Master thesis

Epicutan laser-assisted vaccination of ovalbumin-dextran nanoparticles in mice

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'A poet once said, " The whole universe is in a glass of wine." We will probably never know in what sense he meant that, for poets do not write to be understood. But it is true that if we look at a glass of wine closely enough we see the entire universe. There are the things of physics: the twisting liquid which evaporates depending on the wind and weather, the reflections in the glass, and our imagination adds the atoms. The glass is a distillation of the Earth's rocks, and in its composition we see the secrets of the universe's age, and the evolution of stars. What strange arrays of chemicals are in the wine? How did they come to be? There are the ferments, the enzymes, the substrates, and the products. There in wine is found the great generalization: all life is fermentation. Nobody can discover the chemistry of wine without discovering, as did Louis Pasteur, the cause of much disease. How vivid is the claret, pressing its existence into the consciousness that watches it!'

– Richard P. Feynman
The Feynman Lectures on Physics (1964)
Volume I, 3-10

Preface

Vaccination is generally considered to be an unparalleled triumph for global human health, backed up by a large scientific body [1–5]. The groundwork of vaccination is rooted in the principle of equipping the body with immunological weapons by transferring a safe dose of pathogen. Induced by this challenge, the organism upholds a long-term shielding against the administered infectant.

With the up-rise of molecular biology in the last decades, novel vaccination approaches enriched the traditional pallet. Traditionally, vaccines consisted of pathogen-subunits, attenuated- or inactivated- whole-pathogen agents.

Whole agents excel at mimicry of the pathogen, resulting in a broad range of different antibodies. While inactivated plagues fail to induce cellular immunity, attenuated pathogens can elicit potent infections, hence invoking rich humoral and cellular responses. Subunit vaccination exploit single components of pathogens, like surface molecules. This technique usually produces a humoral-dominant response and requires several booster injections.

More recent approaches aim to lower costs of production while increasing safety and immunogenicity [1]. Peptide based vaccines reduce pathogen related proteins down to the immunogenic MHC-presentable epitopes, in order to drive down costs and reduce allergenic potential. In order to compensate for the low immunogenicity of the stand-alone peptide, formulations are often spiked with adjuvants or incorporated into biopolymer constructs [5]. The dominant methods for drug delivery is intramuscular or subcutaneous injection, targeting anatomical sites low in naturally occurring, professional immunocytes. Alternatively, intra-dermal injection is also performed since 1908 and studied exhaustively since the skin is a highly immunocyte infiltrated tissue. Although a broad range of vaccines was tested, only influenza vaccines are administered intra-dermaly, mainly because of strong local adverse reactions to adjuvants in other vaccines [6]. In contrast, novel epicutan, laser-facilitated approaches, promise direct targeting of immuno-competent cells, combined with a danger-associated stimulus - providing a natural adjuvant effect. [7].

This work focuses on the interplay of innate and adaptive immunity based on *in vitro* cultured dendritic cells and T-cells from *mus musculus*, as well as an *in vivo* vaccination model with *mus musculus* . The treatment consists of in-house produced ovalbumin-dextran conjugates and ovalbumin-gelatine nanoparticles with surface dextrans, produced in the University of Saarbrücken, department of XXXX . The *in vivo* delivery system is laser-assisted epidermal immunization via a P.L.E.A.S.E. ® device.

sounds dumb

add nesma, department, formulate treatments better

get tenses rigth - past/present

The co-culture of T-cells with bone marrow derived dendritic cells yielded

results and data from the experiments

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Grundsätzliche Gedanken

1. Einführung und Motivation: Was ist die Problem- oder Aufgabenstellung und warum sollte sich jemand dafür interessieren?

- wichtigkeit und relevanz von Impfungen (möglicherweise historische Heranführung)

2. Präzisierung des Themas: Stand der Wissenschaft beschrieben, Defizite oder offene Fragen aufzeigen -> Anstoß für eigene Arbeit.

- Vor und nachteile von konventioneller impfung im gegensatz zu präsentierter (allergy, nebenwirkung, schmerz, kosten, lagerung). Hier eine Testreferenz für Fig. 1.1

3. Eigener Ansatz - Vorherige Aufgabe (versuchen) zu lösen - Results

- Übergang zu den Ergebnissen - Punkt 2 und 3 vllt in einandner überfließen lassen

4. discussion: Was wurde erreicht und limitationen, Ausblick in die Zukunft?

