CD8^+$ PE and $CD4^+$ FITC (1:200 dilutions in MACS buffer). Each stained sample was measured on FACSCalibur. 50.000 events were counted from the lymphocyte gate. The data were analyzed by the use of the FCS Express 3 Software to make sure that only $CD8^+$ T cells were used for further analysis.

7.3.5. IFN-γ ELIspot assay

Multiscreen-HTS filter plates (Cat-N° *MSHAS4510,* Millipore*)* were pre-rinsed by adding sterile 1x PBS and coated with rat anti-mouse IFN-γ antibody. The antibody was diluted to a final concentration of 3µg/ml in coating buffer (pH 9.2-9.4) which consisted of 0.1M $NaHCO_3$ in aqua distillate. Plates were incubated over night at 4°C in a wet chamber. Before plating the cells, plates were blocked for 1 hour at 37 °C with blocking buffer, which consisted of 1% BSA in 1x PBS.

In advance to the blocking step 0.25-1.0x10^6 cells were plated (100µl/well) in triplicates and stimuli (100µl/well) were added. As respective controls the cells were restimulated with ConA as positive control (1µg/ml), complete medium as negative control and an irrelevant peptide (1µg/ml) for specifity. Plates were incubated over night at 37°C (5% CO_2).

The next day, cells were discarded from ELIspot plates. Plates were washed three times with washing buffer which consisted of 0.1% Tween20 in 1xPBS. The second biotin anti-mouse IFN-γ antibody was prepared by diluting to a final concentration of 1µg/ml in 1x PBS and adding to each well. Plates were incubated for 2 hours at 37°C (5% CO2). After the washing step Streptavidin-labeled horseradish peroxidase (SA-HPO) was added and incubated at 37°C (5% CO_2) for 30min. After washing, the substrate which consisted of 0.8mg/ml 3,3'diaminobenzidine (DAB) , 0.4mg/ml $NiCl_2$ and 0.015% H_2O_2 in 0.1M Tris, was added.

The reaction was performed at room temperature until the spots became clearly visible. It was stopped by washing plates under running water. The plates were dried at RT. Spots from dry plates were counted by Bioreader 5000.

7.3.6. IL-4 ELIspot assay

IL-4 ELIspot assay was performed as described for IFN-γ ELIspot assay. Multiscreen-HTS filter plates (Cat. N°: MSIPS4510, Millipore) were used for the assay. The primary rat anti-mouse IL-4 antibody was used at a final concentration

of 2µg/ml in coating buffer. The secondary biotin anti-mouse IL-4 antibody, was diluted to a concentration of 1µg/ml in 1x PBS.

7.3.7. Flow cytometry – Intracellular Cytokine Staining

For the Intracellular Cytokine Staining $1x10^6$ cells (50µl) derived from spleen were plated in triplicates in a 96-well U-bottom plate (Nunc). The cells were centrifuged (1200rpm, 5minutes). The supernatants were discarded and the pellet was resuspended in 50µl complete medium without 5% FBS.

In advance 50µl of stimuli were added (1µg/ml). As controls, complete medium without 5% FBS was used as negative control. As positive control 1µg/ml PMA/Iono was added to respective wells. Following 1.5 hours stimulation, Golgi Plug was added in a 1:1000 dilution in complete medium without 5% FBS (100µl) to the cells. After 3.5 hours, cells were washed with MACS buffer and centrifuged 2 min at 2100rpm. Cells were incubated 10minutes at 4°C with FC block (purifies anti-mouse CD16/31 blocks Fc binding) (1µg/$1x10^6$ cells; clone 2.4G2), to block non-specific bindings. Cells were washed once with MACS buffer and centrifuged at 2100rpm for 2minutes. The supernatant was carefully removed by inverting the plate on a paper towel. After the washing step, surface staining was performed by adding 100µl/well of the CD8a antibody. The antibody was diluted to a final concentration of 2µg/ml with MACS buffer. The cells were incubated for 20 minutes at 4°C in the dark. The corresponding isotype control (2µg/ml) was used as well. Cells were washed once again with 100µl MACS buffer and plate was centrifuged at 2100rpm for 2 minutes. Supernatants were discarded. To permeabilize the cell membrane Cytoperm/Cytofix was added for 10-15 minutes. For the next two washing steps 100µl Perm/Wash (1:10 dilution with aqua distillate) was added to each respective well and the plate was again centrifuged at 2100rpm for 2 minutes. Subsequent the intracellular staining was performed by adding 100µl/well cytokine specific antibodies (see table 6). The antibodies were diluted to a final concentration of 2µg/ml with Perm/Wash. Corresponding isotypes for each cytokine antibodies were added at the same time to respective wells (see table 6). The staining took 30 minutes at 4°C in the dark. After finishing the