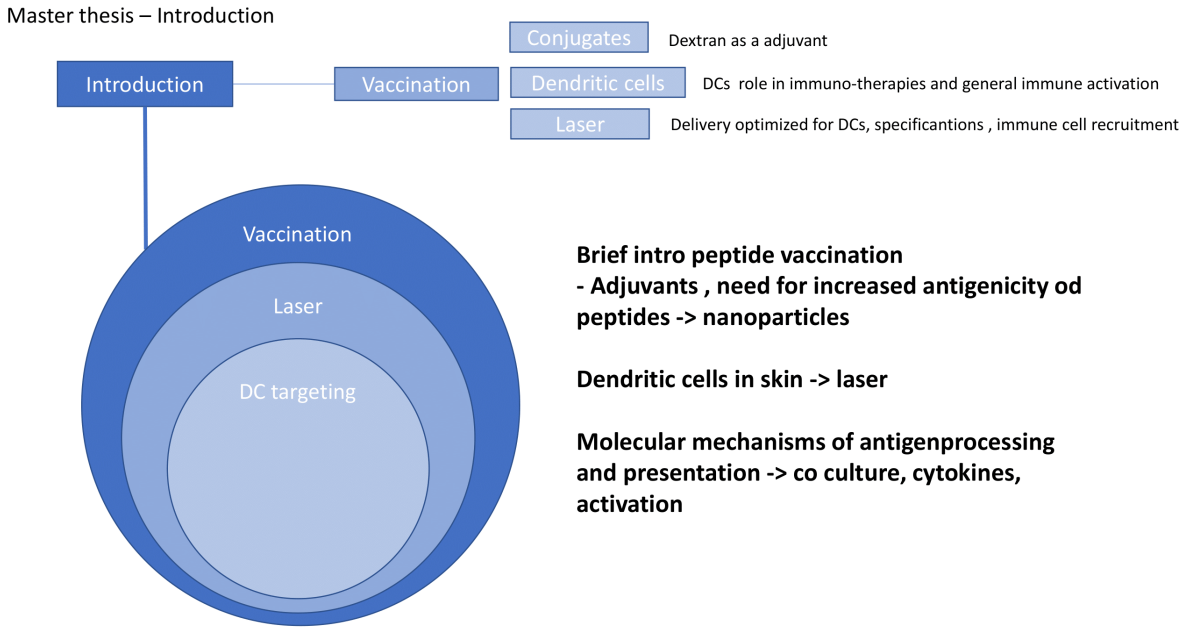


Figure 1.1: Schematische Skizze der Introduction, test des caption layouts

1.1 Vaccinology - a short history

> *"It is said that only those who have seen the beginning of things can understand the present."*
>
> – Stanley Plotkin, *2011*

Documentation of humans, battling infectious diseases via immunization, dates back to ancient China between 1000 - 1500 A.D. It is helpful to know that traditional Chinese medicine relied heavily on the *vis medicatrix naturae*, the body's inherited healing process. Based on two essential principles - *kung* ("attack")[1] and *pu* ("revitalization")[2] a core guideline was *i tu kung tu* - "using poison to combat poison". This phrase is linked to the original attempts of early medicos to protect patients against smallpox by controlled inoculation with the pathogen itself. The remains describe different immunization practices, either by transferring grinded smallpox scarbs from infected people into the patient´s nostrils, or by transmission of infected lymph fluid into the epidermis of the patient. While the former was the dominant procedure in China, the latter technique spread over to Turkey and further then to Europe in the 17th century [9]. The method was known under the name of variolation, derived from latin *varus*,[3] "mark of the skin". It got introduced to England in 1715, triggered by the hunt for a treatment of the english aristocrat Lady Mary Wortley Montague. She commissioned Charles Maitland to variolate her five year old son, at that time in turkey, in 1718. Three years later, back in England, Charles Maitland performed the smallpox inoculation also on Lady Montague´s four year old daughter successfully - sparking her to advocate and popularize variolation. The death rate of the variolation procedures itself, is not precisely historically confirmed, but was probably around 1-3% - approximately ten times lower than the disease itself. The first documented statistical approach was conducted by Mather and Boylston in Boston, 1721 - covering 12000 individuals, amongst whom the case fatality rate of non-immunized persons was 14% contrary to 2% in variolated beings. The death toll was indeed dramatically lower, but safer procedures were desired - a procedure later known as vaccination [10, 11].

1: meaning the active combat of pathogens

2: meaning the strengthening of internal defense mechanisms

3: inoculation was often used interchangeably, derived from the latin word *inoculare*, "to graft", which explains another popular term for the procedure in europe "engrafting"

In the late 18th century, Edward Jenner and Benjamin Jesty pioneered in the development of the first smallpox vaccine - through protection offered by cowpox viruses. At that time there was a known connection between cowpox exposed milkmaids and a resistance against smallpox. Jetsy successfully vaccinated his family, while Jenner pursued further testing and delivered scientific confirmation,[4] leading to a rapid spread of the vaccine.[5] The exact contents of Jenner´s *vaccinia* remain unknown, speculations circle an extinct strain of horsepox or a genetic hybrid between smallpox and cowpox, namely *variola*, a virus with no natural host. The publication eventually gave rise to the global eradication of smallpox [9, 13].

4: Variolae Vaccinae in 1798 [12]

5: Jesty was honored in 1805 by the Original Vaccine Pock Institute in an open statement, recognizing Jesty's cowpox vaccination. Furthermore his portrait arranged in the institute. Nevertheless Jenners paper is accepted as the first scientific shot to regulate a extensively present disease, without transmitting the disease itself [4].

Louis Pasteur characterized 80 years later, the monumental process of attenuation. Whether on purpose, or by accident, Pasteur documented the mitigation of *Pasteurella multocida* by exposure to adverse conditions. Subsequently, he continued to work on Anthrax and rabies, shedding more and more light into the mysteries of vaccination. Decisive discoveries of bacterial toxins, antitoxins, viruses, inactivation of whole

bacteria or toxin- and replication- neutralizing agents in serum,[6] followed between 1890 and 1900. Shortly after, inactivated whole-cell vaccines against typhoid, cholera and plague were created. Antibodies, were described in animals surviving exotoxin-secreting *diphtheria bacilli* infections. Further investigation of the toxin, led to the formulation of a stable non-toxic formalin-inactivated diphtheria antigen toxoid. Further insight arose in the selection of avirulent strains by passage through unnatural hosts. Strikingly, the development of a *Mycobacterium bovis* vaccine through 230 serial passages, took 14 years, offering protection against tuberculosis. Being smaller than bacteria and parasites, filterable agents,[7] were described and the yellow fever strain 17D was attenuated through serial passage. Simultaneously, whole-cell *Bordetella pertussis* vaccine and *influenza virus* vaccines were employed, whereby the original pertussis formulation became refined to only the antibody-inducing bacterial proteins. Influenza - although successfully immunizable initially - emerged as greatly variable in antigens, swinging the original vaccine impotent. Countering this variability, still remains subject to research, in a quest to identify conserved, antigenic domains.

6: antibodies

7: virus; The first reported specimen, *Tobacco mosaic virus* was described by Ivanoski in 1892, showing that extracts from infected leaves remained infectious upon filtration. (typically removing bacteria and parasites). Seven years later, Beijerinck coined the term *"virus"*, describing the tobacco mosaic virus as a soluble, infectious agent [14].

Viral cell culture,[8] was certainly a milestone in vaccine development, taken advantage of by Jonas Salk and Albert Sabin, in developing a *poliovirus* vaccine. Awareness, that neutralizing antibodies correlated positively with immunity, built the credo to reduce reactivity of viruses, while maintaining immunogenicity. These findings also paved the road for measle, mumps, and rubella vaccines (MMR).[9] Protein - polysaccharide conjugation was already discovered but failed to deliver potent results, until Robbins and Rachel Schneerson successfully coupled diphtheria with the type b capsule protein of *H. influenza*. Successive experiments, also with tetanus toxoid, spawned conjugate vaccines for *menigococci* and *pneumococci*.

8: Pioneered by John F. Enders, Thomas H. Weller and Frederick C. Robbins, who tested conditions for extraneural replication of a *poliomyelitis virus* strain. The culture media consisted of a salt solution, and ox serum ultra-filtrate supplemented with various human embryo tissue [15].

9: MMR controversy (Box):

Excursion: Vaccine safety

Myths, that MMR vaccination leads to autism has been among the most harmful controversies in vaccine safety. Reported in 1998 - "Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children" - the article was retracted by the publisher (The Lancet), because of suspicious subject recruitment and financing. The authors observed twelve children with pervasive developmental disorder (9 with autism) and intestinal problems. Additionally, the central message of the study was based on the note of the parents or doctors, observing a worsening of pervasive developmental disorder in eight children. Even though a great number of studies demonstrated no correlation between autism and MMR vaccination, the large initial panic still lingers in the heads of many parents and resulted in a reoccurrence of the diseases in some places [16].

Stanley Cohen and Herbert Boyer have launched the protein production in various cell types via the means of genetic engineering, at that time with using baculovirus vectors. This enabled the development of the first engineered vaccine - a hepatitis B surface antigen. Subsequent protective antigens were expressed for *human papillomavirus*, *Borrelia burgdorferi* and *rotaviruses*. Other than the humoral component of immunity, T cell activation also plays a crucial role in various infections and ensures a long-time protection into the future. The classification of cellular and molecular orchestration is currently object of scientific research and advances the creation of high precision formulations [13]. In the following sections, a coherent outline of the relevant immune machinery is given, leading to the techniques and scientific foundation for the latter experiments.

diversify sources for this section - looks pretty bad with only one source

1.2 Molecular Immunity

Immune components can be roughly divided into adaptive and innate

- cellular - humoral
- communication (cytokines)

- immunocytes
- classification of CDs and T cells
- maturation of B cells
- antibody classes ???
- receptors

1.3 Novel vaccine designs

With ever growing insight into molecular immunology, it is clear that a protective immune response, is in many instances only dependent on a few immunogenic proteins. Whole-pathogen vaccines or their subunits, may not only contain unnecessary components but also allergenic ones [5]. This reductionist approach - creating hypoallergenicity while maintaining immunogenicity - demanded an addition of safe danger signals into the formulations. Attractive techniques have evolved around protein and peptide based vaccines, coupled to polysaccharides, spiked with soluble immune-stimulants or water-oil emulsions. Not only drug design, but also method of delivery is under investigation, ensuring high safety profiles with optimal efficiency[1, 2, 7, 17]. In the following subsections, the mentioned components of vaccination relevant to this work, are explored in greater detail.

1.3.1 Proteins and Peptides - Too gentle?

The mild immunogenicity associated with protein and peptide based vaccines, called for the development of boosters - enhancing the magnitude and and duration of these vaccines. So called adjuvants fulfill additional tasks, depending on the specific requirement. Generally speaking, they can be optimized for distinct immune responses, population targeting and reduction of antigen load. Aluminum salts were the first adjuvants used and licensed by the FDA in 1926, followed by water in oil emulsions. Much time passed before novel formulations came to market, due to high safety regulations and a lack of understanding of the underlying mechanisms. 2009 the FDA approved a cervarix vaccine for human papilloma virus (HPV) containing a new mixed-adjuvant formulation of monophosphoryl lipid and aluminum salt. This collection of adjuvants acts thought release of damage associated patterns (DAMPs), thereby largely reinforcing the humoral immune response via IL-1β production. As previously mentioned, cellular immunity is the key to tackle diseases like tuberculosis, HIV and malaria, and is therefor an interesting target for adjuvant design [2].

hier zurückkommen auf die vorher beschriebenen prozesse der antigenprozessierung und die damit verbundene T und B zell antwort

1.3.2 Molecular Archery - Identifying and hitting the right targets

hier zurückkommen auf die spezifischen rezeptor typen , eintritt in die Zelle sehr wichtig - Wie kommen die konjugate in die zelle?

Since molecular patterns in a wide variety of immune responses are described, subsequent treatments are designed exploiting the known pathways. It is well established, that innate immunity contains specialized cells, capable of recognizing foreign substances via pattern recognition receptors (PRR), processing and further presentation. Professional antigen presenting cells, notably dendritic cells are capable of processing invasive substances via a pathway and subsequently presenting / activating cells of the adaptive immunity . The ability of dendritic cells to function as a sentinel as well as an activator, shapes immune responses in their intensity and manner.

cluncky

name exact pathway

arm?

Research during the past decade has identified a fundamental role for the innate immune system in sensing vaccines and adjuvants and in programming protective immune responses. The innate immune system can sense microbes through pattern-recognition receptors (PRRs), such as the Toll-like receptors (TLRs), which are expressed by various cells, including dendritic cells (DCs)4,5. In addition to TLRs, other types of PRRs, including the C-type lectin-like receptors6 and the cytosolic Nod-like receptors, sense a broad range of microbial stimuli, and the cytosolic RIG-I-like receptors sense viral nucleic acids8. There are many subsets of functionally distinct DCs, and it is now clear that the DC subset, as well as the nature of the PRR, have a key role in determining the magnitude and quality of adaptive immune [18].