intracellular cytokine staining the cells were washed once with Perm/Wash and once with MACS buffer. The cell pellets were diluted in 100µl MACS buffer and the triplicates were pooled in the FACS tube. The samples were kept at 4°C and on the next day the cells were measured on FACSCalibur. 500.000 events were counted from the lymphocyte gate. The data were analyzed by the use of the FCS Express 3 Software and CXP Software.

7.3.8. Flow cytometry – Granzyme B staining

The Granzyme B staining was performed like the Intracellular staining. After plating, the cells were stimulated for 16 hours at 37°C (5% CO_2).In advance to this incubation time, 100µl Golgi Plug (1:50 diluted in complete medium without 5% FBS) were added to each respective well and cells were again incubated for 4 hours at 37°C (5% CO_2). Before washing with MACS buffer the triplicates were pooled and afterwards the procedure for the surface staining was performed like described in 8.3.6.. The antibody for the surface staining was diluted to a final concentration of 1µg/ml with MACS buffer (see table 6). For the Intracellular staining Granzyme B antibody (see table 6) was diluted with Perm/Wash to 1µg/ml.

7.3.9. FlowCytomix^{TM}

For the FlowCytomix^{TM}, cells from spleens, lymph nodes and lungs were plated and stimulated for 48 hours at 37°C (5% CO_2). ConA was used as positive control, complete medium as negative control and antigens as relevant or irrelevant peptide (1µg/ml). Cells were centrifuged at 2100rpm for 2 minutes at 4°C. Supernatants were transferred into a new plate and stored at -20°C until they were used.

For the FlowCytomix^{TM} a Mouse Th1/Th2 10plex Kit (eBioscience) was used. First, all solutions were prepared. The Assay Buffer (10x) was diluted with distilled water to 1x. Afterwards the lyophilized Standards were reconstituted by

adding respective amount of distilled water and incubated them for 30 minutes before pipetting at RT. In the meanwhile Bead Mixture was prepared. All vials were vortexed well, before 150µl were added to 1500µl Assay Buffer. The beads were centrifuged with 4000rpm for 5 minutes at room temperature. 2950µl of the supernatant were removed and the same amount of Assay buffer was used to resuspend the pellet. Afterwards, the Conjugation Mix was prepared. Every vial was vortexed very well and 300µl of each was added to 3000µl Assay Buffer. The 30 minutes of incubation were over and a standard dilution series were twice prepared.

In advance, the filter plate was pre-wetted with 50µl Assay buffer. By using the vacuum filtration manifold (Millipore) the Assay buffer was aspired. Subsequent 25µl of the standard dilution series and the samples were plated. Two wells were used as blanks, were only Assay buffer was added, and one well was used for the Setup Standard 1, which was designated for cytometer setup. 25µl of Bead Mixture were added to each well and 50ml of Biotin-Conjugate Mixture. The plate was wrapped with aluminium foil and put on the shaker for 2 hours at RT.

After these two hours incubation time, the Streptavidin-PE solution was prepared. The wells were emptied using the vacuum filtration manifold. The plate was washed two times with 100µl Assay Buffer and again emptied with the vacuum filtration manifold. Afterwards 100µl Assay Buffer was added to each respective well, as well as 50µl Streptavidin-PE Solution. The plate was wrapped with aluminium foil and incubated for 1 hour on the shaker at RT. Subsequent, the wells were again emptied and washed two times as done before. 300µl Assay Buffer was put in every prepared FACS tube. To each well of the plate 200µl Assay Buffer were added. The 200µl were transferred into a separate acquisition tube for the flow cytometer. The setup beads were designed for cytometer setup and 500µl were put into one separate labeled tube.

Afterwards the samples were measured on FACSCalibur. 1500 events were counted. The data were analyzed by the use of the FlowCytomix Pro Software.

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