1.3.3 Injection - a necessary evil?

review von sandra und richard perfekt für den vergleich zwischen den delivery methods

1.4 framework and construction of this work

Condense information from the previous sections to reflect the thought process of these experiments, I think it would be cool to add the theoretical foundation of the experiments - LPS removal, liquid chromatography, anthrone, cell culture etc here.

1.5 Additional todos

– Auf logischen Fluss achten !!
– erst adjuvants oder antigenprozessierung ???
– dextran-DC interaction [8]

Materials and Methods 2

Chemicals and experimental procedures conducted during the thesis. The majority of the the work was performed in the University of Salzburg, Hellbrunnerstr. 34, Division of Allergy & Immunology. Dynamic light scattering (DLS) analysis was performed by Mark Geppert in Itzling . Microscale thermophoresis (MST) was performed by Elfriede Dall in the Research Centre of Biosciences and Health, Billrothstraße 11, Salzburg. Amino acid analysis (AAA) was perfmored by Sabrina Wildner in the University of Salzburg, Hellbrunnerstr. 34, Division of Allergy & Immunology.

ask Mark

2.1 Materials

2.1.1 Chemicals

- Triton X-114 sigma
- Dextran 15 kDa -
- Dextran 100 kDa
- Dextran 450 kDa -
- Anthron
- Sodiumperiodate
- Sodiumcyanoborhydrate
- Sodiumborohydride
- etc.

2.1.2 Equipment

- pipettes
- Agilent HPLC
- BioCAD
- platereader
- dialysis filter
- etc.

reformulate into a brief text with the exact information

2.2 Glycoconjugate production

2.2.1 Endotoxin removal of Ovalbumin

400 mg commercial Ovalbumin (Sigma-Aldrich, Lot 038K7012) was weighed in and diluted in 20 ml dH_2O (2 stocks). 200 μL Triton-X114 (5%, Sigma-Aldrich, 8x washed) were added drop-wise to the solutions while vortexing. Next, the samples were incubated at 4 °C for 60 mins while shaking, then centrifuged at 10^4 rpm for 30 mins at 37 °C. Supernatants were collected carefully and processed as described above for three additional times. For purification, the samples were treated as above for another two times, but without further addition of Triton-X114. Finally, the samples were passed through a 0,22 μm pre-warmed (37 °C) syringe filter (Starlab). The concentration of the solutions was assessed via Bicinchoninic acid assay while the residual LPS[1] was quantified via Limulus amebocyte lysate assay. The samples were stored at stored at +4 °C in the dark.

1: Lipopolysaccharide

2.2.2 Bicinchoninic acid assay

Protein quantification was performed via Pierce™ BCA Protein Assay Kit (Thermo Fischer). Thereby samples and standards were diluted in ef-H_2O and 25 μL pipetted into a 96-well flat-bottom plate (Greiner). 50 parts of Reagent A were mixed with 5 parts of Reagent B, then 200 μL of this working reagent were added to each well. The plates were incubated for 30 min at 37 °C before measuring the absorbance at 562 nm (Tecan infiniteM200PRO).

2.2.3 Limulus amebocyte lysate assay

50 μL of samples and LPS standards (Pyroquant, Cat Nr 32500A) were diluted in endotoxin-free Water (Sigma-Aldrich) and loaded onto a clear 96-well v-bottom plate (Greiner). Then, 50 μL of LAL reagent (lysate 2018) were carefully added to each well and stirred 20x with pipette tips before measuring the absorption at 405 nm a time series at 37 °C for 30 minutes (9 time points, Tecan infiniteM200PRO).

2.2.4 Oxidation of Dextrans

15-25 kDa, 100 kDa and 450-650 kDa Dextrans (from *Leuconostoc spp.*, Sigma-Aldrich) were oxidized 40%, 20% and 20% respectively, using sodium periodate ($NaIO_4$) (99%, Fischer Scientific U.K.). Briefly, 800 mg of Dextrans were dissolved in 20 mL endotoxin-free Water (Sigma-Aldrich) by vortexing and mild heating (< 75 °C). The solutions were oxidized by addition of 2350 μL (100 kDa, 450 kDa) and 4700 μL (15 kDa) of 90 $\frac{mg}{ml}$ $NaIO_4$ (in ef-H_2O). After incubating the samples for 75min while shaking at 450rpm (dark, RT), dialyzations (Cut-Off_{MW}: 3500 Da, Roth) against 2L PBS were carried out overnight (stirring, 3x PBS change, final dilution 1:5x10^5). The generated solutions were measured via Anthrone assay and stored at +4 °C in the dark.

2.2.5 Anthrone assay

50 μL of samples and standards are added to a 96-well flat-bottom plate (Greiner) and incubated at 4 °C for 15 minutes. Next, 200 μL of anthrone reagent (2 $\frac{mg}{ml}$ anthrone in ice cold H_2SO_4 (95%, Merck)) are added to the wells, while keeping the plate on ice. The plate was shaked briefly, then transferred to a boiling water bath (microwave, 5 min 600 W) for three minutes, where carbohydrates are hydrolysed to hydroxymethyl furfural. Afterwards, the plate is cooled to room temperature in a water bath for 5 min, before measuring the absorption of the samples and standards at 620 nm (Tecan infiniteM200PRO)

2.2.6 One-pot conjugation

For conjugation, to ≈200 mg of oxidized Dextrans (27, 30, 32 $\frac{mg}{ml}$), 1.5 mL of 2-picoline-borane [0.25M, solved in MetOH] (pic-BH_3, Merck) was added while vortexing. Next, 1 mL of acetate buffer [pH 5.8] was added,

before 588 μL of efOVA (17 $\frac{mg}{ml}$) was added and mixed. The final solution with a ratio of 20:1 (DEX:OVA) was rotated for 72h in the dark at 23 °C. The solutions were then stored at 4 °C in the dark.

2.2.7 Size exclusion chromatography

Size exclusion chromatography (SEC) was performed on a Agilent (1050 Series) HPLC in order to identify optimal coupling conditions for the respective Dextrans efficiently. Purification of the desired conjugates was executed with a BioCAD 700E FPLC on a sephacryl 200 size exclusion[2] column equipped with SF-2120 (Advantec) fraction collector.

2: SE

2.2.7.1 Coupling control via SE-HPLC

Coupling efficacy was assessed via high performance liquid chromatography[3] (Agilent) with a size exclusion column (Viscotek - v = 14 mL) . The mobile phase KH_2PO_4(filtered through 0.2 μm (Starlab) and degasssed) flow was set to 1 $\frac{ml}{min}$, with a runtime of 30 min, a injection volume of 70 μL and detection set to 280 nm. Differently oxidized Dextrans (0-40%) were coupled to OVA in a 20:1 and 10:1 ratio under various buffer conditions. Highest coupling efficiency was determined by largest shifted peak area. Optimal conditions were subsequently chosen for production of glycoconjugates an were the following: Buffer - Acetate pH 5.8, Ratio (Dex:OVA) - 20:2, Oxidation - 15 kDa Dex = 40%, 100 kDa & 450 kDa = 20%, Oxidizing agent: 15% ($\frac{v}{v}$) pic-BH_3 [0.25M] .

3: HPLC

name of the column

2.2.7.2 Purification via SE-FPLC

To purify the glycoconjugates, fast protein chromatography[4] was performed on a BioCAD 700E (Perspective Biosystems), equipped with a sephacryl 200 size exclusion[5] column. 5 mL of the conjugates in dPBS were loaded into the loop and eluted with degassed and sterile filtered (Starlab, 0.2 μm) PBS at a flow rate of 1 $\frac{ml}{min}$. 2 mL fractions were collected and analyzed with Anthrone assay and BCA assay. Two fractions of each conjugate were pooled (Tab. 2.1).

Conjugate	Fractions
15F1	F38-F46
15F2	F66-F74
100F1	F37-F46
100F2	F60-F72
450F1	F38-F46
450F2	F55-F72

Table 2.1: Summary of the pooled fractions generated by SE-FPLC

4: FPLC

5: SE

name of filter

2.2.8 Dynamic light scattering

Zetasizer Nano ZS with a DTS1070 capillary cell (Malvern Instruments).

2.2.9 Microscale thermophoresis

using a Monolith NT.115 RED instrument (Nanotemper, Munich, Germany)

2.2.10 Amino acid analysis

2.3 Cell culture

2.3.1 Murine bone marrow isolation

C57BL/6 mice were sacrificed via cervical dislocation. Under steile conditions, femur and tibia bones were isolated, extricated from residual flesh and placed in a 10 cm sterile dish (Greiner) with DPBS (Sigma-Aldrich). Next, the bones were stilized in 70% EtOH, washed with DPBS and placed in fresh DPBS. Finally, the ends of the bones were cut open and the bone marrow was washed out with cold DPBS, using a 27G needle. The solution was passed multiple times through a 22G needle before being filtered through a 70 μm cell strainer (Starlab). The cell mix was centrifuged for 5 mins at 1200 rpm, 20 °C. Supernantants were discarded by suctioning, then the pellet was resuspended in 5 ml BMDC Medium (Tab. 2.2) and counted manually in a Neubauer chamber or though electronic current exclusion (CASY - OMNI Life Science).

Reagent	Content	Manufacturer
RPMI-1640		Sigma-Aldrich
β-MeOH	50 μM	Sigma-Aldrich
Pen-Strep	1x	Sigma-Aldrich
L-Glutamine	0.3 $\frac{mg}{ml}$	Sigma-Aldrich
FBS	10%	
Optional:		
GM-CSF	200 $\frac{ng}{ml}$	ImmunoTools

Table 2.2: Recipe for bone marrow derived dendritic cells medium. For the maturation of GM-CSF stimulated dendritic cell, the stated reagent, listed under 'optional' is supplemented. FBS was heat inactivated at 56 °C for 30 mins prior to use. *Abb:* Pen-Strep: Penicilin-Streptomycin, FBS: Fetal bovine serum, β-MeOH: β-Mercaptoethanol, RPMI: Roswell Park Memorial Institute

2.3.2 FLT3-L stimulated DCs

Cell suspensions were prepared as described in Methods-2.3.1. The solutions were diluted to 2.5x10^6 $\frac{cells}{ml}$ and 3 mL dispensed into non-tissue-treated 6-well plates (Greiner). The wells were spiked with human fms-like tyrosine kinase 3 - Ligand[6] (AcroBiosystems) to a final concentration of 200 $\frac{ng}{ml}$, gently resuspended and incubated at 37 °C 5% CO_2 and 95 % relative humidity. After five days, BMDC Medium (Tab. 2.2) equal to 50 % of starting volume was added. After nine days of maturation, cells were pooled and centrifuged at 1200 rpm for 7 mins at room temperature. Supernatants were discarded via a vaccum pump and pellets resuspended in 1 mL BMDC or T cell medium (Tab. 2.2,**??**). Afterwards, cells were counted manually in a Neubauer chamber or via electronic current exclusion (CASY - OMNI Life Science), appropriately diluted and seeded.

6: FLT3-L

2.3.3 GM-CSF stimulated DCs

Cell suspensions were prepared as described in Methods-2.3.1. The solutions were diluted to 2x10^5 $\frac{cells}{ml}$ in GM-CSF spiked BMDC medium (Tab. 2.2/optional), 10 mL dispensed into 10 cm, non-tissue treated plates () and incubated at 37 °C 5% CO_2 and 95 % relative humidity. After three days GM-CSF spiked BMDC medium equal to 100 % of the starting volume was added. After four or five days 50 % of total volume was removed carefully, centrifuged at 1200 rpm for 5 min at 4 °C resuspended in fresh GM-CSF medium and evenly distributed on the dishes. After seven days of maturation, cells were pooled, centrifuged at 1200 rpm for 7 mins at room temperature and resuspended in 5 ml BMDC or T cell medium (Tab. 2.2,**??**). Afterwards, cells were counted manually in a Neubauer chamber or via electronic current exclusion (CASY - OMNI Life Science), appropriately diluted and seeded.

manufacturer

2.3.4 Dendritic cell activation

Harvested BMDCs as previously described in Methods-2.3.2 and /2.3.3 were diluted to 8x10^5 $\frac{cells}{ml}$, so 1x10^5 $\frac{cells}{well}$ could be seeded out. Treatments were diluted in T cell medium (Tab. **??** based on OVA equivalent concentrations and ranged typically from 20 - 0.08 $\frac{\mu g}{mL}$ (specified in **??**

2.3.5 Naive CD4$^+$ T cell activation

2.3.6 Cell sorting

BD FACS Aria™ III (BD BioSciences),

2.3.7 Flow cytometry

Cytoflex S flow cytometer (Beckman-Coulter)

2.4 In vivo vaccination

C57BL/6 mice were housed in the animal facility of the University of Salzburg according to the local animal care guidelines. All animal experiments were approved by find out approval

2.4.1 Immunization procedure

2.4.2 IgG ELISA

2.4.3 IFN-γ IL-4 ELISPOT

2.4.4 Immunocyte restimulation

Lymph nodes and spleens were harvested for restimulation with OVA and SIINFEKL. After erythrocyte lysis in Ammonium-Chloride-Potassium (ACK) lysing buffer, splenocytes were resuspended in T-cell medium (RPMI-1640, 10% FCS, 25 mM Hepes, 2 mM L-Glu, 100 µg/mL streptomycin, 100 U/mL penicillin) and 0.6 × 106 cells/well were stimulated with 0.1 mg/mL EndoFit™ OVA and incubated for 3 days at 37°C, 5% CO2. Supernatants were harvested and analyzed by LEGENDplex immunoassay using the mouse T helper 13-plex cytokine panel. Cells were stained for CD4, CD25, CD44, FoxP3, GATA3 and analyzed by flow cytometry on a Cytoflex S flow cytometer (Beckman Coulter).

2.4.4.1 Flow cytometry

2.4.4.2 Cytokine analysis

Results 3

"Most people use statistics like a drunk man uses a lamppost; more for support than illumination".

– Andrew Lang, *1937*

3.1 Characterization of Dextran - Ovalbumin Glycoproteins

3.1.1 Endotoxin content of OVA < XX EU

Treatment of commercial OVA with Triton-X114, as described in Methods/ [2.2.1] resulted in

ask for conversion of ng/ml into EU

3.1.2 Fractionized purification of OVA-DEX

Upon coupling, aliquots are drawn from the samples and loaded on the SEC-HPLC for quality control, before separating 2ml fractions on the BIO-CAD (SEC-FPLC). Each specimen produced two distinct, size-shifted peaks compared to pure OVA (OD280), reflecting complexed DEX-OVA. To exclude signal bleeding from the Dextrans into the 280 channel, a control run with plain Dextrans was performed respectively. The collected fractions were analyzed via Bradford assay and Anthrone method in order to asses the dextran content.

Add bioCAD plots

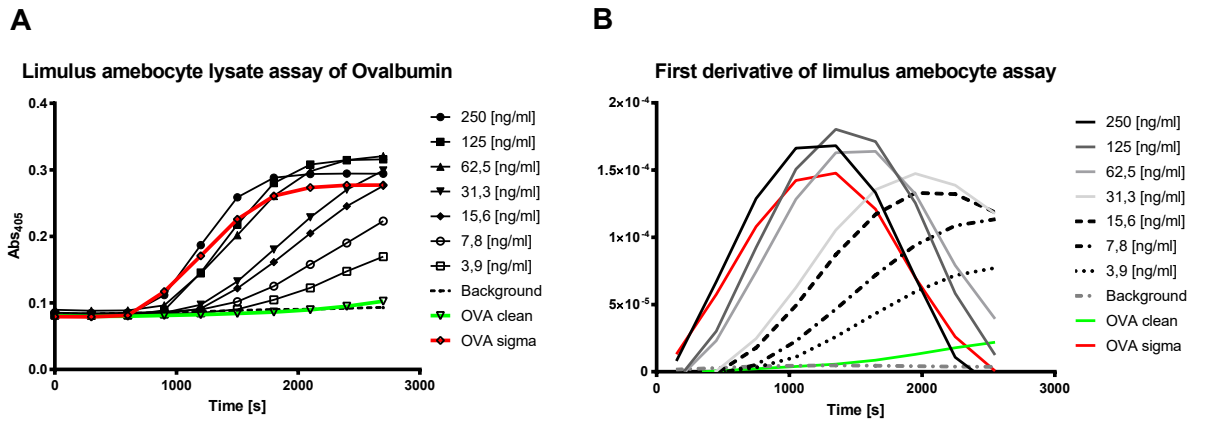


Figure 3.1: (A) Time series of LAL assay of OVA before and after Triton-X114 treatment. **(B)** Mathematical transformation of the time series via first order derivation, in order to illustrate the inflection points.

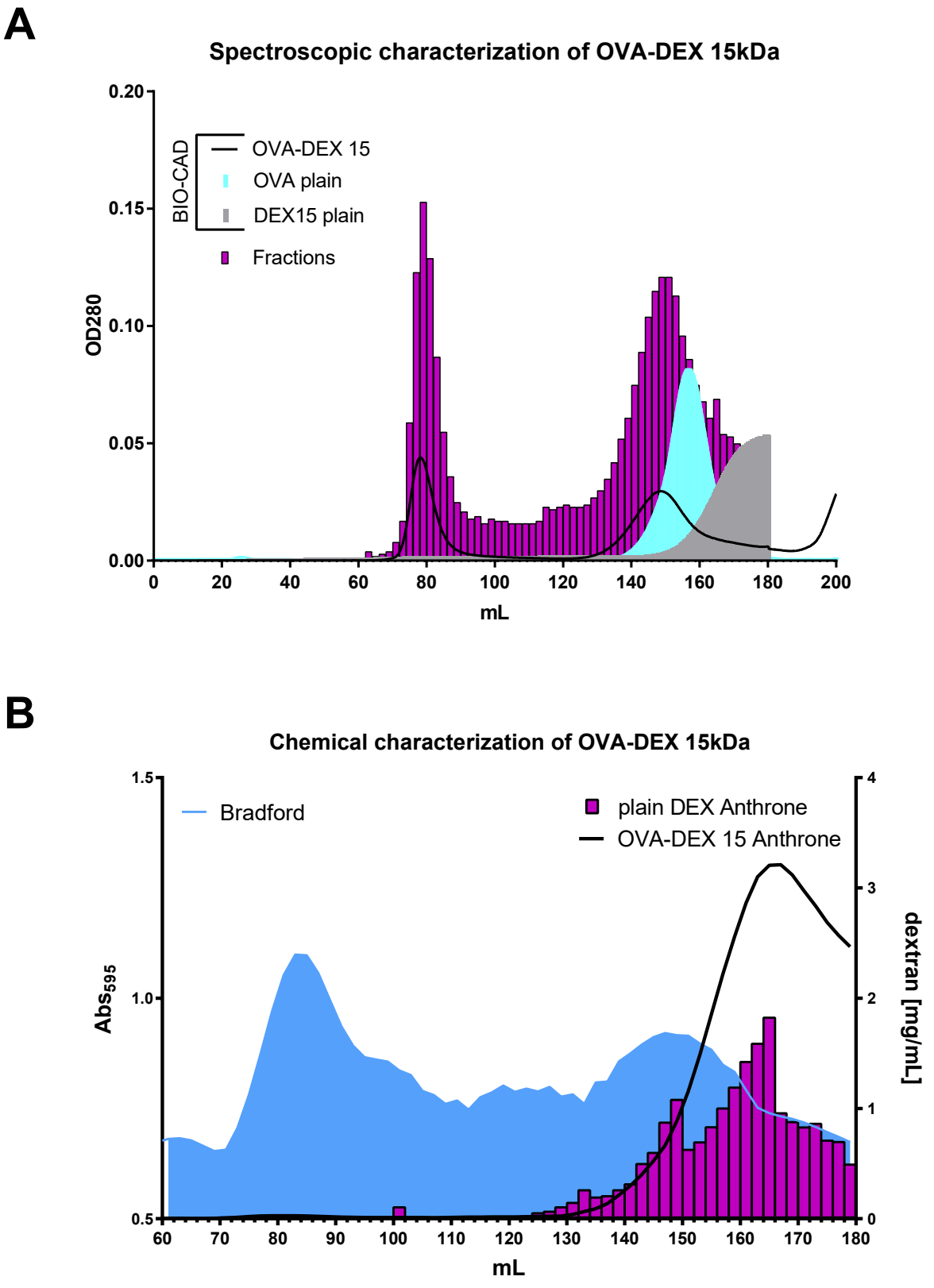
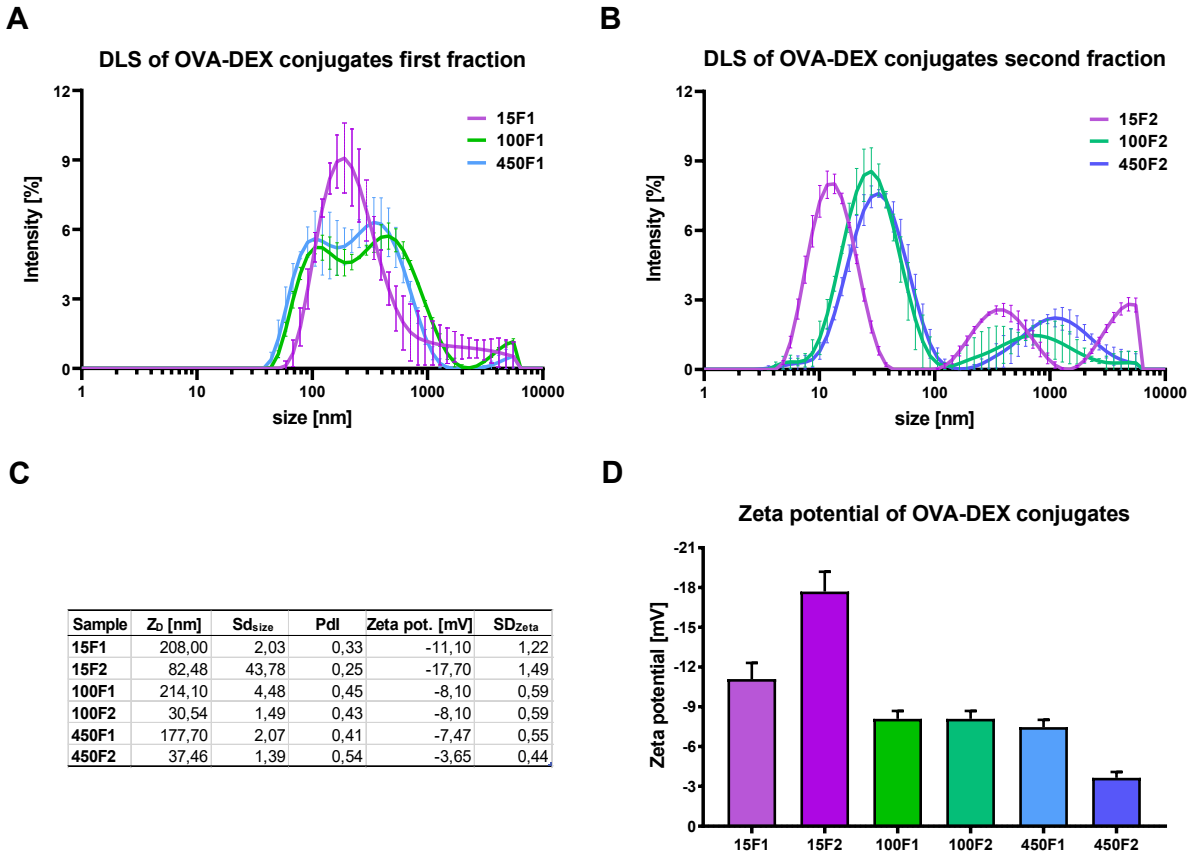


Figure 3.2: Caption



Sample	Z_D [nm]	Sd_{size}	PdI	Zeta pot. [mV]	SD_{Zeta}
15F1	208,00	2,03	0,33	-11,10	1,22
15F2	82,48	43,78	0,25	-17,70	1,49
100F1	214,10	4,48	0,45	-8,10	0,59
100F2	30,54	1,49	0,43	-8,10	0,59
450F1	177,70	2,07	0,41	-7,47	0,55
450F2	37,46	1,39	0,54	-3,65	0,44

Figure 3.3: Size distribution of OVA-DEX conjugates as determined by dynamic light scattering shown in (**A**) and (**B**). Zeta potential of the conjugates displayed in (**D**), where all information is summarized in (**C**). Data is shown as the mean of three measurements + standard deviation. *Abb. PdI = polydispersity index*

3.1.3 Hydrodynamic radii of OVA-DEX conjugates

Z_D values calculated via the intensity-weighted mean yielded good quality results for OVA-DEX conjugates, except for 15F1 (see. Fig. 3.3C). In this case, the giant aggregates (40+μm, see. Fig.3.3B), skew the Z_D value and increase the standard deviation - mathematically grounded in the susceptibility of Equation 3.1 to large agglomerations or contaminants. Among the 15F2 peaks, 62% ± 3 of the particles lie in the first fraction (Z_D: 14.2nm ± 0.5) in contrast to 100F2 (79.5%±5.2 - 31.37nm±3.048) and 450F2 (72.3% ± 5.2 - $\bar{x}$=36.14nm ± 2.408). Among the first fractions, 15F1 is more homogeneous than, 100F1 and 450F1, which appear to be relatively congruent - with two indicated major peaks (see. Fig. 3.3A). All samples have a polydispersity index (PdI) < 1, which indicate suitable compositions for DLS measurements.

$$\bar{x} = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i} \tag{3.1}$$

Equation: 3.1 Intensity-weighted mean with w_i as weighting factor

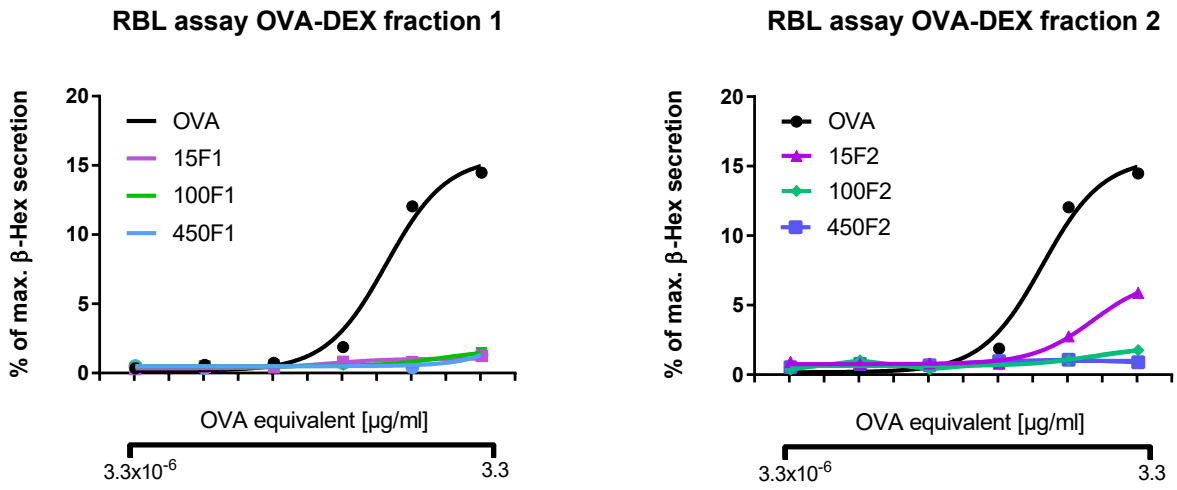


Figure 3.4

3.1.4 Microscale thermophoresis

3.2 In vitro assessment of Dextran - Ovalbumin conjugates

3.3 LPS

LPS[1] , often found on cell walls of gram-negative bacteria, are long known to interfere with various cell types, most prominently immune related cell species. Depending on cell fate, reactions range from proliferation, differentiation and phagocytosis to mediator production. Upon contact, monocytes typically produce proinflammatory cytokines, including IL-l,[2] IL-6, IL-8 and TNF-α [3] .

Murine T-cells are also known to proliferate *in vitro* in response to LPS. In addition, LPS-stimulated $CD8^+/CD4^-$ murine T-lymphocytes can dampen the humoral immune response to bacterial polysaccharides [19] .

1: Lipopolysaccharide; endotoxin

2: Interleukin-X

3: Tumor necrosis factor - alpha

[19]: Holst et al. (1996), 'Biochemistry and cell biology of bacterial endotoxins'

unfinished

3.3.1 OVA-DEX conjugates are hypoallergenic

3.3.2 ELISA

T_H1 and T_H2

Discussion 4

Appendix

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Special Terms

I

Interleukin wichtig für zell zell interaktion lymphocyten bla bla . 14